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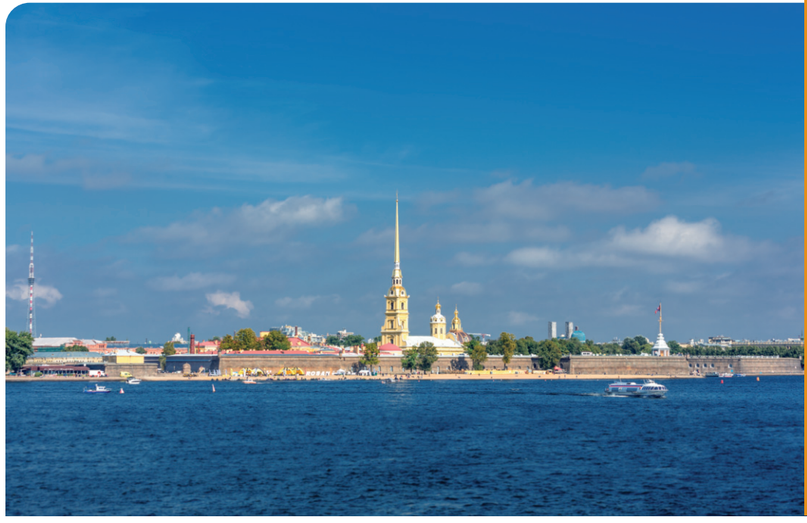
Special Issue

Plant Signalling: From Molecules to Behaviour

Guest Editors: Vadim Demidchik, Frans Maathuis and Olga Voitsekhovskaja



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Cytokinins regulate root growth through its action on meristematic cell proliferation but not on the transition to differentiation

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This paper originates from a presentation at the Fourth International Symposium on Plant Signaling and Behavior, Komarov Botanical Institute RAS/Russian Science Foundation, Saint Petersburg, Russia, 19–23 June 2016.

Abstract. Contrary to the wide-spread view that cytokinins change the rate of root growth and meristem size by regulating the cell transition to elongation (differentiation), our data showed that cytokinins affected the cell cycle duration in the meristem. The rate of meristematic cell transition to elongation itself is regulated by two groups of independent processes, through influence on (i) the life-span of cells in the meristem, and (ii) the cell proliferation rate in the meristem. Trans-zeatin slows down the root growth rate and the cell transition to elongation as a result of prolongation of mitotic cycles. The life-span of cells in the meristem does not change. The number of meristematic cells in one file decreases due to inhibition of cell proliferation but not to an acceleration of cell transition to elongation. Roots of triple mutant *ipt3ipt5ipt7*, in which cytokinin synthesis is slowed down, behave in an opposite way such that the rate of cell transition to elongation and cell proliferation is speeded up. Their peculiarity is that the life-span of cells in meristem becomes shorter than in control roots. In both cases, a change in concentration of endogenous cytokinin or in its signalling are associated with a change in mitotic cycle duration.

Additional keywords: *Arabidopsis thaliana*, cell transition to differentiation, cytokinin, mitotic cycle, root apical meristem, root cell growth.

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Introduction

Root growth rate depends on cell production in the root apical meristem and rapid cell elongation taking place in the elongation zone. To elucidate how any external or internal factor affects the root growth rate it is necessary to know if and how it affects cell proliferation and elongation; specifically, the length of fully elongated cells (Webster and MacLeod 1980; Ivanov 1994).

Cytokinins (CK) play an important role in the regulation of plant growth and morphogenesis (Mok and Mok 2001; Kieber and Schaller 2014). However, our knowledge of the mechanisms involved in their action at the cellular level is still poor and contradictory. Beemster and Baskin (2000) showed that 1μM trans-zeatin (TZ) slowed down the growth of *Arabidopsis* roots by increasing the cell cycle duration (*T*) and slightly decreasing the length of fully-elongated cells, but did not change the rate of cell differentiation. An opposite conclusion, that TZ does not change the cell cycle duration but accelerates the cell differentiation that leads to a shortening of the root apical meristem, was obtained (Werner *et al.* 2003; Dello Ioio *et al.* 2007). However, the mentioned authors did not measure the durations of mitotic cycles by direct methods, whereas we had measured them.

To elucidate the mechanism of CK action, researchers have recently used *Arabidopsis* mutants and transgenic lines in which the synthesis, breakdown and the signalling of CK are changed. Among them were a *ipt3ipt5ipt7* triple mutant of CK biosynthesis genes (Dello Ioio *et al.* 2007), conditional IPT (isopenthenyltransferase)-dependent CK overproduction lines (Kuderová *et al.* 2008), lines that overexpress different members of the cytokinin oxidase/dehydrogenase (*AtCKX*) genes (Werner *et al.* 2003), some mutants in CK signalling genes (Dello Ioio *et al.* 2007; Peng *et al.* 2013; Takahashi *et al.* 2013; Kinoshita *et al.* 2015) and others. Growth analyses at the cellular level in these lines in comparison with the wild type represent a powerful tool to uncover the mode of action of CK. However, in the mentioned studies the root growth

analysis at cellular level was incomplete. The main and now widely accepted conclusion (Skylar and Wu 2011) contradicts to that obtained by Beemster and Baskin (2000).

We reinvestigated this problem with the aim of understanding the action of CK on root growth at cellular level. Specifically, the root growth and cell proliferation of wild type *Arabidopsis* growing with and without TZ, as well as roots of *ipt3ipt5ipt7* triple mutant were studied. We found that CK slowed down root growth as a result of increased cell cycle duration and did not change the rate of transition of meristematic cells to differentiation.

Materials and methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. Wild-type (Columbia-0) seeds were received from Professor Ju Dolgikh (Institute of Plant Physiology, Russian Academy of Sciences, Moscow). Seeds of *ipt3ipt5ipt7* triple mutant were donated by Professor S Sabatini (Università La Sapienza, Rome, Italy). The seeds were sterilised in 5% solution of sodium hypochlorite and then washed three times in sterile distilled water, 5 min in each. Seeds were incubated in Petri dishes on a medium containing quarter- strength MS, 1% sucrose, 0.8% agar, and 0.5 g L^{-1} 2-(N-morpholino)ethanesulfonic acid (MES), pH, 5.8. To some Petri dishes TZ (0.02–1.0 μM) was added. Seeds were stratified at 4°C in the dark for 2 days. Then the seedlings were grown in vertically-maintained Petri dishes in a controlled climate chamber at light period of 16 h and temperature regime of 22/18°C (day/night).

Fixation of material and microscopy

Prior to root fixation, Petri dishes with the seedlings were scanned for root length measurement using an Epson Perfection V300 Photolight scanner (Epson) at a resolution of 600 dpi. The roots were fixed in 4% formalin in phosphate buffer (pH, 7.2) for 4 h at room temperature. Then the seedlings were transferred to 30% glycerol in 2% dimethylsulfoxide for 30 min at room temperature. Before examination under the microscope, the seedlings were cleared using a modified method by Dubrovsky *et al*. (2009) in which instead of NaI we used KI. Briefly, seedlings were placed into a clarifying solution (61 g of KI and 198 mg of $Na_2S_2O_3$ in 100 mL of 2% dimethylsulfoxide), with 35 mL of this solution mixed with 65 mL of glycerol. The seedlings were incubated in the clarifying solution for at least 1.5 h. For microscopy, roots were mounted in 50% glycerol on glass slides.

Measurement of root length, size of meristem and length of fully-elongated cells

The preparations were examined under an Imager D1 microscope (Carl Zeiss) equipped with Nomarski optics. The length of primary roots was measured using an IMAGE J program. The length of the main root was measured from the boundary of the cap and quiescent centre to the base of the root. The cells in the meristem and elongation zone of the primary root were counted using a Carl Zeiss AxioVision program. The upper boundary of the meristem was determined as the point, where cell lengths become obviously greater. Final length of elongated cells was measured on the micrographs by means of Carl Zeiss AxioVision software (application Length).

All measurements were taken along one longitudinal file of cortex.

Calculation of root cell growth parameters

The duration of the cell cycle (T, h) was calculated according to a formula proposed by Ivanov (Ivanov 1994; Ivanov and Dubrovsky 1997):

$$T = (\ln 2 \times N_m l)/V, \quad (1)$$

where N_m is the number of meristematic cells in a file, l is the final length of fully elongated cells and V is the rate of root growth. Applicability of this formula to the calculation of average duration of cell cycle in root meristems was shown in several works (Ivanov and Dubrovsky 1997). We have shown the root apical meristem comprises two domains: the proliferation domain (PD) and the transition domain (TD) (Ivanov and Dubrovsky 2013). In Eqn (1) N_m corresponds to the number of cells in the meristem portion, where cell proliferation is maintained, i.e. this is the PD. The number of cells in the PD was successfully used for estimation of cycle time (López-Bucio *et al*. 2014; Napsucialy-Mendivil *et al*. 2014). Our preliminary results showed that similar results can be obtained when all meristematic cells are included in N_m, as per Eqn (1). This is because the time cells spent in the TD is very short. So, here, as in the study of Rodriguez *et al*. (2015), for Eqn (1) we used data for N_m from the whole meristem.

Life-span of the cells in the meristem (T_{sp}, h) was calculated as:

$$T_{sp} = pT, \quad (2)$$

where T is the duration of one cell cycle (h) and p is the number of division cycles, through which an initial cell derivative at the proximal border of the quiescent centre will pass while in the root apical meristem: p may be calculated according to the formula by Filin and Ivanov (2016):

$$p = \ln(N_m - 1)/\ln 2. \quad (3)$$

The number of cells that completed elongation (N) was calculated according to the formula:

$$n = \Delta L/l, \quad (4)$$

where ΔL is an increment in root length and l is the final length of the elongated cells.

The number of cells in the cell file of cortex may be directly counted on a micrograph along a cortical cell file, from the quiescent centre boundary to the beginning of the elongation zone, or may be estimated if p is known (Ivanov 1994):

$$N_m = 2^{p+1} - 1, \quad (5)$$

where p is the number of division cycles.

The rate of cell production in a file (V_m, cells h^{-1}) was calculated according to the formula.

$$V_{m(1-2)} = N_{(1-2)} + (N_{m2} - N_{m1}) + (N_{e2} - N_{e1}), \quad (6)$$

where $N_{(1-2)}$ is the number of cells per file that completed elongation between the first and the second day. N_{m1} is the

number of meristematic cells in a file on the first day; N_{m2} is number of meristematic cells in a file on the second day; N_{e1} and N_{e2} are numbers of cells in a file in the elongation zone on the first and second day.

The rate of meristematic cell transition to elongation (V_{me}, cells h^{-1}) in a file was calculated according to the formula:

$$V_{me(1-2)} = V_{m(1-2)} + (N_{m2} - N_{m1}), \quad (7)$$

where $V_{me(1-2)}$ is the rate of cell production between the measurements (cells h^{-1}); N_{m1} is the number of meristematic cells on the first day; N_{m2} is the number of meristematic cells on the second day.

Statistics

All experiments were performed three times or more, 40 seeds per treatment. Data are presented as the average % of control and its standard error.

Results and discussion

CK inhibits the root growth

TZ slowed down root growth at concentrations exceeding 0.02 µM (Fig. 1). Roots of a *ipt3ipt5ipt7* triple mutant grew at a higher rate than the roots of wild-type plants (Fig. 1). Similar results were described for TZ (Beemster and Baskin 2000; Dello Ioio *et al.* 2007) and other CKs (Kato *et al.* 1995). Enhanced growth of *ipt3ipt5ipt7* roots was also reported (Dello Ioio *et al.* 2007; Kinoshita *et al.* 2015). In IPT-dependent CK overproduction lines, roots grew slower than those of wild type plants (Kuderová *et al.* 2008). Roots of transgenic plants that overexpress CKX genes and had an increased CK breakdown grew faster than those of the wild type (Werner *et al.* 2003).

Thus, an increase in CK level attained by exogenous application or overexpression of the bacterial IPT inhibits root growth. Accordingly, lower endogenous CK levels, resulting either from CKX overexpression or from the *ipt3ipt5ipt7* triple mutation of CK synthesis genes, showed enhanced root growth. All these results are consistent with the conclusion presented by Ferreira and Kieber (2005), that the endogenous CK concentration in the root is supraoptimal for growth. However, it may be optimal for other functions. Importantly, a certain concentration of CK is necessary for root growth because roots with triple mutations in CK signalling genes practically do not grow (Higuchi *et al.* 2004).

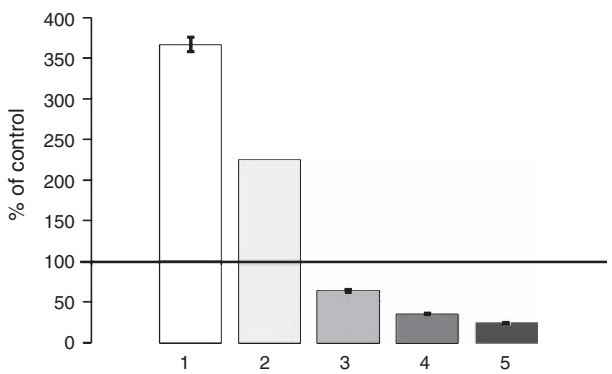

Fig. 1. Root growth rate on 5–7 days post germination (dpg). Data are presented as % of control, i.e. wild-type roots growing without TZ. 1 is roots of *ipt3ipt5ipt7* mutant; 2 is roots of CKX1 (data from Werner *et al.* 2003); 3 is TZ 0.02 µM; 4 is TZ 0.1 µM; and 5 is TZ 1.0 µM.

Scheme of root growth at the cellular level

We studied the effect of CK on root growth at the cellular level and, further, we first describe briefly how cell proliferation in the root meristem is organised, and then we will consider cell elongation and the methods of root growth analysis at the cellular level.

The growing part of root consists of two zones – the meristem, where cells divide, and the zone of elongation in which cells grow at a higher rate and reach their final length. In addition, in roots, a short transition zone can be identified before cells transition to elongation (Mancuso *et al.* 2005; Verbelen *et al.* 2006; Baluška *et al.* 2010; Kagenishi *et al.* 2016). After a cell is formed as a result of cell division at the proximal boundary of the quiescent centre, it divides only few times (p) (in *Arabidopsis* root $p < 5$; Ivanov and Dubrovsky 1997) and leaves the meristem. For roots of some species, it has been shown that the cell cycle duration is constant along the meristem (Ivanov 1994). In this case, the time between the cell formation at the proximal boundary of the quiescent centre and the cell transition to elongation, i.e. the life-span of a cell in the meristem (T_{sp}) equals T_p, where p is the number of cell cycles along the meristem and T is the cell cycle duration.

The number of meristematic cells in a file (N_m), T and T_{sp} parameters are related by the following equation:

$$N_m = 2^{T_{sp}/T_{+1}}, \quad (8)$$

since $N_m = 2^{p+1} - 1$ (Ivanov 1994).

Any factor that accelerates the cell transition to elongation will reduce T_{sp}. In the opposite case, T_{sp} will be longer. However, N_m depends on changes in T as well. The cell transition to elongation is the first sign of a beginning of cell differentiation. Therefore, transition to cell elongation and transition to cell differentiation are often used as synonyms.

Some days after germination, a root grows at a constant rate, and the size of the growing zone and the number of meristematic and elongating cells do not change with time since, per unit time, the same amount of cells divide and the same number of cells begin to elongation and reach their final length.

Growth rate (V) of a root growing at a constant rate is equal to:

$$V = V_m \times l, \quad (9)$$

where V_m is the rate of cell proliferation in one file (number of new cells produced per time unit), l – length of fully elongated cells. In roots growing at a constant rate, V does not depend on the relative growth rate (k_e).

The root growth rate equals $V = L_e \cdot \times k_e$, where L_e is the length of the elongation zone and k_e is the relative growth rate. However, in roots growing at a constant rate, L_e is inversely related to k_e. Therefore, the greater is k_e, the less is L_e.

During a time equal to one cell cycle duration, if N_m does not change, the proximal half of meristematic cells transits to elongation. However, since a fraction of the cells still have a

chance to divide at this proximal position, the number of meristematic cells transiting and beginning to elongate is close to 70% instead to 50% of meristematic cells (as discussed by Ivanov and Dubrovsky 1997).

The rate of cell proliferation depends on the cell cycle duration (T) and the number of meristematic cells in file (N_m). The cell cycle time (T) is calculated according to Eqn 1.

The clear organisation of the root at the cellular level permitted us to analyse the CK effects on root growth with the aid of simple measurements. To this end, we collected the following parameters: (1) root growth rate, (2) length of fully elongated cells, (3) number of meristematic cells in a file, (4) rates of cell proliferation and transition to elongation, (5) cell cycle duration, and (6) life-span of cells in the meristem.

Effect of CK on the length of fully-elongated cells

TZ reduced the length of fully-elongated cells by 20%, but only at 1 µM concentration (Fig. 2). Beemster and Baskin (2000) obtained similar results. According to our results and data presented by Beemster and Baskin (2000), TZ reduced the root growth rate to a much greater extent than the length of fully-elongated cells. Therefore, the growth was slower due also to a decrease in cell production.

In *ipt3ipt5ipt7* roots, the length of fully-elongated cells was the same as in wild type roots (Fig. 2). Other authors did not measure cells in these roots. Similar data were obtained in plants overexpressing CKX genes (Werner *et al.* 2003). Thus, a decreasing endogenous level of CK does not affect the final, fully-elongated cell length, and the enhanced root growth is caused by an increased number of mature cells. In this case, the rate of cell transition to elongation is greater than in wild type roots. If the number of mature cells in the root length increment (the portion of root formed during a certain time) decreases, as occurs in roots growing in TZ solution, the rate of cell transition to differentiation (elongation) must be lower than in untreated roots. Thus, contrary to the conclusion by Dello Ioio *et al.* (2007), CK did not stimulate the rate of the cell transition to differentiation, but reduced it. To clarify the mechanisms of the retardation of cell transition to elongation, it was necessary to measure CK effects on several meristematic cells in a file, the rates of cell proliferation and the transition to elongation, the duration of the mitotic cycles, and the life-span of cells in the meristem.

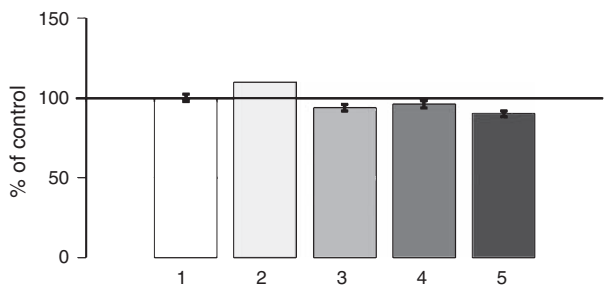

Fig. 2. Length of fully-elongated cells on 7 days post germination (dpg). Data are presented as % of control, i.e. wild-type roots growing without TZ. 1 is roots of *ipt3ipt5ipt7* mutant; 2 is roots of CKX1 (data from Werner *et al.* 2003); 3 is TZ 0.02 µM; 4 is TZ 0.1 µM; and 5 is TZ 1.0 µM.

Effect of CK on the number of meristematic cells in a file

TZ decreased N_m (Figs 3, 4). In *ipt3ipt5ipt7* mutant *roots*, N_m was higher than in wild-type roots (Fig. 4). Similar results were described for the effect of TZ by Beemster and Baskin (2000) and Dello Ioio *et al.* (2007), and, for mutant *ipt3ipt5ipt7* roots by Dello Ioio *et al.* (2007) and Takahashi *et al.* (2013). In roots of plants overexpressing *IPT* genes, in which the endogenous CK level was higher than in wild type roots, N_m was lower (Kuderová *et al.* 2008). However, in roots of transgenic plants overexpressing *CKX* genes, in which the endogenous CK level was lower than in wild-type roots, N_m was greater than in wild-type roots (Werner *et al.* 2003). These results are also presented in Fig. 4.

Thus, in roots growing at a higher rate, N_m is always greater than in roots growing at a lower rate. A decreased endogenous CK level results in a greater N_m and root growth rate; an increased endogenous CK level has opposite effects – root growth was slowed and N_m decreased. As growth rates of roots with reduced CK level were higher than in wild-type roots, but where the final cell length was similar in both roots, the number of cells which completed growth per time unit was greater than in wild-type roots. This effect could only result from higher rates of cell proliferation at a lower endogenous CK level, since N_m was also greater both in the wild type roots in roots treated with CK.

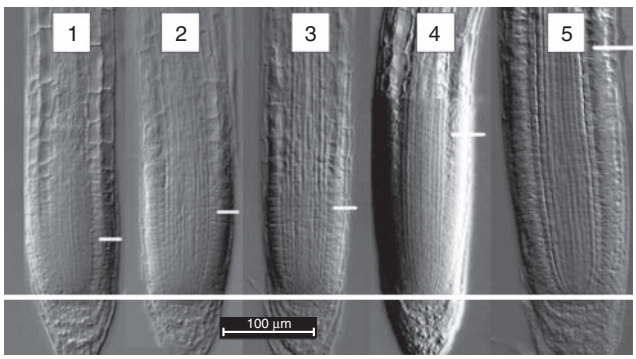

Fig. 3. Root apices on 7 days post germination (dpg). White lines show borders of meristems. 1 is TZ 1.0 µM; 2 is TZ 0.1 µM; 3 is TZ 0.02 µM; 4 is control; and 5 is roots of *ipt3ipt5ipt7* mutant.

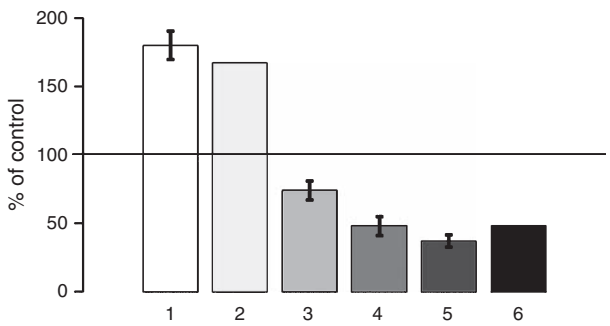

Fig. 4. Number of cells in one meristematic cortex file (N_m) on 7 days post germination (dpg). Data are presented as % of control, i.e. wild type roots growing without TZ. 1 is roots of *ipt3ipt5ipt7* mutant; 2 is roots of CKX1 (Data from Werner *et al.* 2003); 3 is TZ 0.02 µM; 4 is TZ 0.1 µM; 5 is TZ 1.0 µM; and 6 is roots of IPT (data from Kuderová *et al.* 2008).

Effect of CK on the rate of cell proliferation

TZ inhibited the cell proliferation (Fig. 5). In roots of triple *ipt3ipt5ipt7* mutant, cell proliferation rate was higher than in wild-type roots (Fig. 5). Between the 5th and 7th days, N_m did not change. Therefore, the rates of cell proliferation and cell transition to elongation in this period were equal (Fig. 5). These rates were higher in *ipt3ipt5ipt7* roots than in wild-type roots and lower in TZ-treated roots compared with untreated roots.

The decreased N_m in TZ-treated roots was the result of stronger inhibition of cell proliferation rather than a decrease in the rate of cell transition to elongation. Thus, TZ does not stimulate the transition to cell differentiation but, rather, it inhibits it.

Effects of CK on the duration of mitotic cycles

The rate of cell production depends on the number of meristematic cells and the cell cycle duration (T). TZ (0.02 - 1 μM) prolonged T in wild type roots (Fig. 6). Similar data were obtained by Beemster and Baskin (2000). In root treated with 1.0 μM TZ, T increased from 16.9 h (in untreated control) to 24.8 h. In *ipt3ipt5ipt7* mutant roots, T was shorter than in wild-type roots (Fig. 6). However, Dello Ioio *et al*. (2007) did not find differences in T in wild-type roots growing with and without TZ, nor in roots of triple *ipt3ipt5ipt7* mutants. They 'visualised root meristem cells in the G2-M phase in untreated and cytokinin-treated plants harbouring the *D-Box* CYCB1:: GUS construct (Colon-Carmona *et al.* 1999). The percentage of GUS-stained cells in the meristem was the same in cytokinin-treated and untreated roots, suggesting overall division rate. However, such an approach does not always allow us to compare durations of cell cycle between different plants. The durations of the complete cycle and of G_2 and M phases can change simultaneously. Then, the ratio of (G_2 + M) to T will be the same and, hence, does not permit us to estimate whether T changed or not. The duration of the mitotic cycles and their periods were usually determined by a thymidine-labelling method (Grif *et al.* 2002). Data in Fig. 7 show that the dependence of (G_2 + M)/T on T is ambiguous. Unfortunately, no other data on cell cycle duration *ipt3ipt5ipt7* in roots are available.

Federici *et al.* (2012) showed that TZ (0.1 μM) decelerated the growth rate of meristematic cells of *Arabidopsis* root. In this case, the duration of the mitotic cycle should be greater.

Thus, an increased CK concentration results in an increase in cell cycle duration and, hence, an inhibition of cell proliferation,

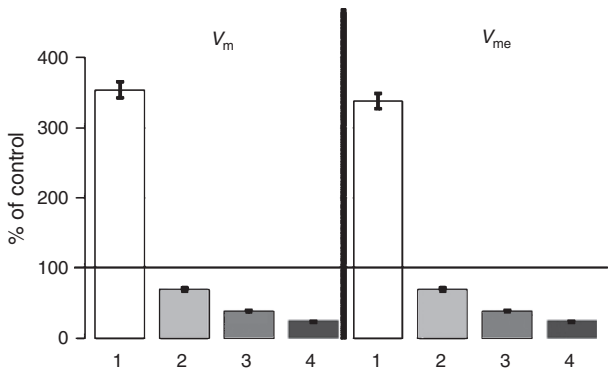

Fig. 5. Rates of cell proliferation (V_m) and cell transition to elongation (V_{me}) on 5 – 7 dpg. Data are presented as % of control, i.e. wild type roots growing without TZ. 1 is roots of *ipt3ipt5ipt7* mutant; 2 is TZ 0.02 μM; 3 is TZ 0.1 μM; and 4 is TZ 1.0 μM.

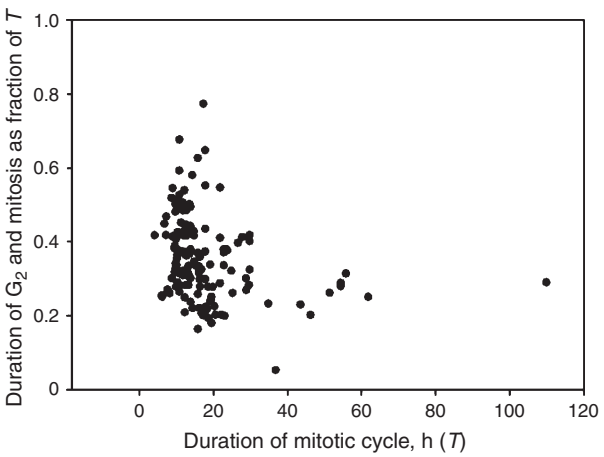

Fig. 7. Ratio between durations of period G_2 + mitosis to whole mitotic cycle (T) as a function of T.

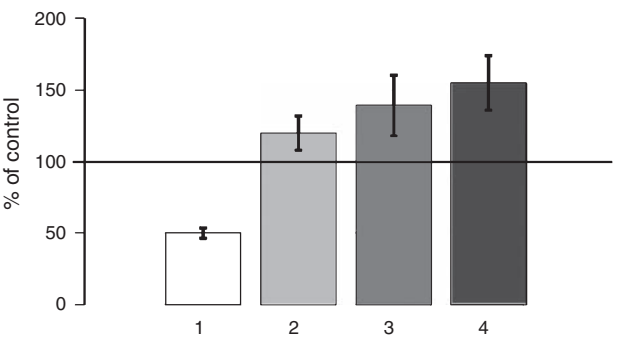

Fig. 6. Duration of mitotic cycles (T) on 5–7 days post germination (dpg). Data are presented as % of control, i.e. wild type roots growing without TZ. 1is roots of *ipt3ipt5ipt7* mutant; 2 is TZ 0.02 μM; 3 is TZ 0.1 μM; and 4 is TZ 1.0 μM.

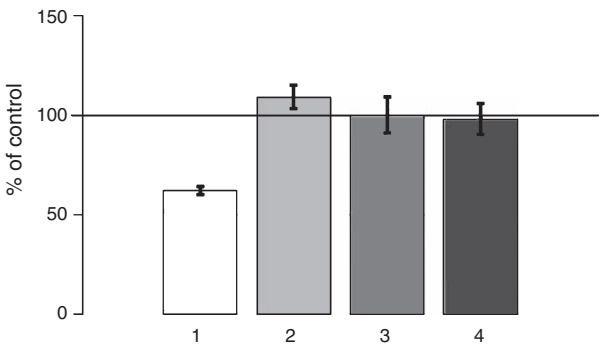

Fig. 8. Time-span of cell in meristem (T_{sp}) on 5–7 days post germination (dpg). Data are presented as % of control, i.e. wild type roots growing without TZ. 1 is roots of *ipt3ipt5ipt7* mutant; 2 is TZ 0.02 μM; 3 is TZ 0.1 μM; and 4 is TZ 1 μM.

whereas a decreased CK concentration will shorten mitotic cycle duration. The mechanism of CK effect on the cell cycle time is still unclear. Often, CK positively regulates cell divisions (Schaller *et al*. 2014). It is possible that a CK-dependent lengthening of the cell division cycle in roots results f not only from direct effect of CK on dividing cells but also from its effect on the transport of IAA within the root meristem. (Benková and Hejátko 2009; Šimášková *et al*. 2015). Other IAA-dependent processes could be involved in regulation of root growth and cell proliferation (Blilou *et al.* 2005). It is possible that a change in IAA concentration can prolong cell divisions.

Effects of CK on life-span of cells in meristem

Beemster and Baskin (2000) showed that 1.0 μM TZ treatment resulted in both a decreased N_m and root growth rate, but *T was* prolonged whereas the life-span of meristem cells (T_{sp}) remained constant. We confirmed this result and showed that in roots of *ipt3ipt5ipt7* mutant, T_{sp} was even shorter (Fig. 8).

Ivanov (1994) suggested that the rate of cell transition to elongation depends on two groups of processes. The first group of processes determines when cells located at various distances from the root tip should reach the proximal boundary of the meristem, i.e. time equal to the life-span of cells in the meristem. The second group determines how many times a cell will divide within the meristem. The analysis of various factor effects showed that the life-span of cell within the meristem is a very stable parameter which rarely changes, for example, in roots treated with 2,4-D (Filin and Ivanov 2016). In contrast, the rate of cell proliferation is very sensitive to several factors, and CK is one of them.

To conclude, our data show that CK slows down root growth and consequently affect the rate of cell transition to elongation due to the inhibition of cell proliferation, but CK seems not to change T_{sp}. For this reason, N_m decreases. With reference to the control roots, CK did not appear to accelerate the cell transition to elongation (differentiation).

Our study clearly shows that a detailed cellular analysis of root growth helps to understand what processes are the main targets of a factor under study. CK-mediated changes in cell proliferation represent an important mechanism of root growth modulation in plants.

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