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PLANT PHYSIOLOGY

Effects of Root Cutting on Cytokinin Content in the Shoot Apex Cells of Arabidopsis Plants

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Abstract—The dependence of cytokinin accumulation in the shoot apexes of Arabidopsis plants on the delivery of these hormones from the roots was studied. For the estimation of cytokinin content in the cells, the immunohistochemical localization method using antibodies against zeatin riboside was used. Differential conjugation of free cytokinin bases and their ribosides was used to prevent washing of cytokinins during the dehydration process. Root cutting decreased the immunostaining of zeatin in the cells of the shoot apical meristem, thereby supporting the hypothesis about dependence of cytokinin accumulation in these cells on the hormone delivery from the roots. The level of cytokinins in the cells of the shoot apex decreased under the effect of protonophore, indicating the important role of the secondary-active transmembrane transport process of cytokinins in the maintenance of their level in the cells of the shoot apex.

Keywords: Arabidopsis, zeatin, zeatin riboside, isopentenyl adenine, shoots apex, transport from the roots, immunohistochemical localization.

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INTRODUCTION

The detection of phytohormones in the xylem sap of plants served as a stimulus for the formulation of an important concept, suggesting their role in the transmission of signals from the roots to shoots (see the references of the review by Kudoyarova et al. [1]). Prof. O.N. Kulaeva first showed the presence of cytokinin-like compounds capable of delaying the aging of leaves in the xylem sap [2]. These findings drew the attention of researchers to the study of the role of cytokinins as root signals. The change in the concentration of cytokinins in xylem sap under drought conditions [3] or an increase in the concentration of nitrates [4], as well as many other effects [1, 5], was shown. These changes were accompanied by a change in both shoot growth and leaf aging rate, transpiration, and expression of cytokinin response genes in leaves. These results confirmed the importance of root cytokinins as signals regulating the functional activity of the shoot. At the same time, the role of cytokinins synthesized in plant roots became a subject of discussion after the expression of *IPT* genes controlling the synthesis of cytokinins was detected not only in roots but also in plant shoots [6]. The local action of cytokinins only at the site of their synthesis, the so-called "paracrine" was suggested [7]. However, this assumption was contradicted by data on the reduced content of cytokinins in cotyledons and inflorescences and their accumulation in the roots of cytokinin transporter mutant plants [8]. Since mutant plants were characterized by impaired shoot functions, these results confirmed the functional role of cytokinin inflow from the roots. It should be noted that insufficient attention was paid to the apex in studies on the role of root cytokinins in regulation of the shoot functions. However, the important role of cytokinins in maintaining the division of the apical shoot meristem is well known [9]. Information gaps regarding this topic are most likely related to the technical difficulties of determining hormones in such small structures as the apical shoot meristem. In this study, we used the immunohistochemical localization of cytokinins using antibodies against zeatin ribosides and isopenteniladenosine (IPA), which proved to be very useful in studying the apical system of the shoot [10, 11]. This approach has an advantage over cytokinin detection by cytokinin biosensors based on the use of transgenic plants in which the reporter gene is under the control of a cytokinin-sensitive promoter. The immunohistochemical localization allows assessing the relative level of hormones in tissues, regardless of the sensitivity of cells to cytokinins, while this sensitivity determines detection by biosensors [12]. In the cited study, an example of an increase in the cytokinin levels during chilling detected using a biosensor was demonstrated, while the cytokinin content did not change according to the results of the quantitative assessment. In addition, the

modification of the experiment developed by us allowed separate identification of free and ribosylated forms of cytokinins on the sections due to their specific fixation, while mainly active cytokinin forms were detected by a biosensor method using the reporter constructs.

Protonophore carbonyl cyanide m-chlorophenyl hydrazone [13], suppressing the function of the transporters due to the destruction of the hydrogen gradient, the energy source for the secondary-active transmembrane transfer, was used in the study for the estimation of the contribution of cytokinin transporters into their accumulation in cells of the shoot apex.

MATERIALS AND METHODS

Cultivation of plants. Seeds of Arabidopsis plants (*Arabidopsis thaliana L. Heynh*) ecotype Columbia (Col) were germinated on wet paper in Petri dishes after stratification for 3 days at 4°C. The seeds were then transferred to the surface of sand, saturated with Hoagland-Arnon nutrient solution in 100 mL containers, and then grown in a climatic chamber (MLR-350H, Sanyo, Japan) at 23°C/19°C (day/night), air humidity of 80%, illumination of 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of PAR and a 16-h photoperiod as described earlier [14]. The plants were supplied with distilled water to compensate for transpiration losses, which were measured by weighing the containers; hydration of sand was maintained at 60% of full water capacity. In experiments with the removal of roots, the plants were grown in containers with sand for 10 days. They were then carefully transferred to polystyrene plates with holes in the bottom, and microplates were placed in 5-L containers, in which the plates floated on the surface of the Hoagland-Arnon solution that was diluted ten times. After 2 days, the roots were cut off with a razor blade from the part of the plants, and the shoots were again placed into the wells of the plate floating on the surface of the nutrient solution. Fixation of shoots for immunolocalization of cytokinins in apexes was carried out 2 h after the root cutting.

Immunolocalization of cytokinins. The procedure, modified in comparison with that previously described [13], was carried out with rabbit antibodies against cytokinins—zeatin riboside and IPA. The sera were highly reactive to the derivatives of that nitrogenous cytokinin base, to the riboside of which antibodies (corresponding to the purine base and its riboside) were obtained and had low cross-reactivity to the derivatives of the nitrogenous bases of another cytokinin: serum against zeatin riboside had a high reactivity to trans-zeatin and low reactivity to cis-zeatin and isopentenyl adenine (IP) and IPA, and serum against IPA had high reactivity to IP and low reactivity to zeatin and its riboside. Shoot apexes were cut for the separate immunolocalization of free bases and their ribosylated forms using the differential conjugation with tissue proteins before dehydration. The ribosylated forms were conjugated with 20 mM sodium periodate, activating the hydroxyl radicals of ribose and providing binding to the proteins. Since postfixation of tissues with an aldehyde mixture was performed after binding of ribosylated cytokinins with sodium periodate [15], binding of not only ribosylated but also free nitrogenous bases to proteins can occur. Therefore, a modified procedure was used, [16] preventing the conjugation of free bases with proteins by increasing the solution pH (up to 9.6) during the fixation of the leaf tissue with an aldehyde mixture. The details of the procedure were as follows. For binding of the ribosylated cytokinins with plant proteins, shoot apexes were treated with sodium periodate and then fixed with a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in 50 mM carbonate buffer (pH 9.6). Purine residues of free cytokinins were conjugated to proteins with a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in 100 mM phosphate buffer (pH 7.2). Ribosylated cytokinins did not bind with proteins with this fixation method (without pretreatment with sodium periodate) [15].

After fixation with an aldehyde mixture, shoot apexes were dehydrated in a series of ethanol dilutions (up to 96%). The tissues were then embedded in resin (JB-4, Electron Microscopy Sciences, United States) as recommended by the manufacturer. Histological sections (thickness of 1.5 μm) were prepared using a rotary microtome (HM 325, MICROM Laborgeräte, Germany) and placed on slides as described previously [13]. Sections were incubated for 30 min in 100 mM phosphate buffer (pH 7.2) containing gelatin and Tween 20 (PGT) and then for 2 h in a humid chamber at room temperature with serum against zeatin riboside or IPA (20 μL per each cut in serum, dilution 1 : 80). The specificity of the immune staining of the sections was verified by a nonimmune serum at the same dilutions. For the detection of antibodies bound to free bases or ribosides (depending on the method of fixation), antirabbit immunoglobulins (Aurion, United States) labelled with colloid gold and diluted with PGT (1 : 40) were placed on each section. Sections were postfixed in 2% glutaraldehyde and incubated with silver enhancer according to the manufacturer's recommendations (R-Gent SE-EM Silver Enhancement Reagent, Aurion, United States) for 30 min. The excess silver ions were removed by washing the glasses with distilled water and the sections were examined using an Axio Imager.A1 light microscope (Carl Zeiss Jena, Germany) equipped with a digital camera (AxioCam MRc5, Carl Zeiss Jena, Germany).

RESULTS AND DISCUSSION

Immune staining of the apex sections of Arabidopsis plant shoots for detection of cytokinins using specific sera with an affinity for zeatin derivatives and IPA (free bases and their conjugates) revealed the presence of derivatives of both forms of cytokinins in the cells of

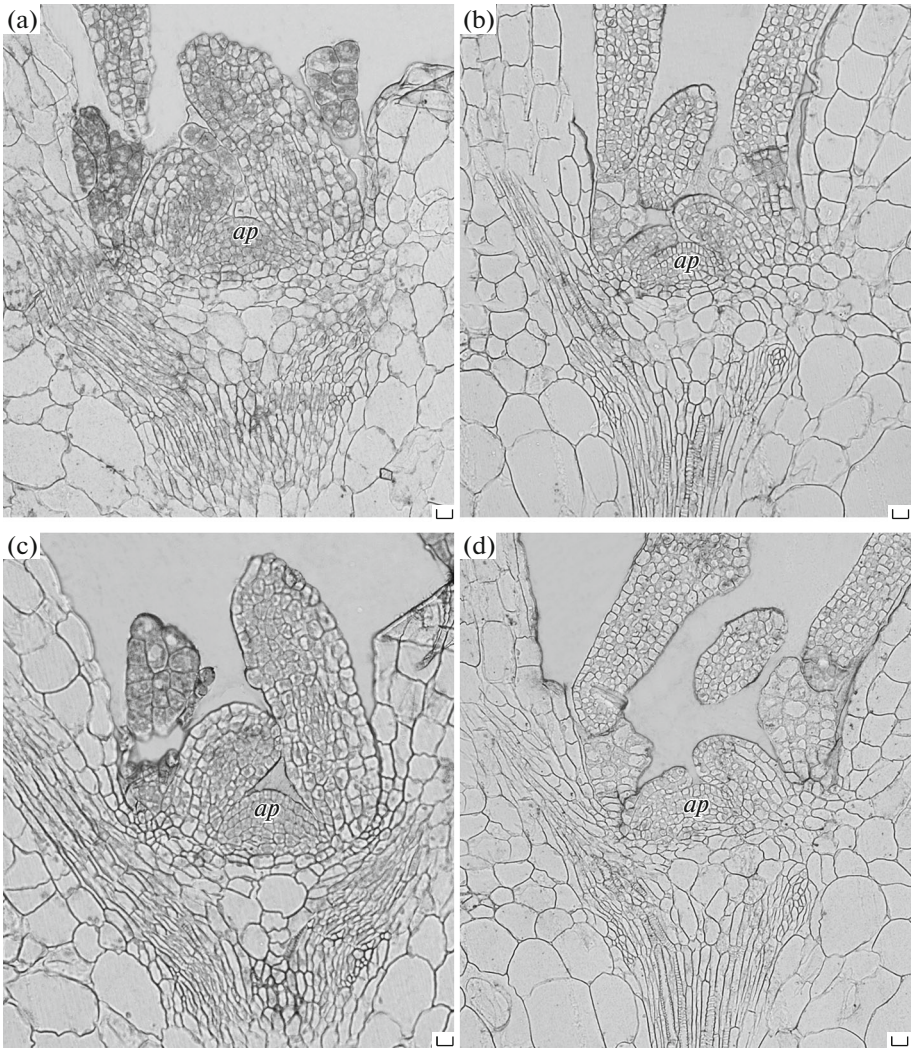

Fig. 1. Immunohistochemical localization of (a) zeatin, (b) riboside zeatin, (c) isopentenyl adenine, and (d) isopenteniladenosine in apex cells of Arabidopsis shoots. *ap*—apex. Ruler is 10 mm.

the apical shoot meristem and leaf primordia (Fig. 1). The staining was somewhat more intense in the case of sections fixed with an aldehyde mixture. This variant of fixation provided conjugation and subsequent detection of free bases but not ribosylated forms of cytokinins. The antibodies used in this study were prepared for a conjugate of protein with cytokinin ribosides. However, due to the specificity of fixation of the free bases of cytokinins with an aldehyde mixture (without pretreatment with sodium periodate) [15], conjugation of ribosylated forms did not occur, and they were washed out of tissues during dehydration. Therefore, for this fixation variant, immunohistochemical localization revealed precisely free forms of cytokinins.

Our data corresponded to information obtained using cytokinin biosensors. In these experiments, increased expression of the GUS-gene under the control of the cytokinin-sensitive *ARR5* gene promoter was detected in the apical meristem, leaf primordia, and shoot vessels [17]. At the same time, the advantage of immunohistochemical localization is the possibility of separate detection of both free and ribosylated forms of cytokinins (due to the fixation specific for these cytokinin forms) as well as differential staining for zeatin and isopentenyl adenine (due to the specificity of antibodies).

Since our task was the verification of the possible dependence of the accumulation of cytokinins in shoot apexes from their inflow from the roots, further studies were performed using antibodies against riboside zeatin (it is known that cytokinins are transported from the roots in the form of zeatin derivatives [18]). As can be seen from Figs. 2a and 2b, the intensity of staining of sections fixed with aldehydes decreased 2 h after the root cutting. These results can be interpreted as evidence of the dependence of the level of cytokinins in the shoot apex of Arabidopsis plants on the

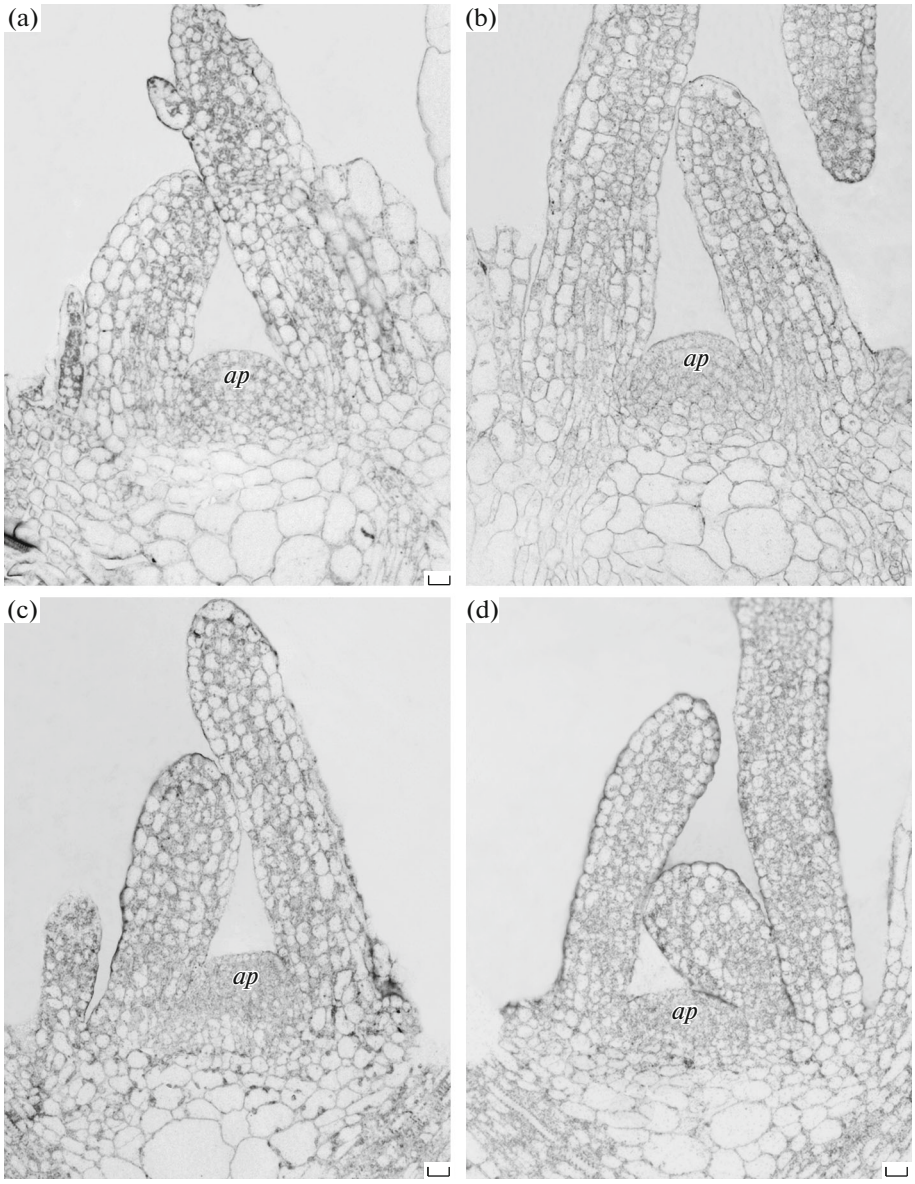

Fig. 2. Immunohistochemical localization of cytokinins (a, b—zeatin; c, d—riboside zeatin) in the apex cells of Arabidopsis shoots in the control and 2 h after removal of the roots: a, b—control (intact) plants; c, d—plants with the removed roots. *ap*—apex. Ruler is 10 mm.

influx of cytokinins from the roots. At the same time, the decreased intensity of staining was not detected on tissue sections fixed with sodium periodate (Figs. 2c, 2d). The absence of changes in the level of ribosylated forms of cytokinins in the shoot apex after root cutting contradicts the assumption about the role of cytokinin transport from the roots in regulation of their level in the apex since the transport function is attributed to the ribosides of cytokinins [18]. An alternative explanation could be that the root cutting affected the ability of shoots to synthesize cytokinins (for example, by reduction in the influx of nitrogen-containing compounds from the roots). For unambiguous interpretation of the results, further experiments are needed (for example, the evaluation of the effect of the removal of roots on the IP and IPA contents in apexes).

We have previously shown that the uptake of exogenous cytokinins by the root cells depends on the secondary active transport [13]. In order to check whether the accumulation of cytokinins in the shoot apex depends on their active absorption by the cells, we treated the shoots of intact plants with protonophore carbonyl cyanide m-chlorophenyl hydrazone. This treatment led to the impaired formation of the proton gradient on the cell membrane. The proton gradient is the source of energy for the secondary active transport of compounds (including free bases [13]) via the membranes. As can be seen from Figs. 3a and 3b, protono-

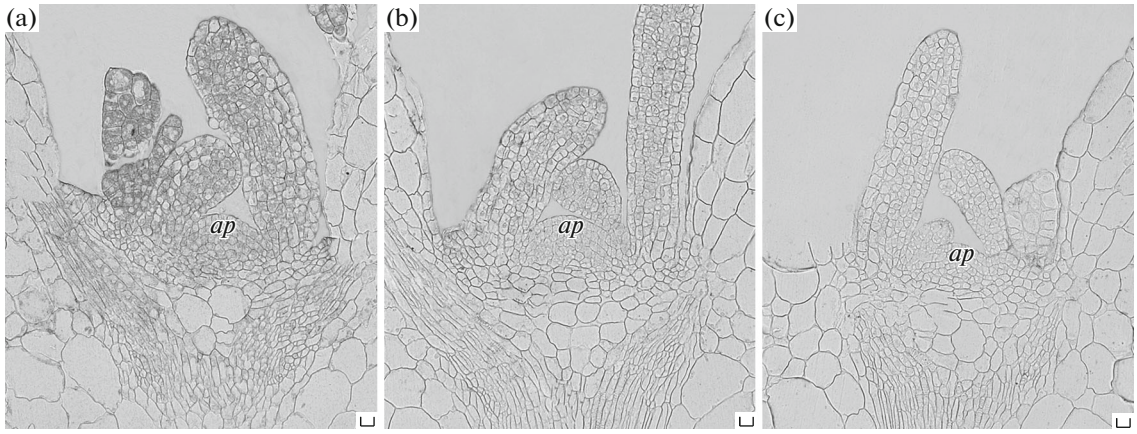

Fig. 3. Effect of protonophore treatment (carbonyl cyanide m-chlorophenyl hydrazone) on immunohistochemical localization of cytokinins (zeatin) in apex cells of Arabidopsis plants using serum against riboside zeatin: (a) control (intact) plant, (b) 30 min after protonophore treatment, (c) section treated with nonimmune serum, without antibodies to the studied hormones. *ap*—apex. Ruler is 10 mm.

phore treatment dramatically reduced the immune staining for zeatin of apical meristem cells fixed with an aldehyde mixture. The absence of staining in Fig. 3c in the case of using nonimmune sera confirms the specificity of the method. These results indicate the dependence of the accumulation of cytokinins on active hormone absorption by cells and correspond to the data obtained by the root cutting.

Recent studies demonstrated the role of transporters of purine bases from the PUP family capable of transferring the free bases of cytokinins, thereby participating in the regulation of their accumulation in cells of the shoot apex [19]. Since the inhibition of activity of these transporters by protonophore carbonyl cyanide m-chlorophenyl hydrazone [20] was demonstrated, these literature data correspond to the results that we obtained.

Thus, our experiments using the immunohistochemical localization method revealed a high level of cytokinins in shoot apexes and a dependence of the accumulation of free zeatin on the presence of roots and the secondary-active transmembrane transfer of cytokinins.

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