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BRIEF COMMUNICATION

Cytokinin oxidase/dehydrogenase activity as a tool in gibberellic acid/cytokinin cross talk

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Abstract

Changes in endogenous cytokinin (CK) content and cytokinin oxidase/dehydrogenase activity (CKX) in response to gibberellic acid (GA_3) in two pea cultivars with different life span were assessed. The control leaves of cv. Scinado, which developed faster, had higher initial cytokinin content and lower CKX activity, while opposite trend was observed in cv. Manuela with longer life span. Increased CKX and decreased CK content were detected in leaves of cv. Scinado after treatments with 0.5, 1 and 5 μM GA_3. Changes in CK content and CKX activity in GA_3-treated cv. Manuela leaves were reciprocal to those in cv. Scinado. CK content and CKX activity in roots were not significantly influenced by the application of GA_3. The slight repression of CKX activity in some of the root samples was accompanied by increased isopentenyladenine and isopentenyladenine riboside content. Obtained results suggest that CKX was responsible for the changes in endogenous cytokinin pool in GA_3-treated plants and most probably this enzyme represents an important link in GA/cytokinin cross talk.

Additional key words: isopentenyladenine, pea, *Pisum sativum*, zeatin.

Gibberellins (GAs) and cytokinins (CKs) control different developmental processes in plants. CKs act early during shoot initiation and control meristem activity, while GAs are responsible for expansion and cell division in shoot elongation, flowering and seed germination. All phytohormones exert their regulatory role in close relation with each other. Hormone signalling pathways form complex interacting network, which enables perceiving of numerous internal and external stimuli and generating respective plant responses. Additionally, exogenously applied growth regulators can alter the content of endogenous phytohormones (Pospíšilová 2003). Thus hormones may influence a wide range of physiological events during plant growth and development acting both in synergism and in antagonism with other phytohormones. Relatively little information is available concerning interaction between GAs and CKs. Both hormones delay senescence in some plant species (Jacob-Wilk *et al.* 1999, Mok and Mok 1994) and induce stomatal opening (Pospíšilová 2003). Huang *et al.* (2003) showed that GAs and CKs promote male development in tobacco and *Arabidopsis*. An antagonistic action of these two hormones was reported by Greenboim-Wainberg *et al.* (2005) in wild type *Arabidopsis* plants, where GA_3 and benzyladenine (BA) had opposite effect on anthocyanin accumulation and root elongation. Earlier

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Abbreviations: ABA - abscisic acid; BSA - bovine serum albumin; *cis*Z - *cis* zeatin; *cis*ZR - *cis* zeatin riboside; *cis*ZRP - *cis* zeatin riboside monophosphate; CK - cytokinin; CKX - cytokinin oxidase/dehydrogenase; GA - gibberellin; GA_3 - gibberellic acid; HPLC - high performance liquid chromatography; iP - isopentenyladenine; iPR - isopentenyladenine riboside; iPRP - isopentenyladenine riboside monophosphate; PMSF - phenylmethylsulfonyl fluoride.
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Werbrouck *et al.* (1996) in a study with *Spathiphyllum floribundum* demonstrated that GA_3 added to the basal medium influenced negatively root number and total root length, and exerted considerable decrease of endogenous CKs. Regarding preliminary information available on CK/GA cross talk it seems that the interaction between the two natural plant growth regulators are under fine-tune control. Greenboim-Wainberg *et al.* (2005) assumed that GAs act on a main branch of the signalling pathway common to most CK responses. They proposed a mechanism explaining the coordination between the two hormones and hypothesized that SPY (a negative regulator of GA responses) was involved in GA/CK homeostasis.

One of the mechanisms for regulation of endogenous CK content is inactivation of the hormones by cytokinin oxidase/dehydrogenase (CKX; EC 1.5.99.12) – the only known enzyme, which performs the degradation of adenine-type cytokinins. CKX performs the cleavage of the N^6-(isopent-2-enyl)-side chain resulting in formation of adenine-type compounds and corresponding isopentenyl aldehyde (Galuszka *et al.* 2001). Under normal growth conditions the enzyme maintains the homeostasis of endogenous CK levels required for plant growth and development (Kamínek *et al.* 1997). It has been detected that exogenously applied purine type CKs resulted in an increase in CKX activity (Redig *et al.* 1997). In several studies it has been stated that *N*-(2-chloro-4-pyridyl)-*N*1-phenylurea (CPPU) acts as an enzyme activity inhibitor by non competitive (Burch and Horgan 1989) or competitive (Bilyeu *et al.* 2001) manner. Response of CKX towards exogenous plant growth regulators, apart from CKs, has not been an object of profound scientific research yet.

Growing evidence supports the existence of relation-ship between hormone signalling and developmental factors (reviewed in Chow and McCourt 2004). This was the reason to choose two pea cultivars which differed in developmental pattern when grown under controlled conditions in order to study the effect of exogenous GA on CK metabolism. The seedlings of the two cultivars were of the same age but they differed in leaf formation rate and number of expanded leaves. Thus, being at different developmental stage the individuals from both cultivars were expected to exhibit diverse cytokinin contents.

Pisum sativum L. cv. Scinado normally requires shorter growing period than cv. Manuela. Pea seedlings were grown in half-strength Hoagland's solution in a growth chamber (12-h photoperiod, photon flux density of 70 μmol m^{-2} s^{-1}, and day/night temperature of 24/20 ºC). GA_3 was applied to nutrient solution in final concentrations 0.5, 1, or 5 μM to 17-d-old seedlings with 4 and 5 fully expanded leaves in cvs. Manuela and Scinado, respectively. Samples for the analyses were collected 48 h after GA_3 application.

Material from the last fully expanded leaves (about 0.5 g) and secondary roots (about 2.0 g) from at least 10 different individuals (19-d-old) was frozen and preserved in liquid nitrogen till the analysis. Specific CKX activity was measured on the basis of 3-methyl-2-butenal production according to Liberos-Minotta and Tipton (1995) protocol. Material was ground in 2.0 cm^3 extraction buffer (pH 6.9), containing 50 mM potassium-acetate, 2 mM $CaCl_2$, 1 mM $MgSO_4$ and 0.5 mM dithiothreitol. The extracts were centrifuged twice: at 12 000 *g* for 50 min and then at 12 000 *g* for 40 min after additional treatment with 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 25 mg cm^3 streptomycin sulphate, and 0.1 % solution of bovine serum albumin (BSA). The CKX activity was measured at 37 ºC for 50 min in a final volume of 1.05 cm^3 100 mM imidazole buffer (pH 6.5) containing $CuCl_2$ and 0.050 mM isopentenyladenine (iP). Absorbance of the samples (λ = 352 nm) was measured on *Shimadzu* (Kyoto, Japan) spectrophotometer *UV-1601*. Soluble protein was determined according to Bradford (1976) using BSA as a protein standard. Chemicals used were purchased from *Sigma-Aldrich* (Shaftesbury, UK). All measurements were made in triplicates and standard errors were calculated with *SigmaPlot for Windows*, version *8.00* software.

Plant material derived from the last fully expanded leaves (about 1.0 g) and secondary roots (1.5 g) was frozen in liquid nitrogen and freeze-dried. Extraction and purification of the freeze-dried samples followed the procedure described in Lexa *et al.* (2003). Material was extracted overnight at -20 ºC with Bieleski solvent (Bieleski 1964). The extracts with added deuterium-labelled cytokinins as internal standards were centrifuged at 15 000 *g* and passed consecutively through a set of two *Sep-Pak* C_{18} cartridges (*Waters Corporation*, Milford, MA, USA) connected in series, and after evaporation to water phase and adjustment of pH to 6.5 through *DEAE Sephadex* column and *Sep-Pak* C_{18} cartridges. CK bases, ribosides and glucosides were eluted twice with 80 % methanol and evaporated to dryness. CK phosphates were eluted with 1 M NH_4HCO_3. These fractions were passed through *Sep-Pak* C_{18} cartridges and eluted with 80 % methanol. Then this fraction was evaporated to water phase and the sample was allowed to react with alkaline phosphatase for 30 min at 37 ºC. After neutralisation and passage trough *Sep-Pak* C_{18} cartridges, CKs (ribosides) released from nucleotides were eluted with 80 % methanol and evaporated to dryness.

CK fractions of *cis* zeatin (*cis*Z), *cis* zeatin riboside (*cis*ZR), *cis* zeatin riboside monophosphate (*cis*ZRP), isopentenyladenine (iP), isopentenyladenine riboside (iPR), and isopentenyladenine riboside monophosphate (iPRP), were separated and quantified by high performance liquid chromatography (HPLC) (*FLUX Rheos 2000* quaternary pump and *CTC Analytics HTS PAL* autosampler with *CSI 6200* series HPLC oven) linked to a mass spectrometer (MS) (*Finningan LCQ*, San José, USA) equipped with an *ESI* source. Data were

processed at MS/MS full scan, two microscans at maximum ion time 100 ms. Values represent the mean of LC/MS/MS measurements in two replications.

The two pea cultivars differed in their initial CK contents and CKX activity before application of GA_3. Lower CK content and higher enzymatic activity were found in cv. Manuela leaves (Fig. 1) compared to those in cv. Scinado (Fig. 3). Root control samples of cv. Manuela (Fig. 2) had higher endogenous CK content and slightly lower CKX activity than those of cv. Scinado (Fig. 4).

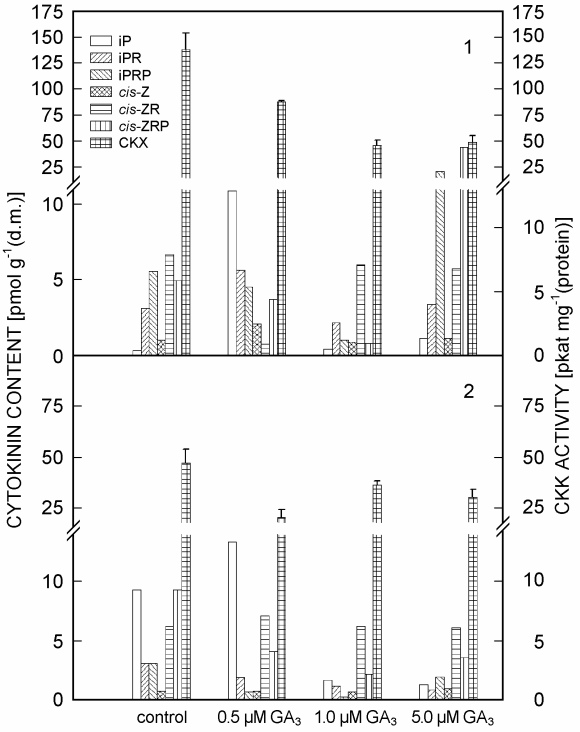

Fig. 1. Changes in specific CKX activity and endogenous CK content in the last fully expanded leaves of cv. Manuela pea plants treated for 48 h in half-strength Hoagland's nutrient solution with 0.5, 1.0 and 5.0 μM GA_3. The control means plants before GA_3-treatment

Fig. 2. Changes in specific CKX activity and endogenous CK content in the secondary roots of cv. Manuela pea plants treated for 48 h in half-strength Hoagland's nutrient solution with 0.5, 1.0 and 5.0 μM GA_3. The control means plants before GA_3-treatment.

The both cultivars responded to exogenous GA_3 in a similar way: increased elongation and after treatment with 5 μM GA_3 more fragile stems were observed. However, changes in CKX activity were different. In leaves of cv. Manuela GA_3 treatment resulted in gradual dose-dependent reduction in CKX activity and after treatment with 5 μM GA_3 CKX activity was almost three times lower in treated plants than in the controls (Fig. 1). Decreased CKX activity was accompanied by increased contents of iP (at 0.5 μM GA_3) and iPRP and *cis*-ZRP (at 5.0 μM GA_3). In roots of cv. Manuela application of GA_3 resulted in slight decrease of CKX activity in comparison with the control (Fig. 2). This was accompanied by an increase in iP content (after 0.5 μM GA_3 treatment) followed by a negligible reduction of all CKs, except *cis*-Z and *cis*-ZR, whose contents remained similar to the control values.

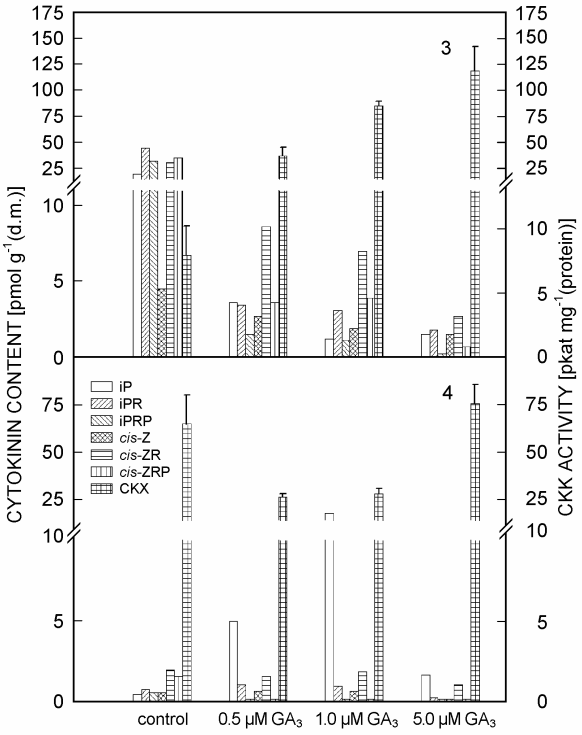

Fig. 3. Changes in specific CKX activity and endogenous CK content in the last fully expanded leaves of cv. Scinado pea plants treated for 48 h in half-strength Hoagland's nutrient solution with 0.5, 1.0 and 5.0 μM GA_3. The control means plants before GA_3-treatment.

Fig. 4. Changes in specific CKX activity and endogenous CK content in the secondary roots of cv. Scinado pea plants treated for 48 h in half-strength Hoagland's nutrient solution with 0.5, 1.0 and 5.0 μM GA_3. The control means plants before GA_3-treatment.

In contrast, GA_3 treatment provoked gradual dose-dependent increase of CKX activity in leaves of cv. Scinado (Fig. 3). Very strong effect was observed after 5 μM GA_3 application, where CKX activity was 25 times higher than in the control. Further, all GA_3 concentrations led to significant reduction of endogenous CK contents. The strongest effect was observed in leaves of plants treated with 5 μM GA_3. CKX activity in roots of cv. Scinado was twice lower after 0.5 μM and 1.0 μM GA_3 application than that of the control and this was accompanied by significant increase in iP content (Fig. 4). The rest of CKs exhibited some insignificant variations after treatment with respective GA_3 concentrations. This effect was not observed in 5 μM GA_3-treated plants (Fig. 4).

Changes in endogenous CK content and activity of CKX caused by exogenous GA_3 (applied in increasing concentrations to leaves and roots of two *Pisum sativum* cultivars characterized with different life span) proved that developmental factors are able to influence hormone signalling as well as hormone cross-talk in plants similarly as shown by Chow and McCourt (2004). Developmental context of hormone responsiveness was also demonstrated in several studies dealing with different *Arabidopsis* mutants (Parcy *et al.* 1994, Beaudoin *et al*. 2000, Ghassemian *et al.* 2000, Suzuki *et al*. 2001). It has been shown that the effect of a given phytohormone could depend on the combination of cell types as well as the developmental status of the cells. In a recent study (Vaseva-Gemisheva *et al.* 2006) on dynamic changes of cytokinin pool and on CKX activity in peas, it was demonstrated that both parameters change considerably during 4 d, which is equal to the period needed for full expansion of the youngest leaf. The shift in development of the two pea cultivars used in this study was estimated to be 3 d. The seedlings of the two cultivars were at the same age, but they differed in their development – cv. Manuela had fully expanded 4^{th} leaf, while cv. Scinado was at the stage of initial development of the sixth leaf pair. Sampling strategy of the experiment was to use material derived from the last fully expanded leaves. Individuals from the two cultivars being at different developmental stage exhibited diverse CK contents in their control tissues – significant differences in the control CK contents as well as CKX activities were witnessed in leaf samples (Figs. 1,3). The effect of the GA_3 treatment there seemed to be strongly dependent on initial values of CKs and CKX. Plants (such as cv. Scinado) with initially high CK content and low enzymatic activity responded to exogenous GA_3 with gradual increase of CKX activity and subsequent reduction in the substrate CKs, and *vice versa* (as seen in cv. Manuela). The only exception of this trend was observed in cv. Manuela leaves treated with 5.0 μM GA_3 where CKX activity remained almost equal to the values measured in the treatment with 1.0 μM GA_3 and this was accompanied by increased CK nucleotide (iPRP and *cis*-ZRP) content (Fig. 1). We presume that this trend was cultivar-specific compensatory response towards the highest exogenous gibberellin concentration with subsequent stimulation of cytokinin biosynthesis (Crozier *et al.* 2000). In roots, GA_3 application did not have any significant impact on CK content and CKX activity (Figs. 2,4). The lack of diverse response of the studied parameters to exogenous GA_3 in roots, where the initial distribution of CKs and CKX activity were similar in both pea cultivars, supports the concept that CK/GA interactions are under precise regulation and depend on initial concentrations of the hormones.

Another study (authors' unpublished data) with the same pea cultivars revealed that treatments with abscisic acid (ABA) significantly influenced CKX activity in roots, which did not result in any CK pool changes in the same tissue. ABA-mediated CK pool alterations were found to be specific to leaves and at the same time, no considerable effect on CKX activity in the same samples was detected. Thus, presented results indicate that the effect of exogenous GA on CKs did exhibit organ specificity and that its influence on their metabolism resulted in differently directed changes, which were determined by initial hormone concentrations in plant tissues. Generally, if endogenous CK content was high, exogenous GA lowered CKcontent through positively influencing CKX activity and *vice versa*.

Obtained results suggest that CKX is responsible for the changes in the endogenous CK pool in GA_3-treated plants and most probably this enzyme represents an important link in GA/CK cross-talk. More detailed studies are needed on other aspects of CK metabolism and signalling in plants grown under exogenous GA_3, which would facilitate further understanding of the mechanisms involved in CK/GA interactions.

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