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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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Functional Plant Biology is abstracted/indexed by: ABOA/Streamline, Agricola, Biological Abstracts, Biology and Environmental Sciences, BIOSIS, CAB Abstracts, Chemical Abstracts, Current Contents (Agriculture, Biology & Environmental Sciences), Current Contents (Life Sciences), Elsevier BIOBASE/Current Awareness in Biological Sciences, Reference Update.

ISSN 1445-4408 eISSN 1445-4416 Issued twelve times per year Journal Compilation © CSIRO 2018

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Functional Plant Biology publishes papers that contain new and significant information about plant functions and their regulation, especially **HIGH IMPACT FACTOR** in relation to changing environments. FPB 2.121 encourages papers on emerging concepts and new tools in plant biology, and studies on the following functional areas encompassing work from the molecular through whole plant to community scale.

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# **Functional Plant Biology**

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# Special Issue: Plant Signalling: From Molecules to Behaviour

| <i>Review</i> : Unravelling the plant signalling machinery:<br>an update on the cellular and genetic basis of plant<br>signal transduction<br><i>Vadim Demidchik, Frans Maathuis</i><br><i>and Olga Voitsekhovskaja</i>              | 1–8   | Signalling is a central phenomenon in biology. It is crucial to all<br>aspects of plant physiology including growth, development and<br>interactions with the environment. Here, novel hypotheses and<br>experimental data regarding signalling hubs, second messengers,<br>programmed cell death and autophagy are presented from the<br>Fourth Plant Signalling and Behaviour Symposium in Saint<br>Petersburg, Russia, June 2016).   |
|--|-------|---|
| <i>Review</i> : Mechanisms of cytosolic calcium elevation<br>in plants: the role of ion channels, calcium extrusion<br>systems and NADPH oxidase-mediated<br>'ROS-Ca <sup>2+</sup> Hub'<br><i>Vadim Demidchik and Sergey Shabala</i> | 9–27  | Transient elevation of cytosolic $Ca^{2+}$ , also referred to as a $Ca^{2+}$ signal, is a central phenomenon of plant signalling. Plants evolved sophisticated systems to initiate, amplify and terminate $Ca^{2+}$ signals. Structure and properties of these systems, including $Ca^{2+}$ -permeable ion channels, $Ca^{2+}$ -ATPases, $Ca^{2+}/H^{+}$ exchangers and 'ROS-Ca <sup>2+</sup> hub' are discussed here. They provide a fine-tuned mechanism for encoding diverse external and internal stimuli.  |
| <i>Review</i> : The role of ion disequilibrium in induction<br>of root cell death and autophagy by environmental<br>stresses<br><i>Vadim Demidchik, Elena V. Tyutereva</i><br><i>and Olga V. Voitsekhovskaja</i>                     | 28-46 | Environmental stresses are main causes for low agricultural<br>productivity. At the cellular level, stresses induce generation of<br>reactive oxygen species (ROS), ion disequilibrium, autophagy<br>and programmed cell death (PCD). Here we propose that these<br>processes interact and that ROS and ion disequilibrium are<br>triggers of autophagy and PCD. Overall, presented data<br>contribute to understanding plant stress physiology.  |
| Review: Cell differentiation in nitrogen-fixing nodules<br>hosting symbiosomes<br>Anna V. Tsyganova, Anna B. Kitaeva<br>and Viktor E. Tsyganov   | 47–57 | Rhizobium bacteria, which live within the root nodules of<br>legumes, allow plants to capture nitrogen gas from the<br>atmosphere and use it for their own growth. Central to this<br>symbiosis is an intracellular structure, called the symbiosome, in<br>which nitrogen-fixing bacterial cells exchange components with<br>the host cells that harbor them. Recent research on the<br>differentiation of symbiosomes and of the infected cells that<br>accommodate them has helped to decipher some general<br>molecular mechanisms of cell differentiation. |

*Cover illustration*: Panorama of Peter and Paul Fortress, City of St. Petersburg, Russia. This Special Issue contains selected peer reviewed papers from the Fourth International Symposium on Plant Signalling and Behavior, Komarov Botanical Institute RAS/Russian Science Foundation, Saint Petersburg, Russia, 19–23 June 2016. Image by Gregory A. Pozhvanov, https://pozhvanov.com.

| Review: Melatonin in plant signalling and behaviour<br>Lauren A. E. Erland, Praveen K. Saxena<br>and Susan J. Murch  | 58–69   | Melatonin is an important hormone and signalling molecule in<br>all forms of life including humans, plants and bacteria. Recent<br>plant physiology and genomic experiments have described the<br>redirection of plant growth and metabolism, and demonstrated a<br>diversity of genes involved in response to melatonin, however,<br>the exact metabolic cascades that translate melatonin signals into<br>physiological responses is not fully understood. This review<br>provides an overview of melatonin mediated signalling<br>manifested as behaviours and its roles in basic and industrial<br>research. |
|--|---------|--|
| <i>Review</i> : Molecular mechanisms accompanying nitric oxide signalling through tyrosine nitration and S-nitrosylation of proteins in plants <i>Prachi Jain and Satish C. Bhatla</i>   | 70–82   | Understanding the molecular mechanisms of plant development<br>constitutes an important field of investigations in the current era<br>of plant biology research. Nitric oxide signalling regulates a<br>variety of biochemical processes in plants. This review provides<br>an in-depth analysis of our current understanding on the subject,<br>particularly with reference to plant growth under stress<br>conditions.   |
| <i>Review</i> : Two-pore cation (TPC) channel: not<br>a shorthanded one<br><i>Igor Pottosin and Oxana Dobrovinskaya</i>  | 83–92   | Large conductance SV/TPC1 channels are ubiquitously and<br>abundantly expressed in the vacuolar membranes of higher<br>plants. They are unique established Ca <sup>2+</sup> -permeable channels in<br>vacuoles, but their activity is strongly negatively controlled, so<br>that they were believed to be inactive or to act only locally.<br>Recent evidence suggests the key role of SV/TPC1 channels in<br>the long-distance Ca <sup>2+</sup> signalling.   |
| <i>Review</i> : cGMP signalling in plants: from enigma<br>to main stream<br><i>Jean-Charles Isner and Frans J. M. Maathuis</i>   | 93–101  | Cyclic GMP (cGMP) signalling in plants is crucial for many<br>physiological processes. Recent analytical and genomic<br>developments now allow detailed studies into the biochemistry<br>and physiological role of cGMP in plants, and the latest findings<br>are reviewed in this article.  |
| <i>Review</i> : Formation mechanisms of superoxide radical<br>and hydrogen peroxide in chloroplasts, and factors<br>determining the signalling by hydrogen peroxide<br><i>Boris N. Ivanov, Maria M. Borisova-Mubarakshina</i><br><i>and Marina A. Kozuleva</i> | 102–110 | Photosynthetic electron transport chain is not the only source of<br>ATP and NADPH for photosynthesis; it is a sensor, informing<br>adaptation systems of plant about environmental changes. An<br>important transmitter of this information is hydrogen peroxide<br>whose mechanisms of formation are presented, laying special<br>emphasis on the formation outside and within thylakoid<br>membrane. It is discussed, that the formation place can ensure<br>definite signal about the specific environmental change.   |
| <i>Review</i> : Plant ion channels and transporters<br>in herbivory-induced signalling<br><i>Shuitian Luo, Xiao Zhang, Jinfei Wang,</i><br><i>Chunyang Jiao, Yingying Chen and Yingbai Shen</i>  | 111–131 | Clarifying herbivory-induced plant cellular signalling is a critical step to push the research of plant-herbivore interaction forward. We review the role of ion channels/transporters in modulating herbivory-induced early signalling events and rapid systemic signal transmission in plants. This work provides a comprehensive source of information about plant defensive strategies upon attack.  |
| Viewpoint: Electrical signalling in Nitellopsis obtusa:<br>potential biomarkers of biologically active compounds<br>Vilma Kisnieriene, Indre Lapeikaite<br>and Vilmantas Pupkis  | 132–142 | The electrophysiological response pattern of <i>Nitellopsis obtusa</i> cell can be assessed to evaluate the effect of many biologically active compounds. We illustrate a variety of electrophysiological approaches for the investigations of electrical signaling after chemical treatment <i>in vivo</i> . The insights about the Characean model system are likely to hold for plants in general and even deepen the understanding of the plant evolution.   |

| Rapid changes in root HvPIP2;2 aquaporins abundanceand ABA concentration are required to enhance roothydraulic conductivity and maintain leaf waterpotential in response to increased evaporative demandDmitry S. Veselov, Guzel V. Sharipova,Stanislav Yu. Veselov, Ian C. Dodd, Igor Ivanovand Guzel R. Kudoyarova143–149                          | The ABA-deficient barley mutant Az34 and wild type (WT) were exposed to air warming. Although transpiration rate of both genotypes increased, leaf water potential decreased in the mutant but was maintained in WT plants. Only WT plants showed increased root ABA accumulation, which increased root hydraulic conductivity and aquaporin abundance, which seems important in maintaining leaf hydration.   |
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| Two native types of phytochrome A, phyA' and phyA",<br>differ by the state of phosphorylation at the <i>N</i> -terminus as<br>revealed by fluorescence investigations of the Ser/Ala<br>mutant of rice phyA expressed in transgenic <i>Arabidopsis</i><br><i>Vitaly A. Sineshchekov, Larissa A. Koppel</i><br><i>and Cordelia Bolle</i> 150–159      | Plants adapt to environmental light conditions with the use of the sophisticated phytochrome system. In this work, polymorphism of its major component – phytochrome A– was investigated. With the use of transgenic <i>Arabidopsis</i> and fluorescence technique, it was shown that two molecular types of the photoreceptor differ by the state of phosphorylation and their existence accounts for its complex functions.  |
| Parameters of electrical signals and photosynthetic<br>responses induced by them in pea seedlings depend<br>on the nature of stimulus<br><i>Vladimir Vodeneev, Maxim Mudrilov,</i><br><i>Elena Akinchits, Irina Balalaeva</i><br><i>and Vladimir Sukhov</i> 160–170  | Plants, like animals, produce electrical signals in response to<br>various external influences. In this study we raised a question<br>whether the electrical signals transmit information about the<br>nature of the stimulus, and found out that different stimuli induce<br>signals of varied parameters. The obtained results explain how<br>plants adapt to changing environment.  |
| Arabidopsis thaliana phytaspase: identification<br>and peculiar properties<br>Nina V. Chichkova, Raisa A. Galiullina,<br>Larisa V. Mochalova, Svetlana V. Trusova,<br>Zulfazli M. Sobri, Patrick Gallois<br>and Andrey B. Vartapetian 171–179  | Although plant proteases of the phytaspase family are important<br>contributors to stress-induced plant cell death, phytaspase of a<br>classical model plant <i>Arabidopsis thaliana</i> has escaped<br>identification thus far. We identified the <i>Arabidopsis</i> phytaspase-<br>encoding gene and characterised the recombinant enzyme.<br>Substrate specificity and properties of the <i>Arabidopsis</i> phytaspase<br>display both important similarities with and distinctions from the<br>already characterised phytaspases.  |
| Spatial distribution of organelles in leaf cells and<br>soybean root nodules revealed by focused ion<br>beam-scanning electron microscopy<br><i>Brandon C. Reagan, Paul JY. Kim, Preston D. Perry,</i><br><i>John R. Dunlap and Tessa M. Burch-Smith</i> 180–191   | Focussed ion bean scanning electron microscopy (FIB-SEM) is<br>a technique that can be used to generate 3D renderings of cells<br>and their contents. Although FIB-SEM has been regularly used<br>to investigate animal cells and tissues, it has rarely been<br>deployed to study plant structures. Here we demonstrate that<br>FIB-SEM can easily be used to study plant samples and have<br>discovered previously unknown arrangements of organelles and<br>membranes in those samples.   |
| Studies of cytokinin receptor–phosphotransmitter<br>interaction provide evidences for the initiation<br>of cytokinin signalling in the endoplasmic reticulum<br><i>Sergey N. Lomin, Yulia A. Myakushina,</i><br><i>Dmitry V. Arkhipov, Olga G. Leonova,</i><br><i>Vladimir I. Popenko, Thomas Schmülling</i><br><i>and Georgy A. Romanov</i> 192–202 | Cytokinin is an important plant hormone and its mode of action<br>has been extensively studied; however, to date, the subcellular<br>localisation of cytokinin perception and signal transduction<br>remains a matter of debate. This study describes cytokinin<br>receptor–phosphotransmitter interaction and its subcellular<br>localisation in living plant cells and it provides several<br>experimental evidences for receptor activity at the endoplasmic<br>reticulum (ER) membrane. It is concluded that intracellular<br>cytokinins within the ER lumen may play an important role in<br>cytokinin signalling, at least in some cell types. |

| Phloem fibres as motors of gravitropic behaviour   of flax plants: level of transcriptome   Oleg Gorshkov, Natalia Mokshina, Nadezda Ibragimov   Marina Ageeva, Natalia Gogoleva   and Tatyana Gorshkova 2   | v <b>a,</b><br>203–214 | Plant fibres with a tertiary cell wall (G-layer) may function as<br>plant 'muscles'. Large-scale transcriptome profiling of isolated<br>flax phloem fibres permitted to identify the major players and<br>regulatory elements that operate during graviresponce<br>specifically in the fibres of the pulling stem side. The suggested<br>mechanisms of phloem fibre involvement in tropisms may<br>considerably renew the concept of herbaceous plant behaviour<br>upon gravistimulation.   |
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| Cytokinins regulate root growth through its action on<br>meristematic cell proliferation but not on the transition<br>to differentiation<br><i>Victor B. Ivanov and Alexey N. Filin</i> 2  | 215–221                | Root growth is controlled by phytohormones, but what cellular<br>processes are regulated and how it occurs is still an open<br>question. Here it is shown that cytokinin affects root growth<br>mainly through its effect on cell proliferation, and does not<br>initiate the transition of cells to differentiation as previously<br>thought. Cellular analysis performed could be applied for the<br>analysis of how any plant hormone influences developmental<br>processes in plant roots.  |
| Sunpatiens compact hot coral: memristors in flowers   Alexander G. Volkov and Eunice K. Nyasani  | 222–227                | Memristors, or resistors with memory, exist <i>in vivo</i> as components of plasma membranes in plants, fruits, roots and seeds. Authors found memristors in an androecium, spur, petals and pedicel in Sunpatiens flowers. The discovery of memristors in Sunpatiens ( <i>Impatiens</i> spp.) creates a new direction in the modelling and understanding of electrophysiological phenomena and memory elements in flowers.   |
| Photochemical activity changes accompanying the embryogenesis of pea ( <i>Pisum sativum</i> ) with yellow and green cotyledons   Galina Smolikova, Vladimir Kreslavski,   Olga Shiroglazova, Tatiana Bilova, Elena Sharova,   Andrej Frolov and Sergei Medvedev 2  | 228–235                | We studied the dynamics of photochemical activity in seed coats<br>and cotyledons during development of yellow- and green-seeded<br>pea cultivars by using the pulse amplitude modulation<br>fluorometric analysis. The fast transients of the chlorophyll <i>a</i><br>fluorescence revealed higher photochemical activity in the coats<br>of yellow-seeded cultivar at the early- and middle cotyledon<br>stages of seed development in comparison to those observed in<br>the green-seeded ones. Photochemical activity in the cotyledons<br>of both cultivars could not be any more detected at the late<br>cotyledon stage. This process was triggered by dehydration of<br>seed tissues. |
| Cyclosis-mediated long distance communications<br>of chloroplasts in giant cells of Characeae<br><i>Anna V. Komarova, Vladimir S. Sukhov</i><br><i>and Alexander A. Bulychev</i> 2   | 236–246                | Intracellular communications in plant cells of large dimensions<br>rely primarily on cytoplasmic streaming, because diffusion is too<br>slow for the transport on mm-scale distances. Illumination of a<br>small cell spot at various distances from the point of chlorophyll<br>fluorescence measurements revealed the wave-like propagation<br>of the fluorescence response along the cell length. The results<br>show that the photosynthetic function of immobile chloroplasts<br>under constant light can be affected by long-distance<br>transmission of a photosynthetically active metabolite from the<br>remote cell parts.  |
| The levels of peroxisomal catalase protein and activity<br>modulate the onset of cell death in tobacco BY-2 cells<br>via reactive oxygen species levels and autophagy<br><i>Elena V. Tyutereva, Ksenia S. Dobryakova, Andreas</i><br><i>Schiermeyer, Maria F. Shishova, Katharina Pawlowsk</i><br><i>Vadim Demidchik, Sigrun Reumann</i><br>and Olga V. Voitsekhovskaja2 | <b>i,</b><br>247–258   | Peroxisomes balance the cellular levels of reactive oxygen<br>species (ROS) and therefore should modulate ROS-regulated<br>programs like autophagy or cell death. We used tobacco<br>suspension cultures to show that degradation of peroxisomes via<br>autophagy was a prerequisite for cell death and depended on the<br>levels of the major peroxisomal protein, catalase. This suggests a<br>role of plant catalase in the regulation of peroxisome turnover<br>and autophagic cell death.  |

| Myotubularins, PtdIns5P, and ROS in ABA-mediated<br>stomatal movements in dehydrated <i>Arabidopsis</i><br>seedlings<br><i>Akanksha Nagpal, Ammar Hassan,</i><br><i>Ivan Ndamukong, Zoya Avramova</i><br><i>and František Baluška</i> | 259–266 | <i>Arabidopsis</i> myotubularins AtMTM1 and AtMTM2 control<br>stomata movements via reactive oxygen species (ROS)<br>homeostasis under drought stress. Acting as a secondary<br>messenger in the ABA-induced ROS production in guard cells,<br>PtdIns5P emerges as an evolutionarily conserved signalling<br>molecule downstream of AtMTMs calibrating cellular ROS<br>levels under stress. AtMTM1 and AtMTM2 activities balance<br>ABA-induced ROS and cellular homeostasis under dehydration<br>stress.       |
|---|---------|---|
| Molecular insights into the functional role of nitric<br>oxide (NO) as a signal for plant responses in chickpea<br><i>Parankusam Santisree, Pooja Bhatnagar-Mathur</i><br><i>and Kiran K. Sharma</i>                                  | 267–283 | Although many studies established nitric oxide (NO) as a signaling molecule in plants, the identification of target molecules of NO has remained elusive due to the lack of in depth molecular studies. Our quantitative proteome analysis suggests the differential regulation of 248 proteins and dynamic regulation of metabolic pathways by exogenous NO donor in chickpea. This is the first report in legumes pointing at the potential candidates that attribute the reported functions of NO in plants. |
| Endoplasmic reticulum stress regulates glutathione<br>metabolism and activities of glutathione related<br>enzymes in <i>Arabidopsis</i><br><i>Baris Uzilday, Rengin Ozgur, A. Hediye Sekmen</i><br><i>and Ismail Turkan</i>           | 284–296 | Prolonged endoplasmic reticulum (ER) stress oxidises the cellular glutathione pool. To elucidate the role of glutathione during ER stress, biosynthesis and degradation of glutathione and activities of related enzymes were evaluated. Our data demonstrated that glutathione biosynthesis and an apoplastic but not cytoplasmic catabolic pathway was induced. In addition, the activities of enzymes that use glutathione as a substrate were increased by ER stress.                                       |

Functional Plant Biology http://dx.doi.org/10.1071/FP16340

# 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 lifespan 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.

Received 30 September 2016, accepted 1 February 2017, published online 28 March 2017

# 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<sup>-1</sup> 2-(Nmorpholino)ethanesulfonic acid (MES), pH, 5.8. To some Petri dishes TZ (0.02–1.0  $\mu$ M) 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<sub>2</sub>S<sub>2</sub>O<sub>3</sub> 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):

$$T = (\ln 2 \times N_{\rm m} l) / V, \qquad (1)$$

where  $N_{\rm m}$  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)  $N_{\rm m}$  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  $N_{\rm m}$ , 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  $N_{\rm m}$  from the whole meristem.

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

$$T_{\rm sp} = pT, \tag{2}$$

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):

$$p = \ln(N_{\rm m} - 1)/\ln 2.$$
 (3)

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

$$n = \Delta L/l, \tag{4}$$

where  $\Delta L$  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):

$$N_{\rm m} = 2^{\rm p+1} - 1, \tag{5}$$

where *p* is the number of division cycles.

The rate of cell production in a file  $(V_{\rm m}, \text{ cells h}^{-1})$  was calculated according to the formula.

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

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:

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

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  $0.02 \,\mu$ M (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



**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  $\mu$ M; 4 is TZ 0.1  $\mu$ M; and 5 is TZ 1.0  $\mu$ M.

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).

# 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  $(T_{\rm sp})$  equals  $T_{\rm p}$ , 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  $(N_{\rm m})$ , T and  $T_{\rm sp}$  parameters are related by the following equation:

$$N_{\rm m} = 2^{T_{\rm sp}/T_{+1}},\tag{8}$$

since  $N_{\rm m} = 2^{p+1} - 1$  (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:

$$V = V_m \times l, \tag{9}$$

where  $V_{\rm m}$  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 ( $k_{\rm e}$ ).

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  $N_{\rm m}$  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  $\mu$ M 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  $0.02 \,\mu$ M; 4 is TZ  $0.1 \,\mu$ M; and 5 is TZ  $1.0 \,\mu$ M.

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

TZ decreased  $N_{\rm m}$  (Figs 3, 4). In *ipt3ipt5ipt7* mutant *roots*,  $N_{\rm m}$  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,  $N_{\rm m}$  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,  $N_{\rm m}$  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,  $N_{\rm m}$  is always greater than in roots growing at a lower rate. A decreased endogenous CK level results in a greater  $N_{\rm m}$  and root growth rate; an increased endogenous CK level has opposite effects – root growth was slowed and  $N_{\rm m}$  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  $N_{\rm m}$  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  $\mu$ M; 2 is TZ 0.1  $\mu$ M; 3 is TZ 0.02  $\mu$ M; 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  $\mu$ M; 4 is TZ 0.1  $\mu$ M; 5 is TZ 1.0  $\mu$ M; 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,  $N_{\rm m}$  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  $N_{\rm m}$  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  $\mu$ M) prolonged *T* in wild type roots (Fig. 6). Similar data were obtained by Beemster and Baskin (2000). In root treated with 1.0  $\mu$ M 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



**Fig. 5.** Rates of cell proliferation ( $V_{\rm m}$ ) and cell transition to elongation ( $V_{\rm me}$ ) 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  $\mu$ M; 3 is TZ 0.1  $\mu$ M; and 4 is TZ 1.0  $\mu$ M.



**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. lis roots of *ipt3ipt5ipt7* mutant; 2 is TZ 0.02  $\mu$ M; 3 is TZ 0.1  $\mu$ M; and 4 is TZ 1.0  $\mu$ M.

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 cytokinintreated 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<sub>2</sub> 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  $\mu$ M) 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. 7.** Ratio between durations of period  $G_2$ +mitosis to whole mitotic cycle (*T*) as a function of *T*.



**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  $\mu$ M; 3 is TZ 0.1  $\mu$ M; and 4 is TZ 1  $\mu$ M.

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  $1.0 \,\mu\text{M}$  TZ treatment resulted in both a decreased  $N_{\rm m}$  and root growth rate, but *T* was prolonged whereas the life-span of meristem cells ( $T_{\rm sp}$ ) remained constant. We confirmed this result and showed that in roots of *ipt3ipt5ipt7* mutant,  $T_{\rm sp}$  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  $T_{\rm sp}$ . For this reason,  $N_{\rm m}$  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.

# Acknowledgements

We thank Professor S Sabatini (Università La Sapienza, Rome, Italy) for seeds of *ipt3ipt5ipt7* mutant of *Arabidopsis* and Professor Ju Dolgikh for *Arabidopsis* seeds of wild type. We thank P Barlow, J Dubrovsky, N Obroucheva, V Mironova, S Vasetsky and V Volkov for useful discussion and help. This work was partially supported by Grant of RFBR no. 15-04-02502. This paper is dedicated to the memory of Professor. Peter W. Barlow.

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