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CYTOKININS AND POLAR TRANSPORT OF AUXIN IN AXILLARY PEA BUDS

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Abstract

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The influence of cytokinin on auxin transport during release of axillary buds from apical dominance was studied. Expression of auxin-carrier coding genes *PsAUX1* (*AUXIN RESISTANT 1*) and *PsPIN1* (*PIN-FORMED 1*) was explored in axillary buds of the 2nd node of 7-day pea plants (*Pisum sativum* L.) cv. Vladan after decapitation or after exogenous application of benzyladenine (6-benzylaminopurine) onto axillary buds of intact plants. Localization of the PsPIN1 protein, the key factor for polar transport of auxin in axillary buds, was visualised by immunohistochemistry. After exogenous application of cytokinin the expression of *PsAUX1* and *PsPIN1* rapidly increased with a simultaneous rapid decrease in *PsDRM1* and *PsAD1* expression – genes related to bud dormancy. The same changes in expression were observed after decapitation, however they were markedly slower. The PsPIN1 auxin efflux carrier in the inhibited axillary buds of intact plants was localised in a non-polar manner. After exogenous application of cytokinin gradual polarisation of the PsPIN1 protein occurred on the basal pole of polar auxin transport competent cells. Despite the fact that direct auxin application to buds of intact plants led to an increase in *PsAUX1* and *PsPIN1* expression, the buds remained dormant (non-growing) what was accompanied by persistent expression of the dormancy markers *PsDRM1* and *PsAD1*. The results indicate a possible effect of cytokinins on biosynthesis, and/or transport of auxin in axillary buds and they highlight the importance of auxin-cytokinin crosstalk in the regulation of bud outgrowth after breaking of apical dominance.

apical dominance, cytokinins, pea, polar auxin transport

Abbreviations: BAP – benzyladenine, CK – cytokinin, IAA – indole-3-acetic acid, RNA – ribonucleic acid, RT-PCR – reverse transcription polymerase chain reaction

Apical dominance is a phenomenon where the stem apex inhibits the growth and development of axillary buds. Removal of the shoot apex is followed by release of axillary buds from inhibition and their outgrowth into lateral shoots. Already Thimann and Skoog (1934) proved that the inhibiting effect of the stem apex could be simulated by an application of the plant hormone auxin (indole-3-acetic acid). From the site of biosynthesis in young leaves of the stem apex auxin is transported to the base of the stem in a polar manner. Active polar transport of auxin from one cell into another is realised in xylem parenchyma, procambium and cambium cells (Gälweiler *et al.,* 1998) and is mediated by the activity of specific auxin carriers. The auxin influx carrier e.g. AUX1 protein catalyses auxin transport into the cell (Bennett *et al.,* 1996), while the efflux of auxin from the cell is dependent on the activity of auxin efflux carriers e.g. proteins of the PIN family (Vieten *et al.,* 2005) and proteins of the PGP group (Geisler *et al.,* 2005). The polarity of auxin basipetal flux in the stem is determined by polar localization of carrier molecules of PIN1 proteins on the basal pole of auxin transport-competent cells (Wisniewska *et al.,* 2006).

The exact mechanism of auxin-induced growth inhibition of axillary meristems has not yet been fully clarified. According to Bangerth (1989) polar transport of auxin from the dominating organ (stem apex) blocks the export of auxin from the in-

hibited organs (axillary buds) into the primary stem. This so-called autoinhibition of polar auxin transport occurs at the sites of junction of the dominant and inhibited organs. According to this hypothesis the export of auxin from the lateral bud to the main stem is essential for its growth.

Another group of plant hormones of crucial importance in regulation of shoot branching are cytokinins. A number of experimental studies proved that cytokinins weaken the apical dominance (Ongaro and Leyser, 2008). Direct application of cytokinins onto axillary buds stimulated their growth even if the apical bud was intact (Sachs and Thimann, 1967). It was also confirmed that after stem decapitation the level of endogenous cytokinins in the axillaries increased significantly and that it is correlated with the onset of axillary bud growth (Turnbull *et al.,* 1997). The effect of decapitation on the CK levels can be eliminated by treating the stem stumps of the decapitated plants with auxin (Blažková *et al.,* 1999). After decapitation the axillary buds can be supplied with cytokinins from two sources. In pea plants auxin regulates negatively the local biosynthesis of cytokinins in the stem by controlling the expression of specific adenosinemonophosphate-isopentyltransferases *PsIPT1* and *PsIPT2* which play a key role in cytokinin biosynthesis and after decapitation CKs are synthesised directly in the stem (Tanaka *et al.,* 2006). Secondly, the cytokinins from the roots are transported into the axillary buds via xylem, what was confirmed by experiments with ^{14}C-labelled cytokinins (Procházka and Jacobs, 1984) and by analyses of the levels of cytokinins in the xylem exudate after decapitation (Bangerth, 1994). Studies of the branching *ramosus* (*rms*) pea mutants proved that the *RMS2* gene was the key gene controlling the level of cytokinins exported by the xylem from the roots to the stem (Foo *et al.,* 2007). Recently also the mobile inhibitory substance strigolactone was identified (Gomez-Roldan *et al.,* 2008; Umehara *et al.,* 2008) that in contrary to cytokinins inhibits axillary bud outgrowth.

The growth activity of axillary buds was correlated with the decrease in the expression of *PsDRM1* (*DORMANCY-ASSOCIATED PROTEIN 1*) and *PsDRM2*. (Stafstrom *et al.,* 1998) and also *PsAD1* (*APICAL DOMINANCE 1*) and *PsAD2* genes (Madoka and Mori, 2000). The role of these genes in apical dominance has not been clarified either, however the *DRM* and *AD* genes are excellent markers of the dormant state of axillary buds.

The objective of the present study was to explore the establishment of polar transport of auxin in axillary buds of intact plants after exogenous application of the cytokinin benzyladenine (BAP), and to compare it to auxin application that does not cause bud outgrowth. Attention was focused namely on the expression of auxin carrier genes *PsPIN1* and *PsAUX1*, the *PsDRM1* and *PsAD1* genes associated with bud dormancy and on localization of the auxin efflux carrier PsPIN1.

MATERIALS AND METHODS

Plant material: Seeds of pea (*Pisum sativum* L.) cv. Vladan (Semo, Smržice) were imbibed for 24 hours in water at room temperature and then sown into perlite. Five days after sowing the plants were transplanted into cultivation vessels and cultivated in a hydroponic culture in Richter's nutrient solution (Richter, 1926) in a growth chamber at 20/18 °C, photoperiod 16/8 h and light intensity 150 µmol.m^{-2}.s^{-1}. All the analysed changes were studied in axillary buds of the 2nd node in 7-day-old plants because at this age the seedlings have not yet developed the functional vascular connection between the axillaries of this node and the vasculature of the main stem. On the contrary, in 9 – 13-day old plants the vascular connection has already been formed (Tepper, 1993).

Experimental setup: A single application of exogenous cytokinin or auxin to the apical part of the axillary bud in the form of lanolin paste containing 1% of benzyladenine or 0.5% IAA, was made. The hormonal paste was applied to the largest axillary bud of the 2nd node. The other accessory buds were not included in the following analyses. Decapitation was performed 10 mm above the 2nd node. Axillary buds of intact plants not treated with hormonal paste were analysed as the control treatment.

The length of the axillary buds was measured daily for 6 days after the application of exogenous cytokinin. For comparison we also measured the length of the axillaries of decapitated plants and as a control the length of axillary buds of intact plants not treated with cytokinin. In each variant 20 plants were measured.

Semiquantitative RT-PCR: At certain time intervals after decapitation or application of cytokinin or auxin to the axillary buds we studied the expression of *PsDRM1*, *PsAD1*, *PsAUX1*, *PsPIN1* genes and the constitutively expressed gene *DEAD box* (Bai and DeMason, 2006) as an internal control. Total RNA was isolated from axillary buds using the RNeasy® Plant Mini Kit (Qiagen, Venlo, The Netherlands). Semi-quantitative two-step RT PCR was carried out using the Enhanced Avian RT-PCR Kit (Sigma-Aldrich, St. Louis, USA). The sequences of specific primers were derived from sequences of the relevant genes published in the GenBank using the Primer 3 programme (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). In the PCR reaction the used primers specify the amplification products of the following sizes: *PsDB1* 1191 bp, *PsDRM1* 352 bp, *PsAD* 173 bp, *PsPIN1* 306 bp and *PsAUX1* 307 bp. After electrophoretic separation the PCR products were assessed using the GelWorks 1D Intermediate programme (UVP, Upland, USA). The obtained data corresponding to the levels of mRNA of the individual genes in the respective sample were expressed as the value relative to the constitutively expressed gene *PsDB1*. Data from two independent series were assessed and each series was repeated twice. For each variant axillary buds from 10 plants were used.

Immunolocalization of the PIN1 protein: Localization of the PIN1 protein was studied on 12 µm thick longitudinal sections of the axillary bud according to (Paciorek *et al*., 2006) using rabbit polyclonal antibody anti-*Arabidopsis*-PIN1 which is also bound to the homologous PIN1 protein of pea (Sauer *et al.,* 2006). In the experiments the following antibodies and dilutions were used: anti-AtPIN1 (1 : 500) and secondary polyclonal Cy3-anti-rabbit antibody (1 : 500) (Dianova, Hamburg, Germany). The objects were scanned on a confocal laser microscope TCS SP2 AOBS (Leica, Mannheim, Germany; using lenses 20×/0.7, or 63×/1.4). The luminance and contrast of the scans was modified by means of the programme Photoshop 7.0 (Adobe Systems Incorporated, San Jose, California, USA).

Statistical evaluation: The programme Microsoft Excel ver. 2002 was used for statistical analysis of the obtained results. From replications of each variant the mean value and standard deviations were assessed. Student's t-test was performed to test the significance of differences between the individual variants.

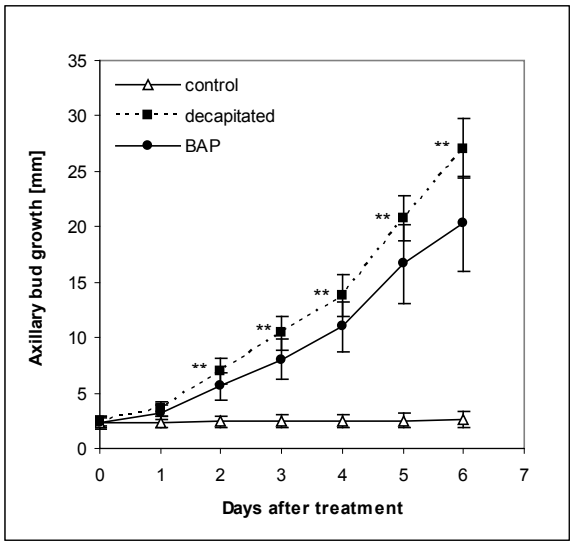
RESULTS AND DISCUSSION

Effect of exogenous CK on axillary bud outgrowth. The growth of the pea axillary bud of the second node after exogenous BAP application was compared with its growth on intact and decapitated plants. During 24 hours after treatment the response of axillary buds to stem decapitation and exogenous application of BAP was similar; in later intervals the growth of the axillaries of decapitated plants was significantly higher (Fig. 1). No significant growth of axillaries of intact plants in the entire experiment was detected. Likewise Sachs and Thimann (1964) stated that 2–3 days after treatment growth triggered by the application of kinetin was comparable to growth after decapitation; however later the elongation growth of axillaries of decapitated plants was faster.

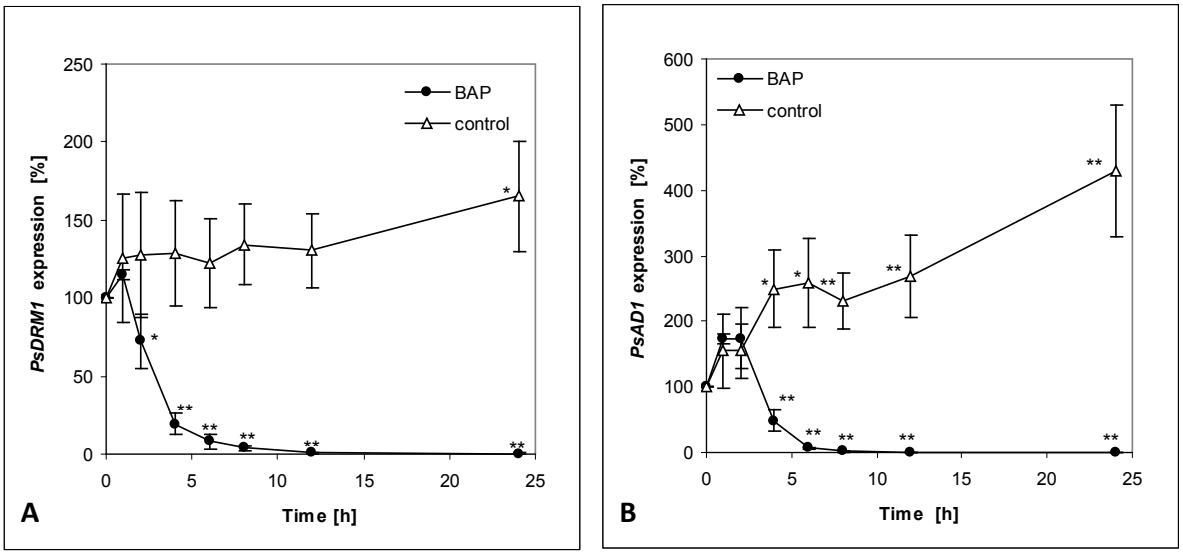
Expression of dormancy associated genes after CK application Although growth of axillary buds after BAP application in longer time intervals was slower than of the decapitated plants, during the 24 hours after treatment the changes at the level of gene expression were considerably faster in the cytokinin-treated plants. As early as 2 hours after BAP application a significant decrease in the expression of the *PsDRM1* gene in axillary buds was observed. Between 8 and 12 hours after treatment the expression almost disappeared (Fig. 2A). 2–4 hours after treatment the decrease in the expression of the *PsAD1* gene was also rapid and significant; after 6 hours the levels of the transcript dropped to zero (Fig. 2B). In axillary buds not treated with BAP the expression of both genes remained high (Fig. 2). By contrast, in axillary buds of the 2nd node Balla *et al*., (2005) observed after decapitation that the expression of *PsDRM1* decreased during 3 hours and the expression of *PsAD1* not until 6 hours. The expression of both genes disappeared completely within 12 hours after decapitation. Similar effect of exogenous BAP application on the *PsAD1* expression in pea axillary buds was observed after 16 h by Madoka *et* Mori (2000).

Influence of CK application on auxin carrier genes. BAP application induces a rapid increase in the expression of genes coding auxin carriers. Between 1 and 6 hours after application we measured a significant increase in *PsAUX1* expression culminating between 2 and 4 hours and slightly later similarly increased also *PsPIN1* expression; later on both gene expressions returned to its original values. The expression of *PsAUX1* also increased after stem decapitation; however it is not significant until after 3 to 24 hours, culminating between 6 and 12 hours (Fig. 3A). In contrary the expression of *PsPIN1* after decapitation decreased at first; however between 3 and 24 hours the expression was temporarily restored approximately to its original level (Fig. 3B). Some recent studies report on the direct effect of CK on the *PIN* genes expression. Růžička *et al.* (2009) described differential effect of CK in Arabidopsis root, where strong negative effect on *AtPIN1* and *AtPIN4* expression, positive effect on *AtPIN7* expression and small negative effect on *AtPIN2* expression were detected. On the other hand Pernisová *et al.* (2009) observed up-regulation of *AtPIN6*, down-regulation of *AtPIN2* and only slight effect on *AtPIN1* expression in Arabidopsis hypocotyl explants after exogenous cytokinin treatment. It is obvious from these results that the effect of CK on *PIN* expression is gene and tissue specific.

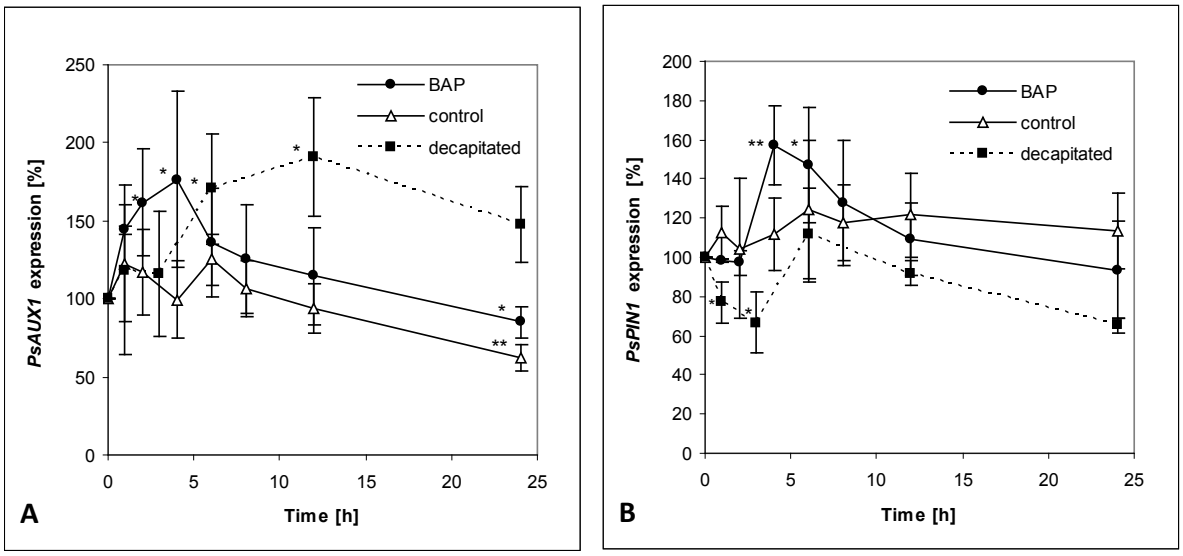
Influence of exogenous CK application on PsPIN1 localization. In inhibited axillary buds of intact plants localization of the auxin efflux carrier protein PsPIN1 was not polar (Fig. 4A, B). Similarly



1: *The response of large axillary bud of the second node to decapitation and exogenous BAP application. The statistical significance of the identified differences between buds of decapitated and BAP treated plants (T-test) at α 0.05 and 0.01 is designated (* and **, respectively). Error bars show standard deviations (n = 20).*



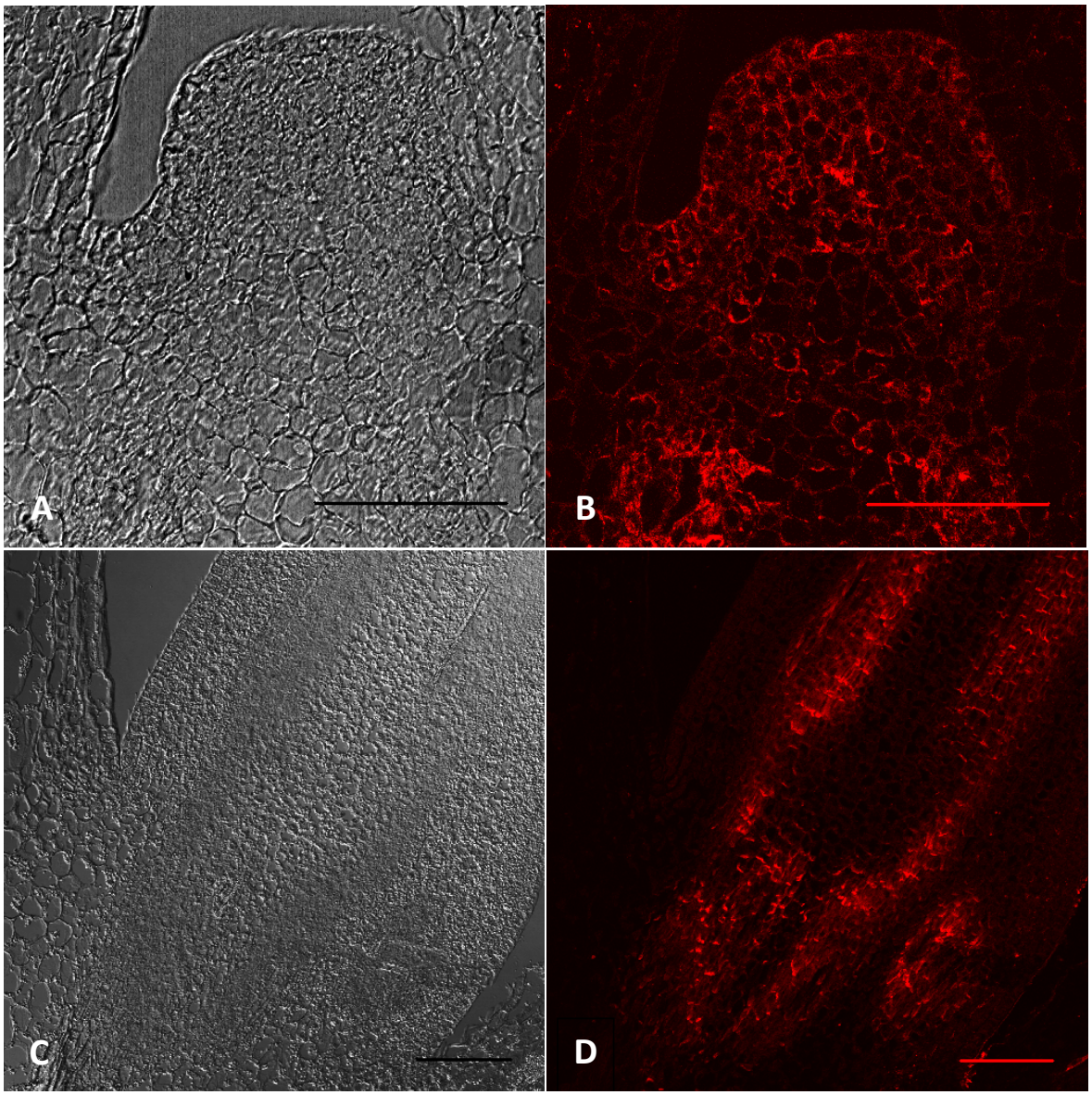
2: *The expression of PsDRM1 (A) and PsAD1 (B) in large axillary bud of the 2nd node of intact plants after exogenous BAP application and control treatment with lanoline. The statistical significance of the identified differences in comparison to buds from intact plants at time 0 h (T-test) at α 0.05 and 0.01 is designated (* and **, respectively). Error bars show standard deviations (n = 4).*



3: *The expression of PsAUX1 (A) and PsPIN1 (B) in large axillary bud of the 2nd node of intact plants after exogenous BAP application, control treatment with lanoline and of decapitated plants. The statistical significance of the identified differences in comparison to buds from intact plants at time 0 h (T-test) at α 0.05 and 0.01 is designated (* and **, respectively). Error bars show standard deviations (n = 4).*

like after decapitation (Balla *et al.*, 2005) also exogenous application of cytokinins on axillary buds of intact plants caused polarisation of the PsPIN1 protein in procambial strands (Fig. 4C, D). The results from decapitated plants indicated, that in the case of PsPIN1 re-localization of already synthesised proteins present in the axillaries of intact plants was obviously more important for the establishment of polar auxin transport in the buds than *de novo* biosynthesis of proteins. With the release of buds from growth inhibition by CK application synthesis of auxin carriers occurred (Fig. 3A, B) and both PsPIN1 *de novo* biosynthesis and re-localization could contribute to the faster response of axillary buds to CK application than to decapitation. It is known that re-localization of PIN proteins plays an important role in the regulation of a whole range of developmental processes in plants (Blakeslee *et al.*, 2004; Friml *et al.*, 2003).

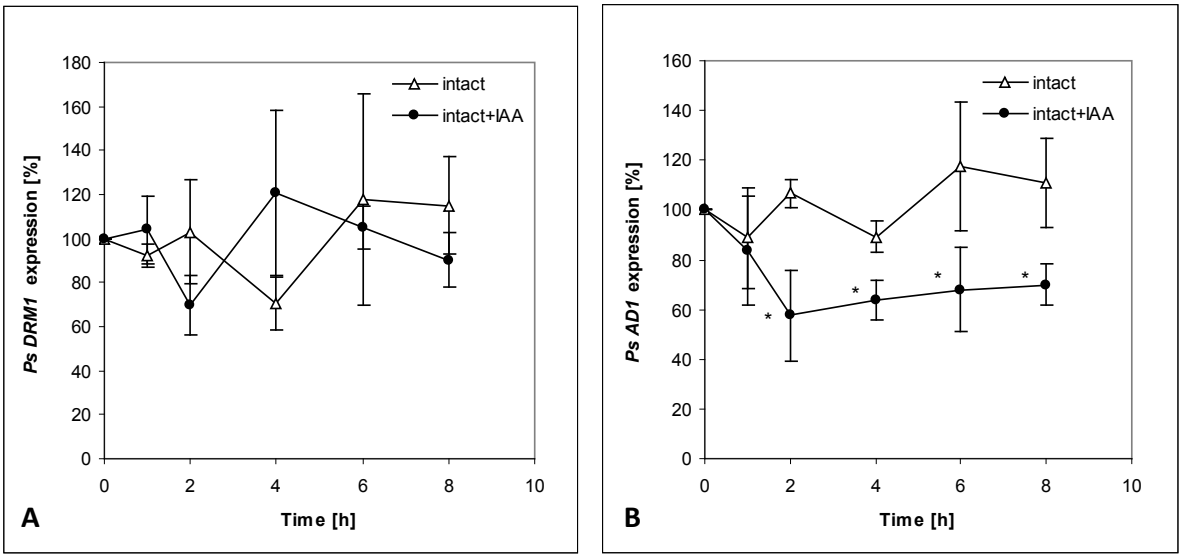
Effect of exogenous auxin on axillary buds. From the obtained data and from other published works is evident, that there is after decapitation an increase of IAA in the buds (Gocal *et al.*, 1991, Balla *et al.*, 2002) that promote its canalization mainly by polarization of PsPIN1 (Balla *et al.*, 2005). Despite this fact direct application of auxin to the buds does not release axillaries from inhibition as documented already by Thimann (1937)



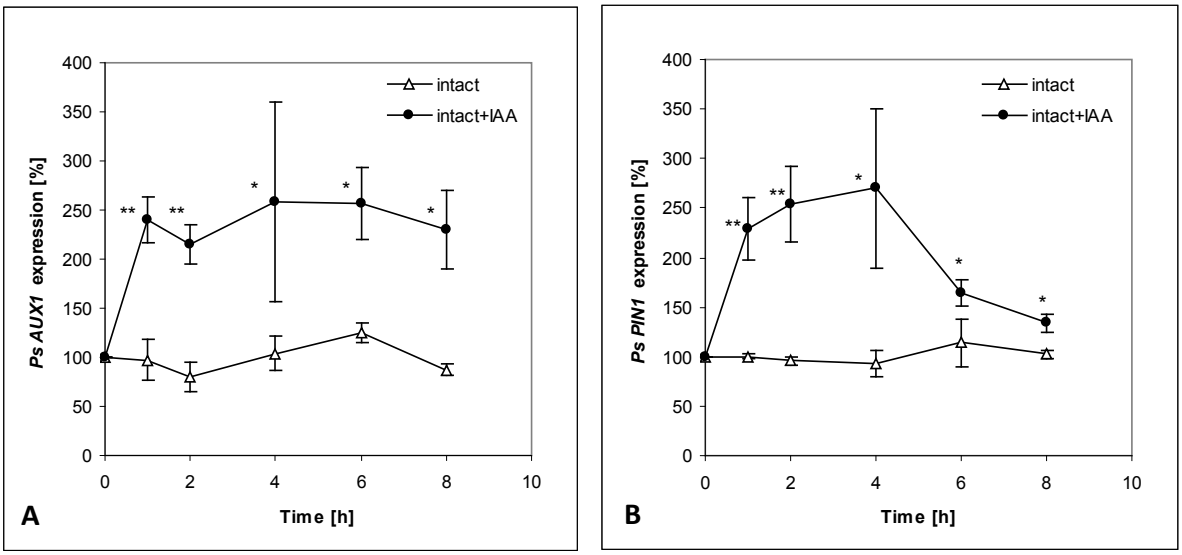
4: *Localization of PIN1 protein in large axillary bud of second node of intact plant (A, B) and 24 hours after exogenous BAP application (C, D). Pictures on the left represent transmitted light overview of objects, pictures on the right show immunolocalization signals of PIN1 (in red). Scale bars = 50 μm.*

and the applied auxin is not exported into the stem (Balla *et al*., 2007). Therefore we tested the influence of application of IAA to the axillary buds on the expression of auxin carrier and dormancy associated genes. As expected the auxin application caused dramatic increase in *PsAUX1* (Fig. 6A) and *PsPIN1* expression (Fig. 6B) already 1 hour after treatment. This is in accordance with the known inducibility of the genes (Hoshino *et al*., 2005; Vieten *et al*., 2005). Importantly only partial decrease in *PsAD1* expression (Fig. 5B) and no significant changes in *PsDRM1* expression were detected (Fig. 5A) proving that they are at least suitable markers of dormant (non-growing) state of the buds. Thus it is evident that increase of auxin levels in axillary buds itself is not sufficient to trigger their outgrowth and mutual interactions between auxin and cytokinin are necessary.

Conclusions. The several fold increase in the expression of auxin carrier genes and the more rapid decrease of dormancy associated gene expression after BAP application than after decapitation suggest that cytokinins are factors of ultimate importance for the activation of axillary bud outgrowth. Increased expression of the auxin–inducible *PsAUX1* and *PsPIN1* genes (Hoshino *et al*., 2005; Vieten *et al*., 2005) after BAP application indeed indicates that the level of auxin in the buds is increasing. There was reported not only the direct effect of CK on auxin carrier genes, but also increased IAA levels due to CK increase. This effect was measured in cytokinin-overproducing transgenic lines of tobacco (Binns *et al*., 1987), in pea root tips after exogenous BAP application (Bertell *et* Eliasson, 1992) and in maize roots after zeatin application (Bourquin et



5: *The expression of PsDRM1 (A) and PsAD1 (B) in large axillary bud of the 2nd node of intact plants after exogenous IAA application and control treatment with lanoline. The statistical significance of the identified differences in comparison to buds from intact plants at time 0 h (T-test) at α 0.05 and 0.01 is designated (* and **, respectively). Error bars show standard deviations (n = 4).*



6: *The expression of PsAUX1 (A) and PsPIN1 (B) in large axillary bud of the 2nd node of intact plants after exogenous IAA application and control treatment with lanoline. The statistical significance of the identified differences in comparison to buds from intact plants at time 0 h (T-test) at α 0.05 and 0.01 is designated (* and **, respectively). Error bars show standard deviations (n = 4).*

Pilet, 1990). On the contrary Nordstrom *et al*. (2004) studied the effect of induced cytokinin overproduction on auxin levels in Arabidopsis plants with glucocorticoid-inducible *ipt* expression. The biosynthesis and overall IAA pool of the plant were unaffected during the first 24 h. Later both the pool size and IAA biosynthesis were substantially reduced.

After exogenous application of cytokinins Li and Bangerth (2003) observed that IAA export from the treated apex and the capacity of polar auxin transport in the stem internode under the apex considerably increased. During the release of buds from apical dominance according to Li and Bangerth (1992) cytokinins could act as modifiers/stimulators of biosynthesis and/or transport of IAA. Our results showing a rapidly increasing expression of the genes of auxin transport carriers after BAP application, along with polarisation of the PsPIN1 protein, support this hypothesis.

SUMMARY

The objective of the present study was to explore the influence of cytokinin on the polar auxin transport in axillary buds during release of buds from apical dominance. All the analysed changes were studied in axillary buds of the 2nd node of 7-day-old pea plants (*Pisum sativum* L.) cv. Vladan. Expression of auxin-carrier coding genes *PsAUX1* (*AUXIN RESISTANT 1*) and *PsPIN1* (*PIN-FORMED 1*) and dormancy associated genes *PsDRM1* (DORMANCY-*ASSOCIATED PROTEIN 1*) and *PsAD1* (*APICAL DOMINANCE 1*) after decapitation or after exogenous application of benzyladenine (6-benzylaminopurine, BAP) onto axillary buds of intact plants was studied by semi-quantitative two-step RT PCR. For comparison, the gene expression in axillary buds of exogenous IAA treated plants was also explored. The *PsDEADbox* expression level was used as internal control. Localization of the PsPIN1 protein, the key factor for polar transport of auxin in axillary buds, was visualised immunohistochemically on longitudinal sections of the axillary bud according to Paciorek *et al.* (2006). The objects were scanned on a confocal laser microscope TCS SP2 AOBS (Leica, Mannheim, Germany). After exogenous application of cytokinin the expression of *PsAUX1* and *PsPIN1* rapidly increased with a simultaneous rapid decrease in *PsDRM1* and *PsAD1* expression. The same changes in expression were observed after decapitation; however they were markedly slower. The PsPIN1 auxin efflux carrier in the inhibited axillary buds of intact plants was localised in a non-polar manner. After exogenous application of cytokinin gradual polarisation of the PsPIN1 protein occurred on the basal pole of polar auxin transport competent cells. Despite the fact that direct auxin application to buds of intact plants also led to an increase in *PsAUX1* and *PsPIN1* expression, the buds remained dormant (non-growing) what was accompanied by persistent expression of the dormancy markers *PsDRM1* and *PsAD1*. The results indicate a possible effect of cytokinins on biosynthesis, and/or transport of auxin in axillary buds and they highlight the importance of auxin-cytokinin crosstalk in the regulation of bud outgrowth after breaking of apical dominance.

SOUHRN

Cytokininy a polární transport auxinu v axilárních pupenech hrachu

V této práci byl sledován vliv cytokininu na polární transport auxinu v axilárních pupenech 2 nodu sedmidenních rostlin hrachu setého (*Pisum sativum* L.) cv. Vladan v průběhu jejich uvolňování z apikální dominance. V axilárních pupenech dekapitovaných, příp. exogenní aplikací benzyladeninu ošetřených intaktních rostlin byla metodou dvoukrokové, semikvantitativní RT PCR studována exprese genů *PsAUX1* (*AUXIN RESISTANT 1*) a *PsPIN1* (*PIN-FORMED 1*), které kódují auxinové přenašeče, a genů *PsDRM1* (DORMANCY-*ASSOCIATED PROTEIN 1*) a *PsAD1* (*APICAL DOMINANCE 1*), jejichž exprese souvisí s dormantním, resp. korelačně inhibovaným stavem pupenů. Pro srovnání byla sledována i exprese těchto genů v axilárních pupenech ošetřených exogenně aplikovaným auxinem. Exprese genu *PsDEADbox* byla použita jako vnitřní kontrola. Lokalizace PIN1 proteinu, klíčového faktoru polárního transportu auxinu v axilárních pupenech, byla vizualizována imunohistochemicky na podélných řezech axilárním pupenem podle metodiky Paciorek *et al.* (2006). Objekty byly snímány laserovým konfokálním mikroskopem TCS SP2 AOBS (Leica, Mannheim, Germany). Po exogenní aplikaci cytokininu na axiláry byl prokázán rychlý vzestup exprese genů *PsAUX1* a *PsPIN1* se současným poklesem exprese genů *PsDRM1* a *PsAD1*. Obdobné změny v expresi byly zaznamenány rovněž v axilárech dekapitovaných rostlin, byly však zřetelně pomalejší. Auxinový přenašeč PsPIN1 protein nebyl v axilárních pupenech intaktních rostlin lokalizován polárně. Po aplikaci cytokininu došlo k postupné polarizaci PIN1 proteinu na bázi auxin transportujících buněk. Přestože přímá aplikace auxinu na axilární pupeny intaktních rostlin vedla rovněž ke zvýšení exprese genů *PsAUX1* a *PsPIN1*, pupeny zůstaly inhibovány, což prokazuje přetrvávající exprese markerů dormantního stavu genů *PsDRM1* and *PsAD1*. Dosažené výsledky ukazují na možný efekt cytokininů na biosyntézu, respektive transport auxinu v axilárních pupenech a vyzdvihují význam vzájemných interakcí auxinu a cytokininů při uvolňování pupenů z lodyžní apikální dominance.

apikální dominance, hrách setý, cytokininy, polární transport auxinu

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