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BRIEF COMMUNICATION

Developmental stage as a possible factor affecting cytokinin content and cytokinin dehydrogenase activity in *Pinus sylvestris*

A.E. VALDÉS*, P. GALUSZKA**, B. FERNÁNDEZ*[1], M.L. CENTENO*** and I. FRÉBORT**

Departamento de Biología de Organismos y Sistemas, *Universidad de Oviedo*, *c/ Catedrático Rodrigo Uría, s/n, E-33071 Oviedo, Spain**
Faculty of Science, Palacký University, Šlechtitelů 11, CZ-78371 Olomouc, *Czech Republic***
Facultad de Ciencias Biológicas y Ambientales, Universidad de León, *Campus de Vegazana*, *León*, *Spain****

Abstract

In the present study cytokinin dehydrogenase (CKX) activity was for the first time found in a conifer species, *Pinus sylvestris*. The activities were correlated with the endogenous cytokinin contents. Several enzyme substrates and two different electron acceptors were used to search for the enzyme activity in the extract from seeds, seedlings and plantlets. The highest specific activity was found in one-year-old plantlets with isopentenyladenine as the substrate and 2,6-dichlorophenolindophenol as the electron acceptor, at pH 8. An enhancement in the CKX specific activity corresponded to increasing contents of cytokinins, mainly isopentenyladenine and isopentenyladenosine, indicating that the enzyme activity is affected by the endogenous supply of cytokinins. CKX affinity for the ribosylated form of isopentenyladenine was dependent on the developmental stage, being higher in seeds than in seedlings, and not detectable in plantlets. The results are indicative of the presence of different isoenzymes throughout the development.

Additional keywords: cytokinin metabolism, conifers, plant ageing.

Tree maturation is modulated by intrinsic factors responsible for age-related changes, in which cytokinins (CKs) are the essential hormones involved throughout plant development, especially in the transition from the vegetative to the reproductive phase (Oka 2003). Control of biologically active CKs occurs through a balance between biosynthesis, interconversion among distinct forms, transient inactivation by conjugation, and catabolic reactions resulting in a complete loss of biological activity (Sakakibara and Takei 2002). Degradation of these molecules occurs by N^6-side-chain cleavage produced by the enzyme cytokinin dehydrogenase (CKX), the only known plant enzyme which catalyses the irreversible degradation of naturally occurring CKs. CKX was believed to be a copper-containing amine oxidase (EC 1.4.3.6) (Hare and Van Staden 1994), but further studies demonstrated the presence of a flavin cofactor, as well as the enzymatic use of a different electron acceptor than oxygen in *in vitro* conditions, classifying it as a dehydrogenase (EC 1.5.99.12) (Bilyeu *et al*. 2001, Galuszka *et al*. 2001). The enzyme has a preferred specificity for the substrates isopentenyladenine (iP) and its ribosylated form (iPR), yielding adenine or adenosine and 3-methyl-2-butenal as reaction products (McGaw and Horgan 1983). This substrate specificity is relevant due to the participation of distinct forms of CKs in the plant growth and development with a differential implication dependent on the developmental stage of the plant (Morris *et al*. 1990, Frugis *et al*. 2001, Zhang *et al*. 2003).

Previous results on ageing and maturation obtained with *Pinus* showed that not all CKs are equally present throughout these processes, being reported the importance of CKs with hydroxylated side-chains in adult states, whereas iP and iPR were associated with juvenile individuals (Valdés *et al*. 2002, 2003). These results,

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Abbreviations: (2-OH)BA - N^6-(2-hydroxybenzyl)adenine; CKX - cytokinin dehydrogenase; DCPIP - 2,6-dichlorophenolindophenol; DHZ - dihydrozeatin; DHZR - dihydrozeatin riboside; iP - N^6-isopentenyladenine; iPR - N^6-isopentenyladenosine; Q_0 - 2,3-dimethoxy-5-methyl-1,4-benzoquinone; Z - zeatin; ZR zeatin riboside.
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[1] Corresponding author; fax: (+34) 985104867, e-mail: bmfernan@uniovi.es

which indicate the physiological state of the plant, might be representative of changes in the activity of some degradative enzyme, such as the CKX, between juvenile and adult states. However, whether variations in the cytokinin content are produced by enzymatic activity regulating interconversions between different cytokinin forms, or by differential *de novo* synthesis remains still unknown.

CKX genes have been identified and cloned in several cereal species in addition to *Arabidopsis* and *Dendrobium* orchid (Bilyeu *et al*. 2001, Yang *et al*. 2003, Galuszka *et al*. 2004, Popelková *et al*. 2004), but information about either the gene or the CKX protein in other species is still hardly available. The objective of the current work was to investigate CKX activity in *Pinus sylvestris* and to establish a method to be used in further experiments for monitoring CKX activity associated with developmental changes produced in the plant.

Pinus sylvestris L. imbibed seeds, 15-d-old seedlings growing in a nursery in natural conditions and 1-year-old plantlets also growing in a natural stand, were used for cytokinin dehydrogenase (CKX) activity assays and determinations of endogenous CKs content. Experiments were carried out with more than one thousand seeds, more than one hundred seedlings and ten plantlets to have similar mass of each kind of material. Several independent pools for each sample were used, considering plant material from different individuals to avoid differences due to genotype. All material was acquired from the Forest Administration of the Czech Republic, Šternberk, Czech Republic.

For protein extraction frozen material was ground to powder with mortar and pestle in liquid nitrogen. Before allowed to thaw, the extraction of proteins was done with 0.5 M Tris-HCl buffer, pH 8, at 4 ºC, and repeated stirring every 10 min for 1 h. After washing twice with the same buffer to obtain higher protein recoveries, plant debris was removed by centrifugation at 10 000 *g* for 10 min. Polyvinylpyrrolidone [18 % (m/v)] was added to the supernatant to remove phenolic compounds, highly present in conifer extracts. After 15 min with every 5-min stirring, the pellet was removed by centrifugation at 12 000 *g*, 10 min, 4 ºC. To concentrate the proteins, $(NH_4)_2SO_4$ was slowly added to 70 % saturation to the supernatant within 1 h, at 4 ºC. Proteins were collected as a precipitate by centrifugation at 12 000 *g*, and suspended in the possible minimum volume of 0.1 M Tris-HCl buffer and stored at 4 ºC. Protein content was estimated by the method of Bradford with BSA as a standard (Bradford 1976).

The end-point enzymatic assay was performed using the method of Libreros-Minotta and Tipton (1995) modified for the use of an electron acceptor (Frébort *et al*. 2002). Under acidic conditions the aldehyde produced by cleaving a cytokinin forms with 4-amino-phenol a coloured Schiff base ((4-hydroxyphenylimino)-3-methyl-2-buten for iP, that has a molar absorption coefficient of 15.2 mM^{-1} cm^{-1}. The reaction mixture (0.6 cm^3 final volume) containing the enzyme extract, a substrate (0.15 mM) and an electron acceptor (0.5 mM) in 75 mM Tris-HCl buffer, pH 8, was incubated at 37 ºC for suitable period (1 - 5 h). The reaction was then stopped by the addition of 0.3 cm^3 40 % trichloroacetic acid. After the addition of 0.2 cm^3 of 4-aminophenol (solution in 6 % trichloroacetic acid), the spectrum over the range 300 - 700 nm was scanned and the absorbance was measured at a specific wavelength (352 nm for iP) on a *DU 7500* spectrophotometer (*Beckman*, Fullerton, CA, USA). In order to select the best substrate for monitoring CKX activity in coniferous species iP, iPR, zeatin (Z), zeatin riboside (ZR) and N^6-(2-hydroxy-benzyl)adenine (2-OHBA) were tested. As electron acceptors 2,6-dichlorophenolindophenol (DCPIP) and 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0) were used.

Seeds, seedlings and plantlets were deep-frozen in liquid nitrogen and lyophilised prior to the extraction and purification of several CKs: the free bases Z, dihydrozeatin (DHZ), iP and their ribosylated forms ZR, DHZR and iPR. Extraction and purification were performed according to Fernández *et al*. (1995) combining overnight-liquid and solid phase extraction with organic solvents and immunoaffinity chromato-graphy techniques. Reverse-phase HPLC on a *Kromasil 100 C18* - 5 μm (150 × 4.6 mm) was used for the separation of different CKs employing kinetin as internal standard to corroborate chromatographic retention times. The mobile phase was acetonitrile and triethylammonium acetate 40 mM, adjusted at pH 7. CKs were eluted from the column by means of a linear gradient from 5 to 20 % acetonitrile (v/v) over 40 min. Collected fractions were tested by ELISA with polyclonal antibodies obtained according to Fernández *et al*. (1995). The radiolabelled standard [8-^{14}C]BA (2.0 GBq $mmol^{-1}$) was added at the beginning of each analysis to determine purification losses.

All hormonal analyses were carried out in three independent samples with three repetitions each. Deviation from normality and homogeneity of variance was tested with Shapiro-Wilk and Barlett-Box tests respectively. For parametric data one-way analysis of variance (ANOVA) with Scheffé post-hoc comparison were used. The primary data on the iP, iPR and total CKs were transformed by the square root to reach parametric assumptions. Non-parametric data were analysed by means of Kruskal-Wallis and Mann-Whitney tests. The level of significance was set at $\alpha = 0.05$ for all tests.

Pinus sylvestris exhibited an enzymatic activity capable of the side-chain cleavage of iP and iPR. The activity was dependent on the plant tissue. In *P. sylvestris* seeds, the values of specific activity with iPR were about 85 % of those obtained with iP independently of the electron acceptor, while in the seedlings the activity with iPR was only about 55 % of the activity with iP (Table 1). Assays using either Z, ZR or (2-OH)BA as substrates of the enzyme did not show any detectable activity.

Differences in the CKX activity were found between the two electron acceptors used, DCPIP and Q_0, independently of the assayed substrate, the enzymatic

activity obtained with Q_0 being only about 11 - 16 % of that obtained with DCPIP in both seeds and 15-d-old seedlings (Table 1). In one-year-old plantlets, no CKX activity was found with Q_0 as the electron acceptor.

Table 1. Cytokinin dehydrogenase activity in the extracts of *Pinus sylvestris* with several substrates and two different electron acceptors. N.D. - no activity detectable. Data presented as mean ± SE.

Electron acceptor/substrate	CKX activity [pkat mg^{-1}(protein)] seeds	seedlings	plantlets
DCPIP/iP	0.624 ± 0.08	4.118 ± 0.72	28.974 ± 2.30
DCPIP/iPR	0.512 ± 0.07	2.411 ± 0.51	N.D.
Q_0/iP	0.070 ± 0.01	0.695 ± 0.07	N.D.
Q_0/iPR	0.061 ± 0.01	0.337 ± 0.04	N.D.

An increase in the total CKs content was observed throughout the germination and development from imbibed seeds to 15-d-old seedlings (Table 2). However, in one-year-old plantlets a reduction in the amounts of these hormones was observed (*ANOVA*, $F_{2,31}$=30.60; $P < 0.001$). Z was the major cytokinin present in the seeds, but its contents were lower in other developmental stages being hardly detectable in one-year-old plantlets. iP and also iPR (hereafter referred as iP-type CKs) were the most abundant CKs in seedlings and plantlets, being the main responsible for the increase in the CKs level detected between these two developmental stages. In the transition from seed to seedling, iP concentration increased more than 18-fold, whereas iPR values were doubled (Table 2). The increasing pattern in iP-type CKs

Table 2. Endogenous cytokinins content in different developmental stages of *Pinus sylvestris*. Data presented as mean ± SE.

Type of cytokinin	Cytokinin content [nmol g^{-1}(d.m.)] seeds	seedlings	plantlets
Z	0.366 ± 0.046	0.135 ± 0.023	0.004 ± 0.001
ZR	0.066 ± 0.012	0.091 ± 0.011	0.018 ± 0.007
DHZ	0.001 ± 0.0001	0.106 ± 0.017	0.074 ± 0.011
DHZR	0.002 ± 0.0001	0.148 ± 0.024	0.092 ± 0.015
iP	0.061 ± 0.017	1.110 ± 0.120	1.075 ± 0.178
iPR	0.253 ± 0.038	0.538 ± 0.054	0.334 ± 0.031
iP-type CKs	0.315 ± 0.050	1.677 ± 0.132	1.409 ± 0.183
Z-type CKs	0.462 ± 0.030	0.447 ± 0.016	0.191 ± 0.015
Total CKs	0.778 ± 0.072	2.125 ± 0.135	1.600 ± 0.180

obtained from these two developmental stages were not persisting in one-year-old plantlets, which showed similar values in these hormones to those found in seedlings (Scheffé post-hoc, $P = 0.1$). Imbibed seeds hardly contained any DHZ and its riboside, although an increase in the content of these CKs was observed in seedlings, they were found in very low content in the entire tested material (Table 2).

CKX activity has been reported in many plant species (Auer *et al*. 1999, Galuszka *et al*. 2001, Bilyeu *et al*. 2003), but to our knowledge this is the first time being described in conifers. *Pinus sylvestris* CKX showed the best affinity for iP and iPR as substrates, as expected from previous results obtained with other CKXs (Hare and Van Staden 1994, Schmülling *et al*. 2003). However, CKX efficiency in the use of iPR as the substrate was strongly dependent on the tissue. Seed CKX was more efficient in using this hormone than the enzyme from seedlings or plantlets, most probably due to variable biochemical properties of enzymes from different plant materials (Motyka *et al*. 2003, Schmülling *et al*. 2003, Werner *et al*. 2003b). Moreover, it has been described that regulation of CKX activity depends directly on the concentration and/or compartmentation of CKs in the cell (Motyka *et al*. 2003) which may vary in different tissues or developmental stages. In *Arabidopsis CKX* gene family, two different groups of CKX with distinct cell compartmentation were described. One type of CKX has an extracellular localisation, and prefers iP in slight basic conditions, while another one with probable targeting to vacuoles is more active under acidic conditions with preference to iPR (Werner *et al*. 2001, 2003b). Our results on *P. sylvestris* could also indicate the presence of different isoenzymes throughout tree development.

Pine CKX did not show activity with Z and its riboside as substrate, which is in accordance with the results obtained with transgenic tobacco plants overexpressing *AtCKX* genes that did not show noticeable alterations in the Z-type CKs content compared to wild-type plants, most likely due to a different accessibility of the substrate or substrate specificity of the protein (Werner *et al*. 2001). However, these CKs have long been reported as possible substrates for the enzyme (Bilyeu *et al*. 2001, Motyka *et al*. 2003). It must be taken into account that the Schiff base formed with 4-aminophenol and the aldehyde formed by oxidation of Z, 4-hydroxy-3-methyl-2-butenal, does not show stable absorption at 352 nm and the reaction is affected by a side reaction with DCPIP (Laskey *et al*. 2003). Further experiments in *P. sylvestris* would be necessary to find out the use of Z and ZR as substrates of the CKX, whose inactivation could also occur through *N*-glucosylation (Werner *et al*. 2003a). The oxidative degradation of (2-OH)BA produces an aldehyde that is able to react with 4-aminophenol and form a Schiff base, but although recent results report some degradation by maize CKX, pine extract did not show conversion of this cytokinin, most probably because of the low turnover rates described for aromatic CKs in comparison to those for iP (Frébortová *et al*. 2004).

CKX from *P. sylvestris* used DCPIP as the most effective electron acceptor, indicating that the degradation of the isoprenoid side chain possibly occurs following a dehydrogenation mechanism, and as described in other CKXs (Galuszka *et al*. 2001). This suggests that the enzyme could be located on biological

membranes using *in vivo* some membrane electron acceptor (Galuszka *et al*. 2000). Other studies have also proposed an extracellular location for the CKX (Werner *et al*. 2001, Haberer and Kieber 2002). Enzymatic activity with DCPIP was about tenfold higher compared with that obtained with Q_0, probably due to the lower redox potential (E_0') of the latter, which reduces its effectiveness (Q_0; E_0'= +0.08V. DCPIP; E_0'= +0.22V). Similarly to *P. sylvestris*, other studies have shown that degradation rates of CKX using DCPIP could be enhanced up to 4000-fold (Laskey *et al*. 2003). Although CKX is catalytically active even at low pH (4.6) optimal values for this parameter has been stablished at pH 8 (Malito *et al*. 2004). However, at alkaline pH quinones undergo spontaneous oxidative side-reactions that might contribute to the lower enzymatic activities observed when Q_0 was used as electron acceptor in comparison to those with DCPIP (Frébortová *et al*. 2004).

CKs are representative hormones affecting tree maturation and ageing with different roles associated to distinct forms of these hormones. Hormonal analyses previously carried out in another pine species (*Pinus radiata*) manifested that iP and iPR predominate in juvenile tissues over the rest of CKs analysed (Valdés *et al*. 2002, 2003, Zhang *et al*. 2003). By contrast, CKs with hydroxylated chain were in a majority in adult tissues (Valdés *et al*. 2002, 2003). Current work shows that, apart from results obtained in seeds in which Z predominate over other CKs, iP and iPR were present in a majority in *P. sylvestris* juvenile tissues similarly to *P. radiata* (Valdés *et al*. 2002, 2003, Zhang *et al*. 2003). The occurrence of Z as the most abundant cytokinin in mature seeds of *P. sylvestris* was also found in *P. pinea* cotyledons isolated from germinating embryos (Valdés *et al*. 2001), and it could allude to its involvement in the protein and sugar catabolism necessary for the new-seedling growth, as it similarly happens in angiosperms (Muñoz *et al*. 1990). DHZ and its riboside were hardly detectable in the same material, possibly because the process of hypocotyl thickening is under control of iP and Z-type cytokinins, but not DHZ (Lexa *et al*. 2003).

In the seedlings the most abundant CKs were iP and iPR, the preferred substrates for CKX (Hare and Van Staden 1994). The increase in the levels of these CKs in the transition from seeds to seedlings may act as an inductor of the CKX activity. Similarly, previous studies on *Zea mays* kernel development disclosed that an increase in CKX activity correlates with a rise in CKs levels in a dependence on the developmental stage (Armstrong 1994), suggesting that enzymatic activity is regulated by endogenous CKs supply (Dietrich *et al*. 1995, Kamínek *et al*. 1997). A tobacco callus overexpressing cytokinin biosynthetic *ipt* gene showed an increase in CKX activity, likely triggered by the accumulation of Z and DHZ derivatives showing as a high degradation rate of iP-type CKs occurred (Motyka *et al*. 1996, Redig *et al*. 1997). Accordingly, pine plantlets, where the CKX activity was much higher than in the other samples, showed a reduction in the content of iP-type CKs.

The increase in the CKs levels found in the photosynthetically active 15-d-old seedlings could also be related to the production of chlorophyll, chloroplast differentiation and the maintenance of the photosynthetic apparatus (Zaffari *et al*. 1998, Haberer and Kieber 2002). In conifers iP has been shown to participate in the process of plastid formation and in the development of chloroplasts (Cholvadová *et al*. 1999). Consistent with the necessity to regulate the active content of CKs is the finding that CKX might be also targeted to chloroplast as indicated the detection of CKX activity associated with these organelles (Benková *et al*. 1999).

Current work established preliminary conditions for the measurement of CKX activity in *Pinus*, which will be used in further experiments to disclose if specific CKX isoenzymes are associated with different developmental and physiological stages of the tree, and if the observed variations in CKX activity are responsible for controlling of distinct cytokinin forms in juvenile and mature trees (Frugis *et al*. 2001, Valdés *et al*. 2002, 2003, Zhang *et al*. 2003).

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