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EMBRYO CULTURE

Changes in distribution of zeatin and indole-3-acetic acid in cells during callus induction and organogenesis *in vitro* in immature embryo culture of wheat

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Abstract Plant organogenesis remains one of the most essential questions of plant developmental biology. Callus tissue *in vitro* is a valuable tool for the studies on hormonal aspects of plant organogenesis, especially its early events, and immunohistochemical analysis is one of the few approaches offering information on the localization and role of hormones during organ development. The localization of endogenous zeatin and indole-3-acetic acid was investigated during simultaneous bud and root formation in calluses derived from immature embryos of wheat (*Triticum aestivum* L.). Calluses were induced on Murashige and Skoog (MS) medium supplemented with 2.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid. To stimulate simultaneous bud and root formation, calluses were transferred onto MS medium supplemented with 0.2 mg L^{-1} kinetin and 0.2 mg L^{-1} indoleacetic acid. Strong immunostaining for both hormones was detected in proliferating callus tissue, in developing meristematic centers and meristematic zones (whose cells were shown to be involved in organ formation), and at the sites of shoot and root apex initiation. During further development, shoot apexes with leaf primordia were heavily immunostained for zeatin, while immunostaining for indole-3-acetic acid was more intense at the sites of leaf primordia initiation and incipient primordia themselves. In the developing roots, immunostaining for both hormones reached a maximum in the root apex and gradually declined with increasing distance from the apex. Cells of developing procambial strands were also strongly stained for both zeatin and indole-3-acetic acid. These data suggest considerable similarity between patterns of hormone distribution in organs *in vitro* and *in vivo*. Thus, callus culture is a convenient and useful model for the study of fundamental biological questions such as how hormones regulate development.

Keywords Callus · Organogenesis *in vitro* · Immunolocalization · Cytokinins · Auxins · *Triticum aestivum* L.

Introduction

An important goal in plant biology is to unravel the mechanisms controlling plant morphogenesis, and the use of cell culture is an important approach to achieve this objective. Addition of different combinations of plant hormones into the culture medium has been shown to be the main factor regulating the process of morphogenesis *in vitro*, including organ formation (Krikorian 1995 and references therein). The concept that hormones play key roles in the control of plant development originated more than 50 years ago from experiments of Skoog and Miller (1957), who showed that the ratio of cytokinin to auxin in nutrient media influences morphogenesis in plant tissue culture.

Despite the great importance of results obtained *in vitro*, it has become apparent that knowledge of the concentrations of hormones added to nutrient medium may not be sufficient to ascertain their levels in plant cells and ignoring these limitations may lead to faulty conclusions. For example, cytokinin-activated auxin uptake resulted in greater accumulation of auxin by cells after their cultivation on a medium with an increase in the concentration of cytokinin but not of auxin itself (Kakani *et al.* 2009), and cytokinins have been shown to modulate auxin-induced organogenesis *via* regulation of

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efflux-dependent intercellular auxin distribution (Pernisova *et al*. 2009). Moreover, recent studies of the expression of genes controlling hormone synthesis showed expression of genes responsible for cytokinin (Cheng *et al*. 2013) and auxin (Bai *et al*. 2013) synthesis in cells cultivated *in vitro*, while auxin-induced organogenesis was accompanied by endogenous cytokinin production (Pernisova *et al*. 2009). Auxin synthesis, in turn, was decreased by a low level of cytokinins *in vivo* (Jones *et al*. 2010). It is obvious from these observations that the concentration of one hormone added to the nutrient medium may influence morphogenesis indirectly by inducing changes in the content of other hormones inside the cultivated cells. Thus, it is impossible to unravel the mechanisms of hormonal control of plant morphogenesis without identifying the location of the phytohormones and the qualitative assessment of their relative amounts, which is difficult to predict but necessary to study.

The most popular approach for revealing hormonal level in cells utilizes transgenic plants transformed with reporter genes (coding for proteins whose expression can be visualized) placed under control of a hormone-sensitive promoter (*e.g.*, the cytokinin-sensitive *ARR5* promoter or auxin-sensitive *DR5* promoter). This approach allowed detection of auxin and cytokinin levels in callus cells during the process of embryogenesis and organogenesis (Gordon *et al*. 2007; Pernisova *et al*. 2009; Cheng *et al*. 2013). It is not easy to use reporter constructs in the case of wheat because of the difficulty of wheat transformation, although due to the importance of this crop plant, the study of its morphogenesis is of great interest.

An alternative to the use of reporter constructs for detection of hormones in cells is offered by immunohistochemical techniques utilizing specific antibodies raised against auxins and cytokinins (Veselov *et al*. 2003; Aloni *et al*. 2005; Kudoyarova *et al*. 2014). Although examples of application of immunolocalization for the study of morphogenesis *in vitro* are still rare, the results obtained with the technique *in vivo* seem encouraging (Karkonen and Simola 1999; Chen *et al*. 2010; Rijavec *et al*. 2011). In the present research, immunolocalization was used to detect the level and distribution of indole-3-acetic acid (IAA) and zeatin in cells undergoing callus induction and organogenesis in immature embryo culture of wheat. The nutrient medium used here enabled induction of both shoot and root organogenesis in callus, allowing one to follow morphogenesis of these organs simultaneously. Special attention was paid to root morphogenesis *in vitro* because it seems that roots have been neglected in previous research, which has more frequently addressed shoot morphogenesis.

Materials and Methods

Callus and organogenesis induction Calluses were induced from immature embryos of *Triticum aestivum* L. (cv. Bashkirskaya 26). Donor plants were grown in the field. The caryopses were harvested 12–14 d after anthesis, sterilized in 70% (*v*/*v*) ethanol for 10 min, and rinsed three times with sterile water. The excised immature embryos were placed embryonic axis-side down in full contact with solid agar medium for callus induction. Callus induction medium (CIM) contained MS salts and vitamins (Murashige and Skoog 1962), supplemented with 30 g L^{-1} sucrose, 100 mg L^{-1} albumin hydrolysate, 2 mg L^{-1} glycine, 100 mg L^{-1} myo-inositol, 10 g L^{-1} agar, 0.2 mg L^{-1} kinetin, and 2 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). The pH of the medium was adjusted to 5.6 before gelling agent was added and medium autoclaved at 120°C for 20 min. The cultures were incubated at 26°C in darkness for 4 wk. To stimulate simultaneous formation of buds and roots in the same callus, calluses were transferred onto the same medium lacking 2,4-D and supplemented with 0.2 mg L^{-1} kinetin and 0.2 mg L^{-1} indole-3-acetic acid (IAA) (medium designated BRIM for simultaneous induction of buds and roots) and incubated for 2–3 wk in a growth chamber (SANYO, MLR-351H [Osaka, Japan]) at 26°C with 16-h illumination of 30 μM m^{-2} s^{-1}, provided by day light fluorescent tubes installed in the growth chamber by manufacturer. All media components for both CIM and BRIM were purchased from Sigma-Aldrich® [Steinheim, Germany]). Every 3 d, calluses were observed under a stereomicroscope (TECHNIVAL-2; Carl Zeiss [Jena, Germany]) and samples were taken for fixation as described below.

Histological analysis of free hormones in callus To prevent washing out of hormones during the process of dehydration, hormones were conjugated to proteins of the cytoplasm. Specifically, free cytokinin bases in callus were fixed in a mixture of paraformaldehyde and glutaraldehyde (Panreac AppliChem [Barcelona, Spain]) while auxins were fixed in carbodiimide (Sigma-Aldrich® [Steinheim, Germany]), as described by Kudoyarova *et al*. (2014) and Vysotskaya *et al*. (2007), respectively. After washing with 0.1 M phosphate buffer, dehydration was carried out in a series of ethanol dilutions. After this, the tissues were embedded in methylacrylate resin JB-4 (Electron Microscopy Sciences [Hatfield, PA, USA]). Immunolocalization of hormones was carried out with the help of sera against either IAA (Vysotskaya *et al*. 2007) or zeatin riboside (ZR) (Kudoyarova *et al*. 2014), depending on the type of fixed hormone. In short, diluted rabbit anti-IAA or anti-ZR sera were placed on the sections. Gelatin (Sigma-Aldrich® [Steinheim, Germany]) was added to the solution to prevent nonspecific binding. After the sections were incubated in a humid chamber for 2 h and then washed with phosphate buffer containing Tween 20 (Panreac AppliChem [Barcelona, Spain]), they were treated with goat anti-rabbit immunoglobulins (Aurion [Wageningen, Netherlands]) labeled with colloid gold. After incubation and washing, the sections were postfixed in glutaraldehyde and incubated with silver enhancer (Aurion [Wageningen, Netherlands]).

Antibodies raised against ZR recognized not only ZR but also free zeatin, and since the procedure of tissue fixation enabled conjugation of free bases and not their ribosides (Dewitte *et al*. 1999), immunostaining with antiZR serum is interpreted as visualization of zeatin. Nonimmune rabbit serum was used as a control, and the absence of immunostaining when anti-ZR serum or anti-IAA serum were substituted with the non-immune serum confirmed the reliability of the technique.

For histological analysis, sections prepared as described above were stained for 30 s with 0.2% (*w/v)* toluidine blue (Belami Fine Chemicals [Mumbai, India]) in 0.1 M phosphate buffer, after which the stain was removed, or with a double-staining technique (with Ehrlich's hematoxylin and alcian blue (BioVitrum [Saint Petersburg, Russia]), as described by Ozerov *et al*. (2006)). All sections were examined with an Axio Imager.A1 light microscope (Carl Zeiss [Jena, Germany]) and photographed with an AxioCam MRc5 digital camera (Carl Zeiss [Jena, Germany]).

Results

Immunolocalization of hormones during callus formation Cell division in the internal layer of the scutellum of immature wheat embryos cultured on CIM resulted in callus formation. After 3 d of cultivation, groups of small heavily stained and actively dividing meristematic cells were detected near procambial strands of the scutellum, while the rest of the scutellum cells were highly vacuolated and were showing evidence of disintegration (Fig. 1*a*). By the 6th day of cultivation on CIM, the size and number of groups of meristematic cells were significantly greater, resulting in the fusion of these cell groups (Fig. 1*b*, unlabeled arrows). Subsequent proliferation led to formation of the callus itself, with the surrounding cells undergoing almost complete destruction (Fig. 1*c*; ~12 d of cultivation on CIM). Formed morphogenic callus was represented by a mass of cells that were rather uniform in their size and structure despite some heterogeneity (Figs. 1*d* and 2; ~15 d of cultivation). The level of cell immunostaining of such callus for either IAA or zeatin was uneven: some cells were intensively labeled, while other cells were not labeled at all (Fig. 2).

In the course of subsequent development (~18 d of cultivation on CIM), two types of cells could be distinguished in the callus (Fig. 3). Cells found in small groups inside the callus belonged to the first type. These cells were rather small and their cytoplasm was strongly immunostained, which facilitated their being identified as meristematic cells and such cells were identified as meristematic centers. Large, vacuolated cells that showed attributes of parenchyma tissue belonged to the second cell type. The cells of meristematic centers were more evenly and heavily stained for zeatin (Fig. 3*a*, *b*) and IAA (Fig. 3*c*, *d*) than were the surrounding callus cells.

In the process of development on CIM, the meristematic centers were initially composed of rather similar cells but later during culture meristematic centers showed distinct zonality. Some of the cells remained meristematic while others appeared parenchymatous. By the 21st day of cultivation on CIM, meristematic centers consisted of three cell layers (Fig. 4): a central part, characterized by weakly vacuolated cells; an intermediate layer of meristematic cells; and a peripheral layer of highly vacuolated parenchymatous cells. Cells of the intermediate layer were notable for intense staining for both zeatin (Fig. 4*a*, *b*) and IAA (Fig. 4*c*, *d*), unlike cells of the central and peripheral layers, which were weakly stained for these hormones.

In the course of subsequent development, the meristematic site appeared larger due to division of the cells of the intermediate layer (periclinal divisions add cells into the zone and anticlinal divisions expand its width). Furthermore, the intermediate layer developed into an extended meristematic zone composed of several cell layers and situated in parallel to the callus surface (Fig. 5*a*, *b*; after 24–27 d of cultivation on CIM). Cells of the meristematic zone stained strongly for both zeatin (Fig. 5*c*, *d*) and IAA (Fig. 5*e*, *f*). Simultaneous with the formation of the meristematic zone was the destruction of cells of peripheral layers of the meristematic zone (Fig. 5*a*, *b*). Where complete destruction of the cells of peripheral layer had taken place, the forming meristem zone appeared on the callus surface.

At 28 d after cultivation on CIM, calluses with meristematic zone were transferred onto BRIM for simultaneous induction of bud and root formation.

Immunolocalization of hormones during organogenesis *in vitro* In the process of subsequent callus development (~10 d of cultivation on BRIM), an epidermal layer formed at the sites where the meristematic zone appeared on the callus surface as a result of destruction of cells located at the callus periphery. It is in these parts of the meristematic zone (as a rule, on the upper callus part) that shoot buds were initiated with anticlinal and periclinal cell divisions of both epidermal and subepidermal callus layers. At the same time, more intense immunostaining for both hormones was detected at the sites of apex initiation than in the surrounding cells within the meristematic zone (Figs. 6*a*, *b* and 7*a*, *b*). Frequently, shoot apex primordial were located very close to each other, resulting in formation of closely located or even-fused buds (Figs. 6*c*, *d* and 7*a*, *b*). Inside the callus at some distance from the shoot apexes, there were files of cells forming vasculature (procambial cells) having a trajectory directed toward the shoot apex (Figs. 6*c*, *d* and 7*a*, *b*). The intensity of immunostaining of procambial cells for zeatin did not differ from that of the surrounding cells forming the shoot apex (Fig. 6*c*, *d*). At the same time, immunostaining of procambial cells for IAA was stronger than that of the cells forming the shoot apex and

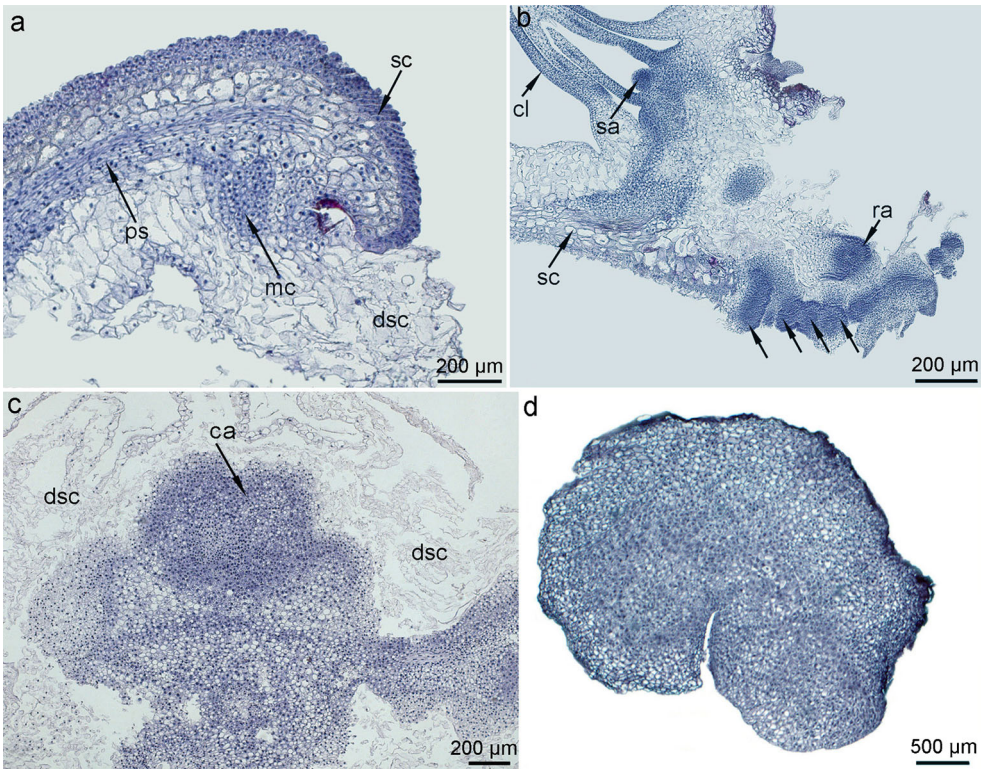

Figure 1. Histological analysis of callus development on callus induction medium. Callus was stained with toluidine blue. (*a*) Groups of meristematic cells (*mc*) forming near procambial strands (*ps*) of scutellum (*sc*). Degenerating scutellum cells (*dsc*) are also visible. (*b*) Groups of meristematic cells (*unlabeled arrows*), fusing at base of scutellum near the region of root apex (*ra*) in cultured embryo with still-intact shoot apex (*sa*) and coleoptile (*cl*). (*c*) Callus (*ca*) enveloped by degenerating scutellum cells. (*d*) Formed callus

much stronger than that of the surrounding callus cells (Fig. 7*a*, *b*). At approximately 15 d of cultivation on BRIM leaf primordia were initiated at the apexes, although at this stage it was sometimes difficult to distinguish between shoot apexes and leaf primordia (Figs. 6*c*, *d* and 7*a*, *b*). Shoot apexes with leaf primordia were heavily immunostained for zeatin (Fig. 6*e*, *f*). Immunostaining for IAA was more intense at the sites of leaf primordia initiation, incipient primordia, and cells of procambial strands (Fig. 7*c*, *d*).

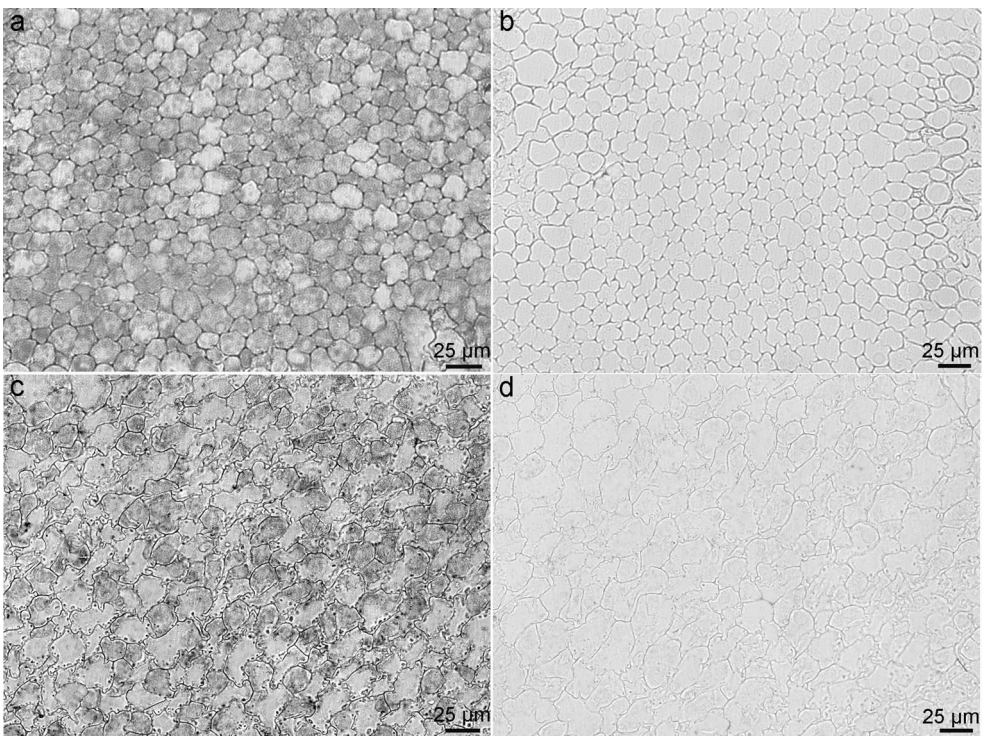

Figure 2. Immunolocalization of zeatin (*a*) and IAA (*c*) in cells of formed morphogenic callus. (*b*, *d*) Control sections (stained with nonimmune serum) for (*a*) and (*c*), respectively

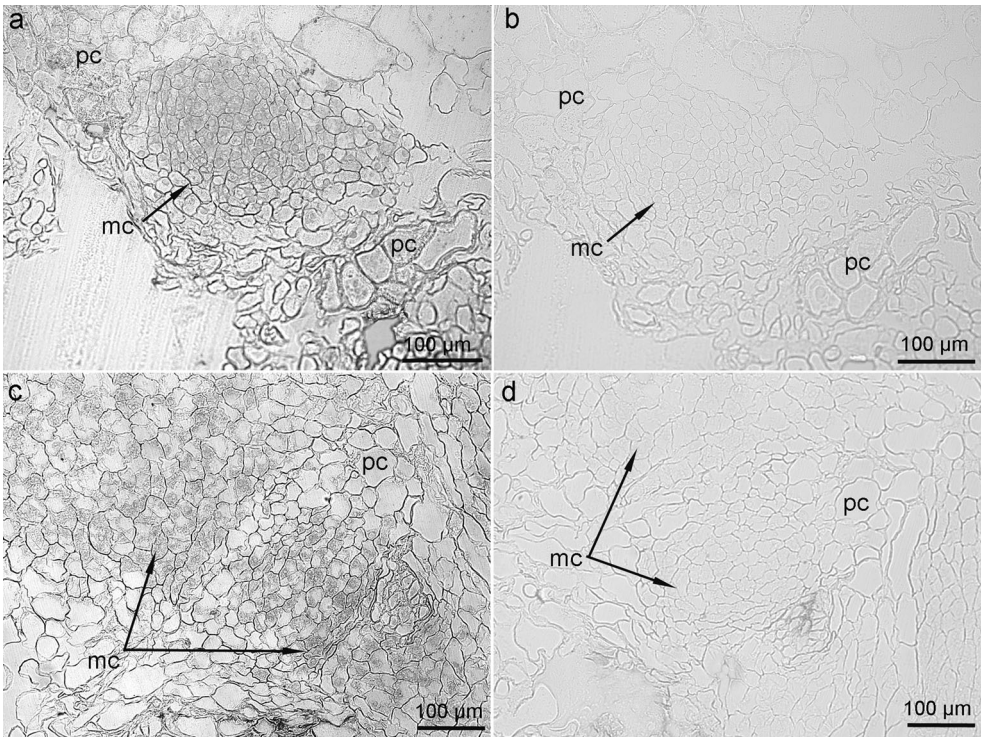

Figure 3. Immunolocalization of zeatin (*a*) and IAA (*c*) in the cells of meristematic centers (*mc*) of callus, surrounded by parenchymatous cells (*pc*). (*b*, *d*) Control sections (stained with nonimmune serum) for (*a*) and (*c*), respectively

Simultaneous with bud initiation (~10 d of cultivation on BRIM), root initiation occurred in the part of meristematic zone remaining inside the callus (as a rule, in the lower part of the callus below the developing buds). This happened due to the ordering and reorientation of the direction of cell division in the meristematic zone, resulting in transformation of its layers into root meristems (Fig. 8*a*, *b*). As in the process of bud formation, sites of newly developing root meristems were frequently located close together, resulting in fusion of closely located root meristems and formation of fused roots (Fig. 8*a*, *c*, *e*). Cells forming root meristem were immunostained strongly for both zeatin (Fig. 8*c, d*) and IAA (Fig. 8*e, f*).

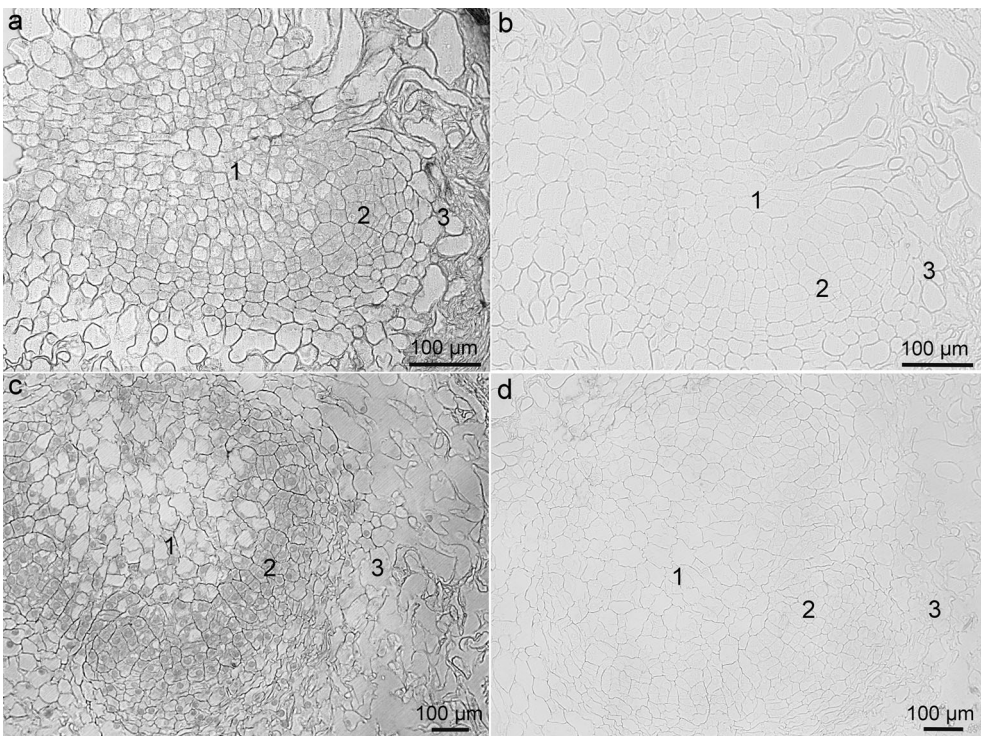

Figure 4. Immunolocalization of zeatin (*a*) IAA (*c*) in the cells of meristematic centers consisting of three layers: central part (*1*), intermediate layer (*2*), and peripheral layer (*3*). (*b*, *d*) Control sections (stained with nonimmune serum) for (*a*) and (*c*), respectively

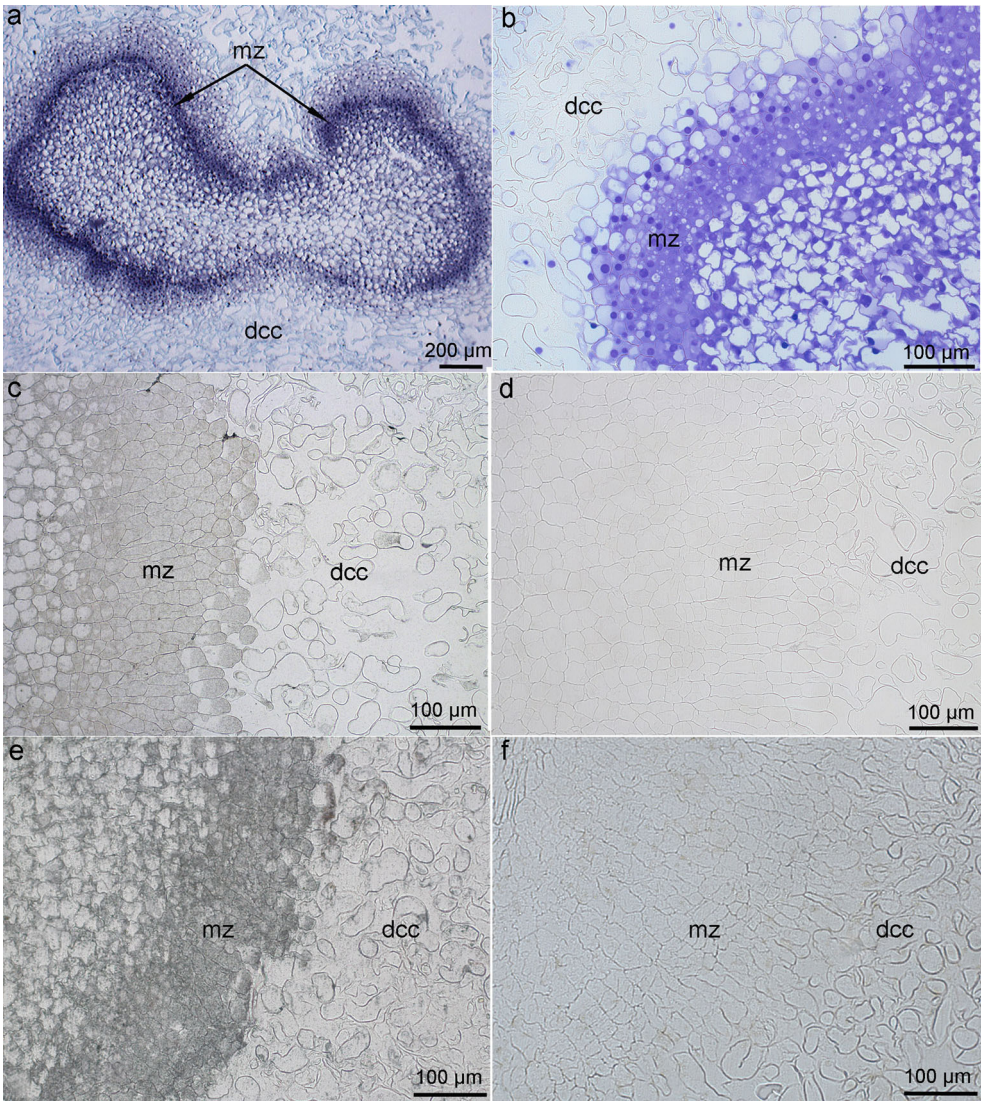

Figure 5. Immunolocalization of zeatin and IAA in the cells of formed meristematic zone (*mz*), surrounded by degenerating callus cells (*dcc*). (*a*) Histological section through callus with formed meristematic zone stained with toluidine blue. (*b*) Enlarged from (*a*). (*c*, *e*) Immunohistochemical staining of sections with immune sera against ZR (*c*) and IAA (*e*). (*d*, *f*) Control sections (stained with nonimmune serum) for (*c*) and (*e*), respectively

During cultivation on BRIM (~15 d), roots acquired a structure that was more or less typical for the roots of gramineous plants. Caps formed at the root apex could be clearly seen in some sections (Fig. 9*a*). A zone of small cells intensively labeled for both IAA (Fig. 9*b*, *c*) and zeatin (Fig. 10*b*, *c*) was detected in the sections of the tips of developing roots.

At a short distance from the root tip, intense immunostaining for both hormones was retained on the sections of several cell layers (Figs. 9*b*, *c* and 10*e*, *f*) apparently corresponding to the root meristem zone *in vivo*. Central metaxylem vessels could be detected, although distinct differentiation of cells of central cylinder and cortex was not observed in the cross sections of this root part (Fig. 10*a–f*).

The intensity of immunostaining for both hormones weakened with further increases in distance from the root tip. The size and number of root cells increased in the cross section of this root region (apparently corresponding to the elongation zone of roots *in vivo*) (Figs. 9*d–i* and 10*a–f*). Only cells of the central cylinder were weakly stained for IAA, while the cortex cells were not labeled (Fig. 9*e*, *f*). Immunostaining for zeatin was more or less even (Fig. 10*b*, *c*, *e*, *f*). Developing sieve tubes were detected in the sections (Fig. 10*d*, *g*) and their cells were heavily stained for zeatin, while cells of the metaxylem were weakly labeled (Fig. 10*e*, *f*, *h*).

At an even greater distance from the tip was the root zone apparently corresponding to the differentiation zone *in vivo* (Figs. 9*a* and 11*a*). This region had differentiating vessels whose cells were stained strongly for IAA (Fig. 9*b*, *c*). Immunostaining for zeatin was observed only in some cells of the central cylinder (Fig. 11*b–d*), while sieve tubes were not stained (Fig. 11*b–d*). Immunostaining for zeatin was also present in some cells resembling root hairs (Fig. 11*b–d*).

It is necessary to emphasize that regular continuous files consisting of cells of the meristem and elongations zones and then of the vasculature were clearly visible in the central part of the longitudinal root sections (Fig. 9*a–c*). However, such

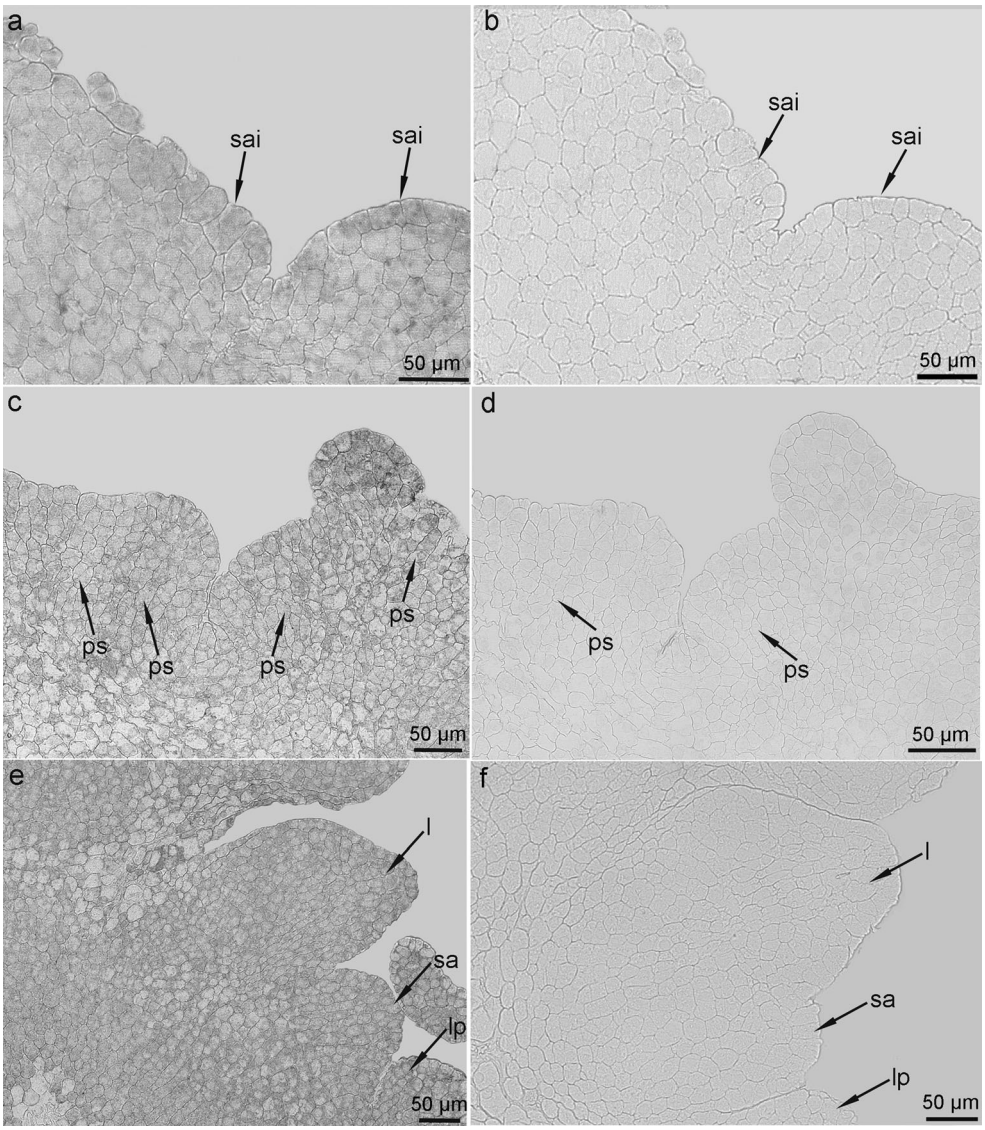

Figure 6. Immunolocalization of zeatin during shoot organogenesis *in vitro*. *(a)* Section through sites of shoot apex initiation (*sai*). (*c*) Emergence of procambial strands (*ps*) oriented toward the developing shoot apex. (*e*) Shoot apex (*sa*) with leaf primordium (*lp*) and forming leaves (*l*). (*b*, *d*, *f*) Control sections (stained with nonimmune serum) for (*a*), (*c*), and (*e*), respectively

files were sometimes absent from the root cortex, which was not developed per se. In these roots, the cells of the central cylinder were surrounded by a disordered group of callus cells (Fig. 9*a*–*c*). Due to the absence of such files in the root periphery, root tip cells formed globular structures (Fig. 9*a*), which is unusual for roots *in vivo*. The absence of a clearly apparent cortex layer was evident in some root cross sections (Figs. 9*g* and 10*a*, *d*). At the same time, cortex was present in other root sections (Figs. 9*d* and 11*a*).

Discussion

Formation and differentiation of meristematic zones in the callus Immunohistochemical analysis showed that callus cells, which were uniform in size and characteristic for the initial stage of callus formation, were unevenly stained for zeatin and IAA (Fig. 2), suggesting heterogeneous distribution of these hormones between the cells. This can be explained by the fact that callus consists of cells that may be at different physiological states, the characteristics of some cells corresponding to actively dividing meristematic cells and those of others corresponding to differentiated parenchymatous cells. Different physiological states are likely to determine different morphogenetic competences, which, in turn, determine different cell fates (Batygina 2011).

Overall, the cells of morphogenic callus were characterized by high content of both hormones. This is partially in accordance with the data of Gordon *et al*. (2007), who used reporter constructs to show high levels of cytokinin and auxin in the cells of callus proliferating on CIM.

Different reagents were used for callus fixation with the aim of either cytokinin or auxin detection, so the same sections could not be stained for both hormones. Consequently, it was impossible to evaluate the frequency of callus cells with elevated levels of both hormones. Nevertheless, cytokinins

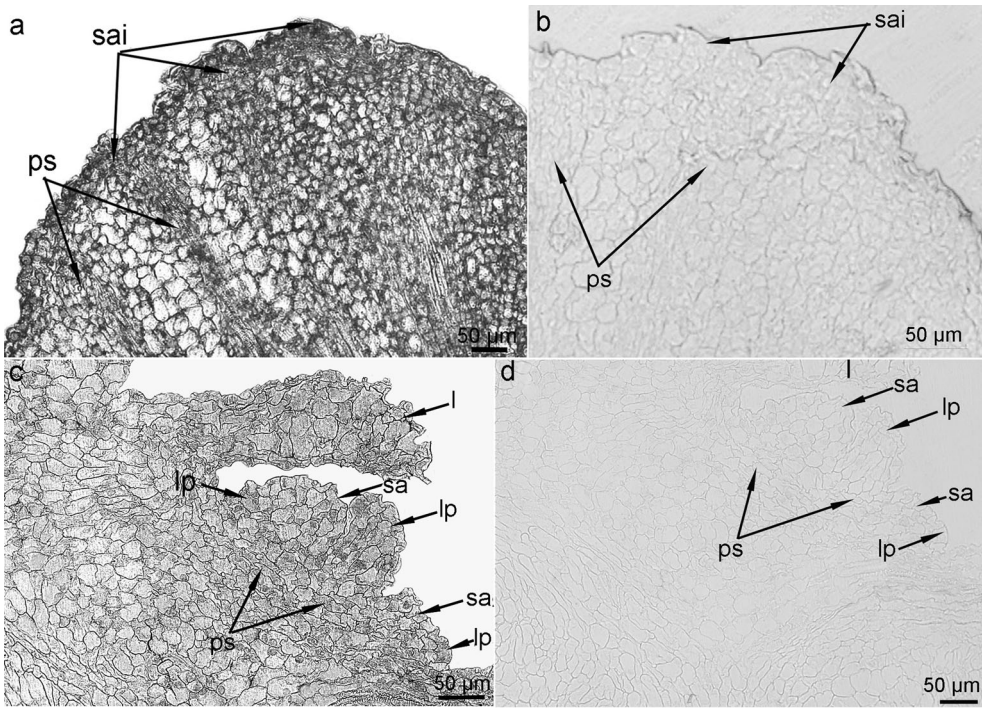

Figure 7. Immunolocalization of IAA during shoot organogenesis *in vitro*. *(a)* Section through sites of shoot apex initiation (*sai*). Procambial strands (*ps*) oriented toward the developing shoot apex are seen. (*c*) Shoot apex (*sa*) with leaf primordium (*lp*) and forming leaves (*l*). (*b*, *d*) Control sections (stained with nonimmune serum) for (*a*), and (*c*), respectively

and auxins are well-known to be necessary for cell division, suggesting that cells with elevated levels of these hormones were likely to divide actively, giving rise to groups of small cells found within the callus after 18 d of cultivation *in vitro* on CIM (Fig. 3). Antibodies used for immunolocalization were obtained against endogenous zeatin riboside and IAA and had low cross-reactivity to synthetic auxins and cytokinins (Veselov *et al*. 1992, 1999). Consequently, elevated levels of cell staining were not likely to be due to the uptake of synthetic hormones from the culture medium used for callus induction (CIM). The high levels of hormones in the groups of small cells may have been maintained by their synthesis in the cells. This possibility is suggested by expression *in vitro* of the genes controlling synthesis of auxins and cytokinins (Bai *et al*. 2013; Cheng *et al*. 2013).

Similar groups of meristematic cells forming in callus were revealed by other authors, and the globular form of such cell groups suggested that these structures were embryoids (Bouamama *et al*. 2011; Ślesak *et al*. 2013; Delporte *et al*. 2014). Such an interpretation is inapplicable in the present study, since under the applied experimental conditions only organogenesis occurred in callus, and embryoids have not been detected. Furthermore, identification of embryoids is complicated in early stages of development. Formation of embryoids may be established with certainty using the criteria suggested by Haccius and Lakshmanan (1969): unlike a bud, an embryoid has a closed radicular pole and no vascular connection with callus. Thus, further development of cell groups resulting in formation of a ring-shaped meristematic zone (Fig. 5), which was parallel to the callus surface and gave rise to numerous roots and shoots, had nothing in common with the formation and development of embryoids. Formation of such a meristematic zone with cell activity linked to the processes of organogenesis has been mentioned in other reports (Verdeil *et al*. 2001; Steinmacher *et al*. 2007; Seldimirova and Kruglova 2013; Ślesak *et al*. 2013). It is reported that formation of meristematic zone is a universal initial stage of different morphogenetic pathways *in vitro* in morphogenic callus, including organogenesis (Seldimirova *et al*. 2011).

Formation and differentiation of shoot apexes and the vasculature below them Cells of the meristematic zone, which participated in formation of either shoot apexes or root meristem, were stained strongly for zeatin and IAA (Fig. 5*c–f*). This agrees with the data of Cheng *et al*. (2013), who detected even distribution of auxins and cytokinins in several superficial callus layers immediately prior to the induction of shoot apexes.

Cytokinins in shoots The strong staining of developing shoots for zeatin (Fig. 6) agrees with previous reports of high levels of cytokinins in shoot apexes detected with the help of reporter constructs (Gordon *et al*. 2007; López-Bucio *et al*. 2007; Cheng *et al*. 2013) and immunohistochemical approaches (Rijavec *et al*. 2011) *in vitro* and *in vivo*.

Evidence of high levels of cytokinins in the developing leaves may be also found in the literature (Caboni *et al*. 2002). According to some authors, cytokinin content decreased at the site of initiation of leaf primordia where accumulation of auxins was detected (Gordon *et al*. 2007).

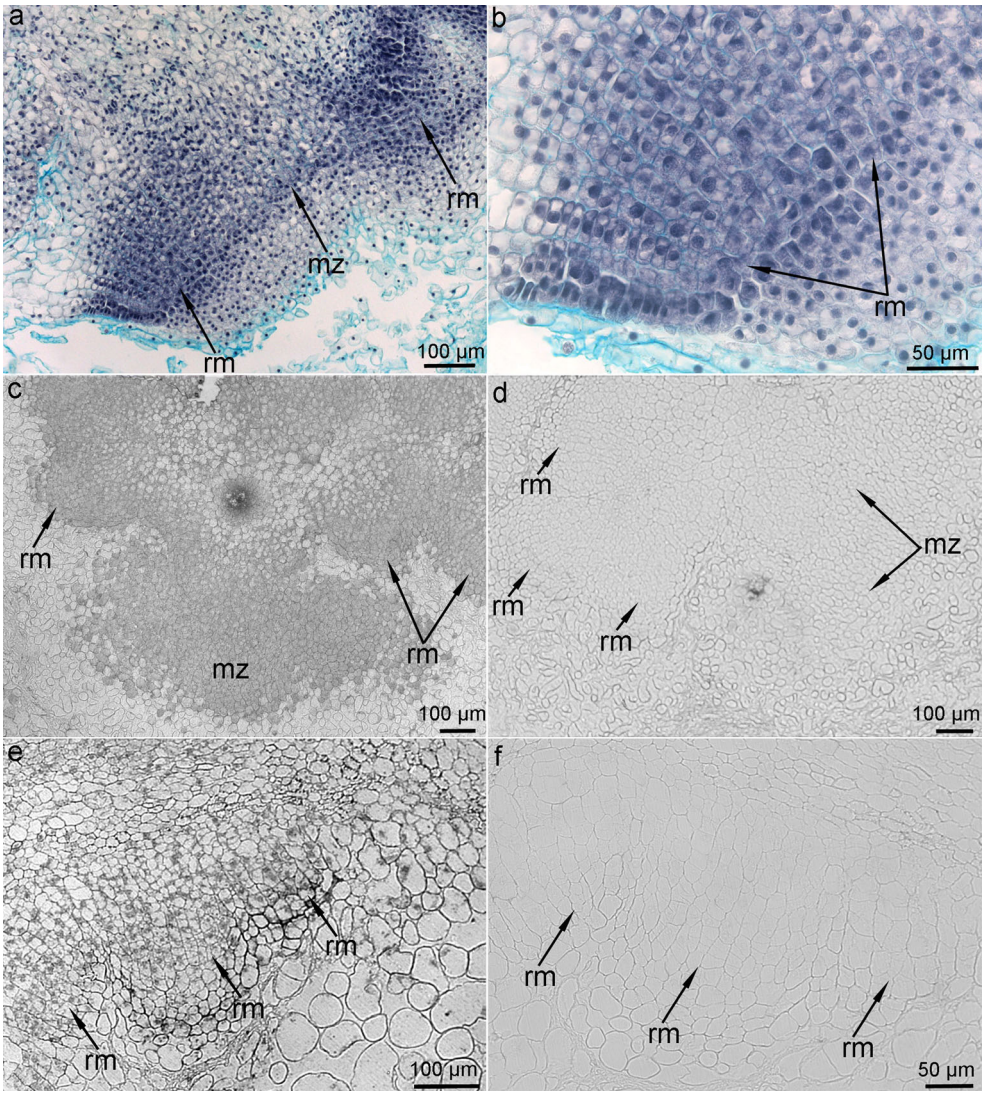

Figure 8. Immunolocalization of zeatin and IAA during initial stages of root organogenesis *in vitro*. (*a*) Section through part of meristematic zone (*mz*) reorganizing into root meristems (*rm*), stained with Ehrlich's hematoxylin and alcian blue. (*b*) Enlarged from (*a*). (*c*, *e*) Immunohistochemical staining of sections with immune sera against ZR (*c*) and IAA (*e*). (*d*, *f*) Control sections (stained with nonimmune serum) for (*c*) and (*e*), respectively

However, this study did not reveal such a distribution of zeatin in developing shoots. It is important to emphasize that distribution of cytokinins may change during the process of leaf development (Cheng *et al*. 2013). It is possible that uneven distribution of cytokinins may be revealed during later stages of shoot development that were not examined in the present experiments.

Auxins in shoots The strongest staining for IAA was detected in meristem cells of developing shoot apexes (Fig. 7*a*, *b*). Later on, shoots apexes were weakly stained for auxins, while in leaf primordia, the level of staining was stronger (Fig. 7*c*, *d*).

Cells of developing procambial strands forming below the shoot apexes were strongly stained for both zeatin and IAA (Figs. 6*c*, *d* and 7*a*, *b*, respectively). Auxin plays a key role in differentiation of conductive tissues *in vivo* (Scarpella and Helariutta 2010; Ohashi-Ito *et al*. 2013) and has been shown to induce formation of xylem elements from isolated mesophyll cells (Fukuda 1997). Consequently, strong auxin staining of the cells of procambial strands should not be surprising. Immunostaining of procambial cells for cytokinins has also been reported (Sossountzov *et al*. 1988; Caboni *et al*. 2002), which is in accordance with data relating cytokinins to the control of vasculature formation (Hejátko *et al*. 2009).

Formation and differentiation of roots Staining for both zeatin (Figs. 8*c, d*, 10, and 11) and IAA (Figs. 8*e, f*, and 9) was revealed in developing roots.

Cytokinins in roots Given the data of Skoog and Miller (1957) showing the absence of root regeneration on medium with a high concentration of cytokinins, the present data showing a high root concentration of cytokinins may seem unexpected. At the same time, roots are considered to be the

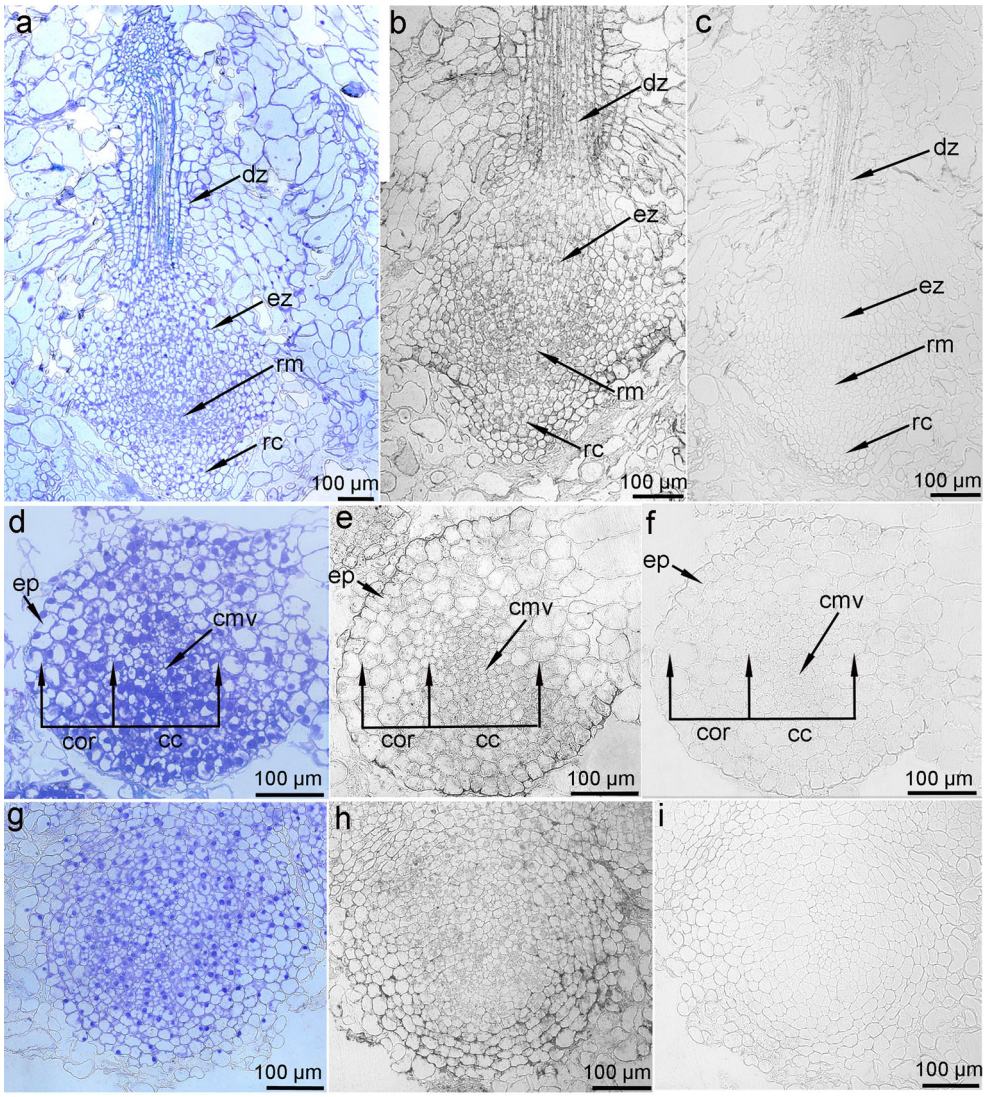

Figure 9. Immunolocalization of IAA in cells of developing root. (*a*) Longitudinal section through root stained with toluidine blue and demonstrating the presence of basic root zones: root meristem (*rm*) with root cap (*rc*), elongation zone (*ez*), and differentiation zone (*dz*). (*d*) Toluidine blue-stained cross section through the root part apparently corresponding to the elongation zone of roots *in vivo*. Root epidermis (*ep*), cortex (*cor*), and central cylinder (*cc*) with central metaxylem vessel (*cmv*) are clearly visible. (*g*) Toluidine blue-stained cross section through the elongation zone of a different root from (*d*), without clearly apparent root internal tissues. (*b*, *e*, *h*) Sections parallel to ones presented in (*a*), (*d*), and (*g*), respectively, and stained with immune serum. (*c*, *f*, *i*) Control sections (stained with nonimmune serum) for (*b*), (*e*), and (*h*), respectively

main site of cytokinin synthesis in plants, which was confirmed by an estimation of root expression of the genes responsible for the synthesis of these hormones (Miyawaki *et al.* 2004). Although exogenous cytokinins are known to inhibit root growth (Laplaze *et al.* 2007), mutants with defects in sensitivity to cytokinins had poorly developed root systems, suggesting the necessity of cytokinins for normal root development (Higuchi *et al.* 2004). Immunofluorescent microscopy of somatic embryos showed that cytokinins were concentrated in cells showing meristematic character, including root apexes (Karkonen and Simola 1999).

As was shown by Vysotskaya *et al.* (2011), initiation and early development of sieve tubes took place in the division zone of wheat roots *in vivo* and was marked by the disappearance of immunostaining for cytokinins. In the present experiments, on the other hand, newly developing phloem cells were not detected in the region corresponding to the root division zone *in vivo* (Fig. 10*d–f*). Strongly stained developing sieve tubes were observed only in the root elongation zone (Fig. 10*d–h*).

Further weakening of immunostaining was detected on the sections of the differentiation zone (Fig. 11*b–d*). It was in this zone that maturing sieve tubes and xylem vessels were found. Cells of the central cylinder and root hairs were more strongly stained than cortex cells. In total, immunostaining of cells in the central cylinder and root cortex was weaker than that of cells in the division zone.

The data reported here are in accordance with results obtained for embryonic and seedling roots with the help of reporter constructs. In those studies, glucuronidase activity (resulting from expression of a *GUS* reporter gene controlled by the cytokinin-sensitive *ARR5* promoter) was detected in the root tips and central cylinder (Aloni *et al.* 2005; López-Bucio *et al.* 2007) in accordance with the present experiments, where a high level of zeatin was observed in the root tips (Fig. 10*b*, *c*, *e*, *f*) and in the region of the central cylinder of differentiation

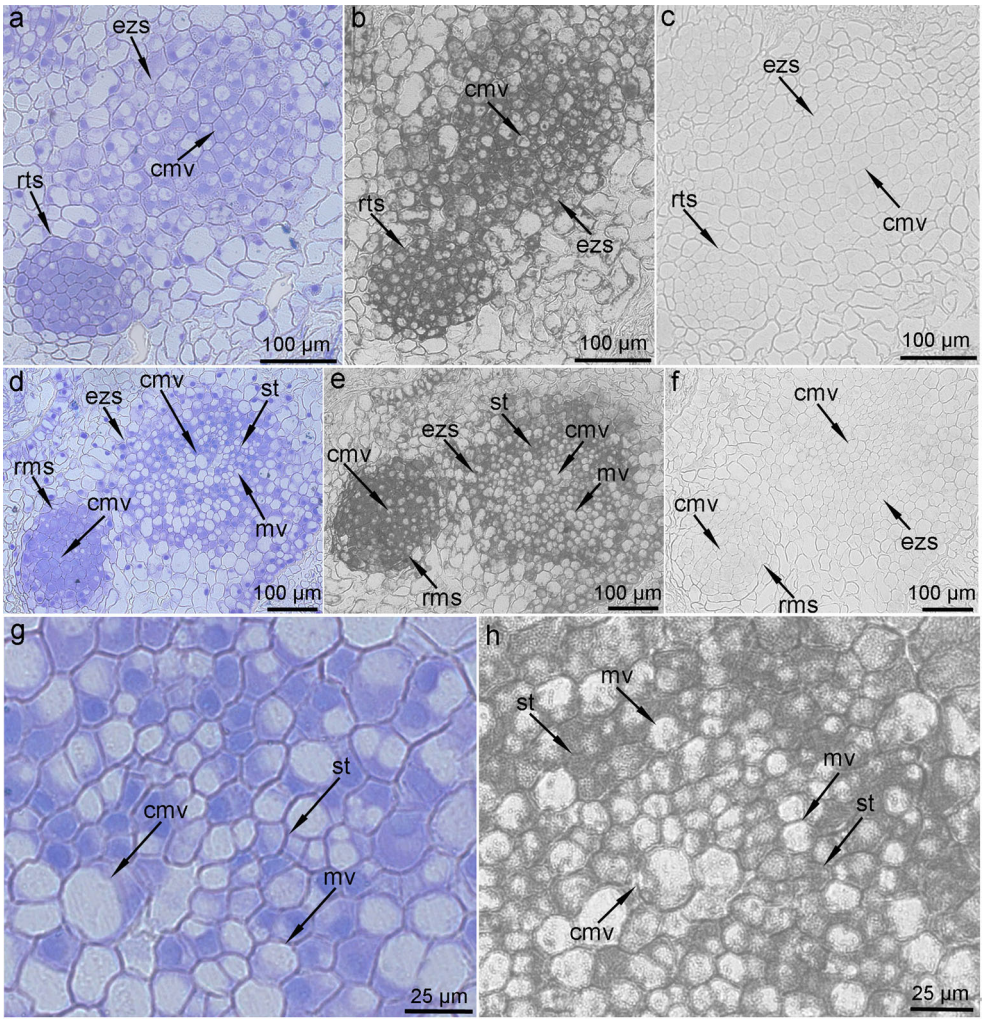

Figure 10. Immunolocalization of zeatin in cells of developing root (cross sections through the same curved root). (*a*) Toluidine blue-stained section through root tip (*rts*) and elongation zone (*ezs*) with visible central metaxylem vessel (*cmv*). (*d*) Toluidine blue-stained section through root meristem (*rms*) and elongation zone with visible central metaxylem vessel, mature metaxylem vessels (*mv*), and developing sieve tubes (*st*). (*b*, *e*) Sections parallel to the ones presented in (*a*) and (*d*), respectively, and stained with immune serum. (*c*, *f*) Control sections (stained with nonimmune serum) for (*b*) and (*e*), respectively. (*g*, *h*) Enlarged from (*d*) and (*e*), respectively

zone (Fig. 11*b–c*). Earlier, immunohistochemical staining of the root cross sections of wheat seedlings revealed a similar pattern: stronger staining of the cells of the central cylinder in the differentiated zone (Kudoyarova *et al*. 2014).

Thus, the present data showed that zeatin content in root cells reached a maximum in the root apex and gradually declined with increasing distance from it. Such a tendency was revealed during the study of root cytokinin distribution *in vivo* with the help of quantitative hormone assays and immunohistochemistry in tomato (*Solanum lycopersicum*; Sossountzov *et al*. 1988), maize (*Zea mays*; Brovko *et al*. 2007), and wheat (*Triticum aestivum*; Vysotskaya *et al*. 2011). Vysotskaya *et al*. (2011) attribute the decline in staining to the high activity of cytokinin oxidase catalyzing the decay of cytokinins during their diffusion from the root apex to its basal part.

Auxins in roots Meristematic shoot tissues are considered to be the main site of auxin synthesis during postembryonic development (Leyser 2009 and references therein). However, since IAA was present in the BRIM, increased accumulation of this hormone in the cells involved in rhizogenesis may be due to specific activity of IAA transporters known to be responsible for the establishment of local auxin gradients in plant tisssues (Benková *et al*. 2003). At the same time, the ability of the root to synthesize auxins has been revealed (Ljung *et al*. 2005), and the expression of several *YUCCA* genes, which control auxin synthesis, was detected in the roots (Chen *et al*. 2014). These reports provide an explanation for the IAA immunostaining in the roots formed independent of shoots observed in the present experiments. Consistent with these results, expression of a reporter gene under the control of the auxin-sensitive *DR5* promoter was detected in the root tip, becoming weaker with distance and strong in the vasculature (Friml *et al*. 2002; Monroe-Augustus *et al*. 2003). Visualization of auxin response patterns by using reporter constructs showed that vascular differentiation occurs at sites of maximum auxin response (Mattsson *et al*. 2003). The present data are also in agreement with those showing the role of auxins in the development of vasculature.

The data reported here on the absence of cortex in some developing roots are also of interest. The root cortex is formed by the division, elongation, and differentiation of peripheral

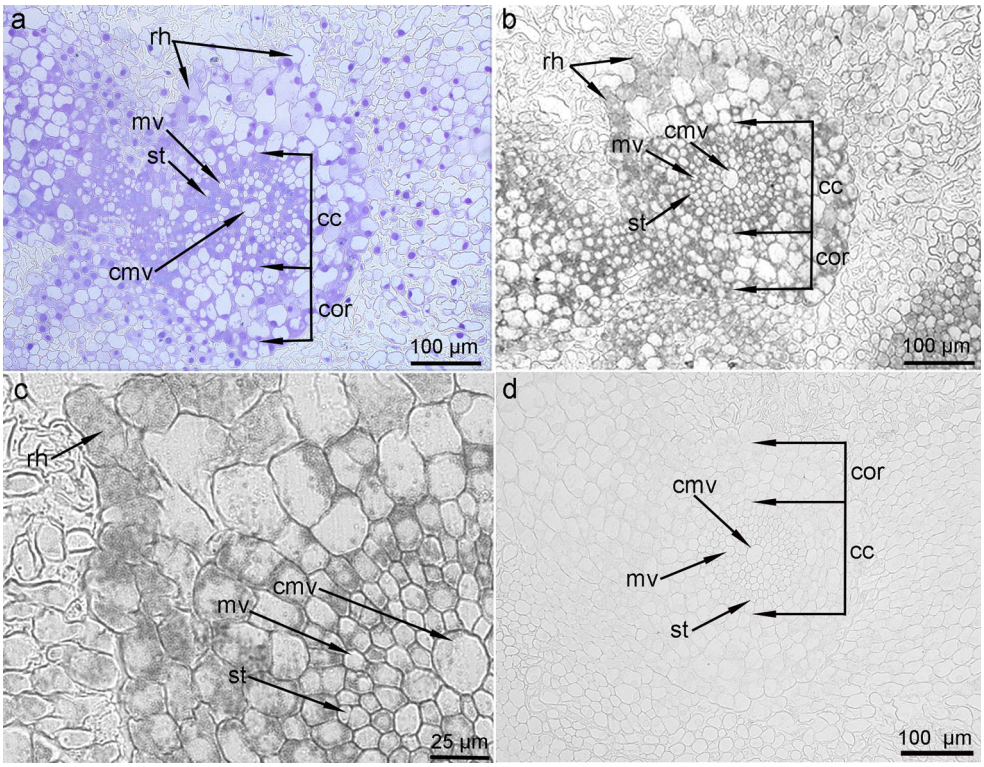

Figure 11. Immunolocalization of zeatin in cells of root part apparently corresponding to the differentiation zone *in vivo*. (*a*) Toluidine blue-stained cross section demonstrating typical internal root structure: central cylinder (*cc*) with apparent central metaxylem vessel (*cmv*), mature metaxylem vessels (*mv*) and sieve tubes (*st*), clearly visible cortex (*cor*), and root hairs (*rh*). (*b*) Parallel section treated with immune serum. (*c*) Higher magnification of part of root section presented in (*b*). (*d*) Control section (stained with nonimmune serum) for (*b*)

cells of the apical meristem. As mentioned above, the continuity of cell files leading from apical meristem to vessels points to the role of the apex in the differentiation of vessels. It remains unclear which cells may be responsible for the spatial differentiation of the cortex cells, but involvement of pericycle cells in this process is not excluded by the present results. In any case, characteristic concentric cortex layers around the central cylinder are found in some root cross sections (Figs. 10*d–f*).

The frequently detected formation of closely located or even fused organs, as well as the globular form of root tips, is apparently not uncommon for culture *in vitro*, as it has also been reported by other authors (Eapen and Rao 1985; Bartok and Sagi 1990; Dong *et al*. 2012).

Orientation of vasculature It is of interest to consider the peculiarities of spatial orientation of the vessels in regard to the centers of shoot and root formation. The present results, revealed a correspondence of vessel trajectories to the position of the meristematic zone, in agreement with the auxin-flow canalization hypothesis, which suggests that canalized auxins flow along a narrow column of cells resulting in the differentiation of strands of procambial cells and, subsequently, vascular strands (Fukuda 2004 and references therein). Intense immunostaining for IAA and zeatin in the centers of shoot and root initiation and the literature on the role of these hormones in the control of vasculature differentiation (Scarpella and Meijer 2004) suggest that hormones may function as these signals.

Conclusions

Together, the present results and the literature serve as evidence of similarity of hormonal mechanisms controlling the processes of organogenesis *in vivo* and *in vitro*, thereby supporting the concept of universality of morphogenetic pathways (Batygina 2013). Furthermore, these data suggest the possible utility of tissue culture for understanding development processes *in vivo*.

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