TWO PEA VARIETIES DIFFER IN CYTOKININ OXIDASE/ DEHYDROGENASE RESPONSE TO UV-B IRRADIATION

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Summary. Cytokinin oxidase/ dehydrogenase (CKX EC: 1.5.99.12) and cytokinin changes after UV-B irradiation (302 nm) in two pea varieties with different vegetation periods have been studied. Treatment caused total inhibition of CKX and reduced endogenous cytokinins in the slower-growing "Manuela", while it induced the enzymatic activity and positively influenced hormonal content in leaves of the faster-growing cultivar – "Scinado". Results suggest presence of diverse *ckx* alleles in the genomes of both varieties, which are characterized with different basal endogenous cytokinin concentration.

Key words: cytokinins, cytokinin oxidase/ dehydrogenase, *Pisum sativum*, UV-B.

Abbreviations: BSA – bovine serum albumin, *cis*Z – *cis* zeatin, *cis*ZR – *cis* zeatin riboside, *cis*ZRP – *cis* zeatin riboside monophosphate, CK – cytokinins, CKX – cytokinin oxidase/ dehydrogenase, HPLC – high performance liquid chromatography, iP – isopentenyl adenine, iPR – isopentenyl adenine riboside, iPRP – isopentenyl adenine riboside monophosphate, PMSF – phenylmethylsulfonyl fluoride.

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INTRODUCTION

Cytokinins play an important role in several aspects of plant growth, metabolism, and development at normal growth conditions, but they are also involved in the plant responses to adverse environments. CK titers alter at low and high temperature, drought, water deprivation, excess salinity, changes in nutrient solutions, pathogen infection and wounding (Hare et al., 1997), high metal concentration (Atanasova et al., 2004), and herbicide treatment (Atanasova et al., 2005). When plants are submitted to unfavorable growth conditions they usually express senescence-like symptoms (Buchanan-Wollaston et al., 2003) due to increased levels of reactive oxygen species after environmental stress (Merzlyak and Hendry, 1994). CKs delay senescence and, it has been suggested that probably the antisenescence properties of these phytohormones are related to their antioxidant activity (Pauls and Thompson, 1982). The mechanisms by which environmental changes affect CKs are still not clear, but their adaptive function is undoubted.

Approximately two-thirds of some 300 species and cultivars tested appear to be susceptible to damage from increased UV-B radiation (Teramura and Sullivan, 1991), and among them is pea. Tremendous variability in plant species sensitivity to UV-B radiation exists. This issue is complicated further by equally large response differences among cultivars of a species (Biggs et al., 1981; Teramura and Murali, 1986).

One of the mechanisms for regulation of endogenous CKs is known to be cytokinin oxidase/dehydrogenase – the only known enzyme, which performs the degradation of adenine-type CKs (Galuszka et al., 2001). Under normal growth conditions CKX maintains the homeostasis of endogenous CK levels required for plant growth and development (Kaminek et al., 1997). Relatively little information is available concerning CKX activity response toward unfavorable environmental factors (Li et al., 2000; Manju et al., 2001; Brugiere et al., 2003; Vaseva-Gemisheva et al., 2004, 2005). For understanding the UV-B stress response and plant sensitivity it is helpful to study the changes in plant hormones and the enzymes involved in their metabolism.

MATERIALS AND METHODS

Plant material and treatments

Young (11-day-old cv. “Scinado” and 13-day-old cv. “Manuela”) pea plants with fully developed 3rd leaves were grown as water cultures in half-strength Hoagland’s solution in a growth chamber (12/12-h photoperiod; 24/20^{o}C day/night; photon flux density 70 μmol m^{-2} s^{-1}). Under these conditions cv. “Scinado” normally requires a shorter growing period than cv. “Manuela”. For UV-B treatment, part of the plants

was subjected to supplementary UV-B irradiation with UV lamp UVM-28 (230 V/ 50 Hz) (Ultra-Violet Products Ltd., Cambridge) with emission λ = 302 nm. The irradiation dose was measured with a radiometer UVX supplied with a 302-nm sensor and was 15.2 μW cm $^{-2}$. Treatment lasted 4 h and was performed at midday for a period of two consecutive days. The distance between the lamp and the top leaves of the plants was 35 cm and they received a daily dose of 0.218 J cm $^{-2}$ of UV-B irradiation. Plant material for CKX assay was a bulk sample (from at lest 10 different individuals) obtained from the last fully developed 3rd leaf (0.5g) and secondary roots (2.0g). Plant material (1g leaves and 1.5g roots) for endogenous CK determination was frozen in liquid nitrogen and freeze-dried.

Cytokinin oxidase/dehydrogenase activity assay

Specific CKX activity was measured in the roots and the last fully expanded leaves on the basis of 3 methyl-2-butenal production after Liberos-Minotta and Tipton (1995) protocol. Material was ground in 2.0 ml 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 15000 rpm for 50 min and at 15000 rpm for 40 min (after additional treatment with 0.5 mM PMSF, 25 mg/ml streptomycin sulphate and 0.1% solution of bovine serum albumin (BSA). The reactions for CKX activity were carried out at 37 ºC for 50 min in a final volume of 1.05 ml 100 mM imidazole buffer (pH 6.5) containing $CuCl_2$ and 0.050 mM isopentenyl adenine (iP). Absorbance of the samples (λ=352 nm) was measured on Shimadzu spectrophotometer UV-1601 (Shimadzu Corporation). 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 error was calculated with SigmaPlot for Windows Version 8.00 Software.

Cytokinin extraction, purification and determination

Extraction and purification of the lyophilized samples followed the procedure described by Lexa et al. (2003). Briefly, material was extracted overnight at -20 ºC with Bieleski solvent (Bieleski, 1964). The extracts with added deuterium-labeled CKs as internal standards were centrifuged and passed consecutively trough connected in series two Sep-Pak C_{18} cartridges (Waters Corporation, Milford, MA, USA), DEAE Sephadex column (after evaporation to water phase and adjustment of pH to 6.5) 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 trough Sep-Pak C_{18} cartridges and eluted with 80 % methanol. Then this fraction was evaporated to water phase and the sample was treated for 30 min at 37 ºC with alkaline phosphatase. After neutraliza-

tion and passage trough Sep-Pak C_{18} cartridges, CK nucleotides were eluted with 80 % methanol and evaporated to dryness.

CK fractions were separated and quantified by HPLC (FLUX Rheos 2000 quaternary pump and CTC Analytics HTS PAL autosempler with CSI 6200 Series HPLC Oven) linked to a mass spectrometer (Finningan LCQ) equipped with an ESI source. Data were processed at MS/MS full scan, two microscans at maximum ion time 100ms. Values represent the mean of LC/MS/MS measurements in two replications.

RESULTS AND DISCUSSION

The quality of crop yield in regard to the effectiveness of UV-B irradiation on plant growth varies seasonally and is affected by microclimate, soil fertility, as well as depends on specific characteristics of certain varieties. Usually UV-B light influences plant growth and development and these effects originate from the reduced cell divisions in leaf tissues (Tevini and Teramura, 1989). This subsequently reflects in inhibited growth of the above-ground part of the plant. One of the reasons for this phenomenon could be inactivation of physiologically active endogenous cytokinins.

Two pea cultivars were chosen based upon differences in their phenotype for the study. Cv. "Scinado" is a taller and faster growing variety, while cv. "Manuela" exhibits slower growth, shorter stems and broader leaves. The aim of the study was to assess the response of the two cultivars to UV-B irradiation in relation to changes in cytokinin metabolism. It is worth noting that initial CK content in the leaves of both varieties differed significantly (Figures 1A and 2A). At this developmental stage "Manuela" was characterized with lower cytokinin concentration in the control leaves compared to "Scinado". Simultaneously the controls of both cultivars exhibited similar CKX activities in their leaves. After UV-B irradiation the enzyme responded in a different manner. UV-B completely inhibited CKX activity and decreased CK content with exception of phosphorilated forms in "Manuela" leaves (Fig. 1A) while the opposite trend was observed in "Scinado" leaves (Fig. 2A). – CKX activity increased and this was accompanied with higher CKs concentration.

Irradiation did not affect cytokinin ribosides, *cis*Z and iPRP in "Manuela" roots, but it provoked a considerable decrease of iP titre (more than 2.5-fold compared to the control). Increased *cis*ZRP content and CKX activity were measured in irradiated roots while in the controls no enzymatic activity was detected (Figure 1B).

UV-B irradiation resulted in decreased CK content in "Scinado" roots (Fig. 2B) with the exception of *cis*Z. This was accompanied with very low CKX activity compared with controls – almost 10-fold inhibited activity after exposure to UV-B irradiation.

Since cytokinins have been characterized as plant hormones with certain antioxidative properties, increased CK content and CKX activity in cv. "Scinado"

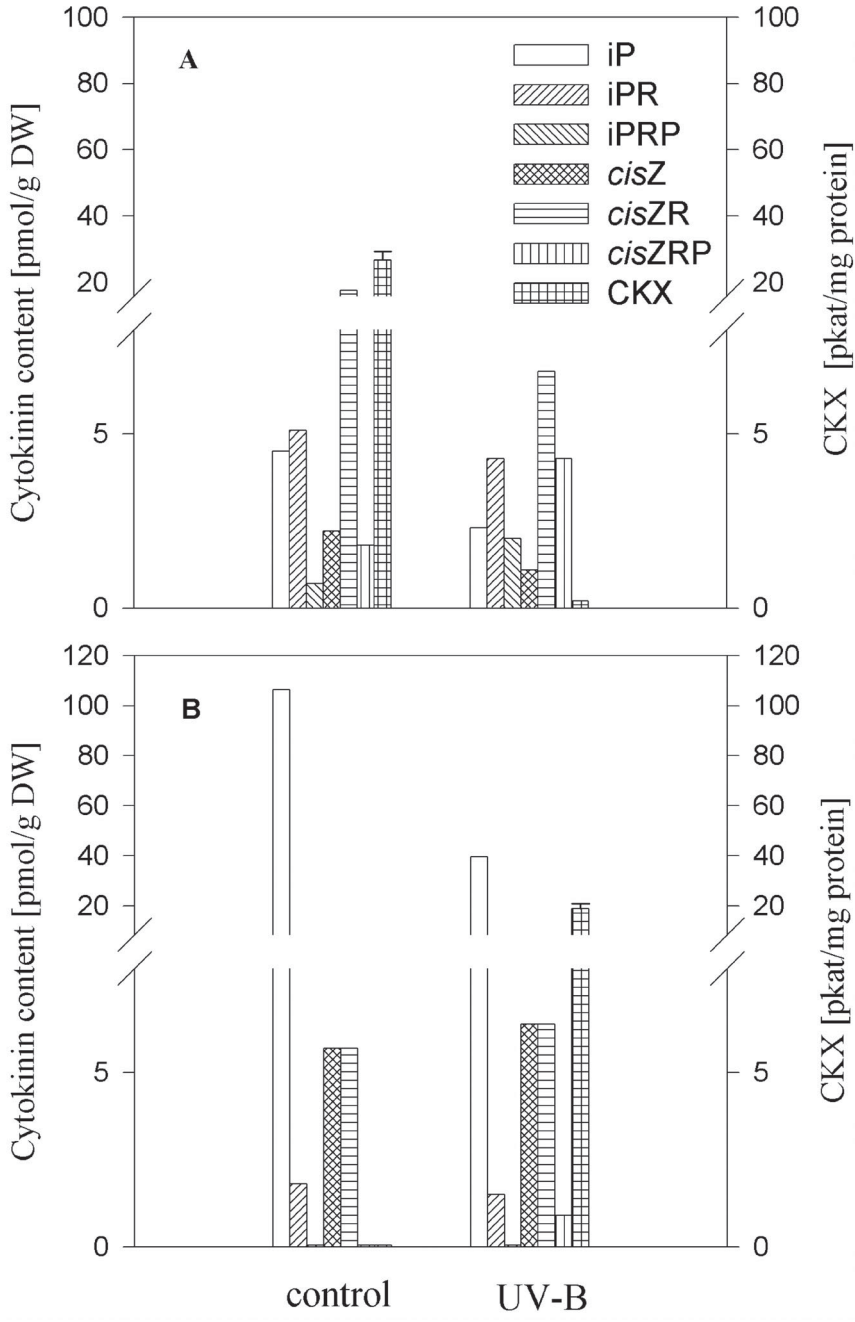

Figure 1. Changes in specific CKX activity and endogenous cytokinin content in the last fully expanded leaves (A) and secondary roots (B) of cv. "Manuela" irradiated with UV-B (λ = 302 nm).

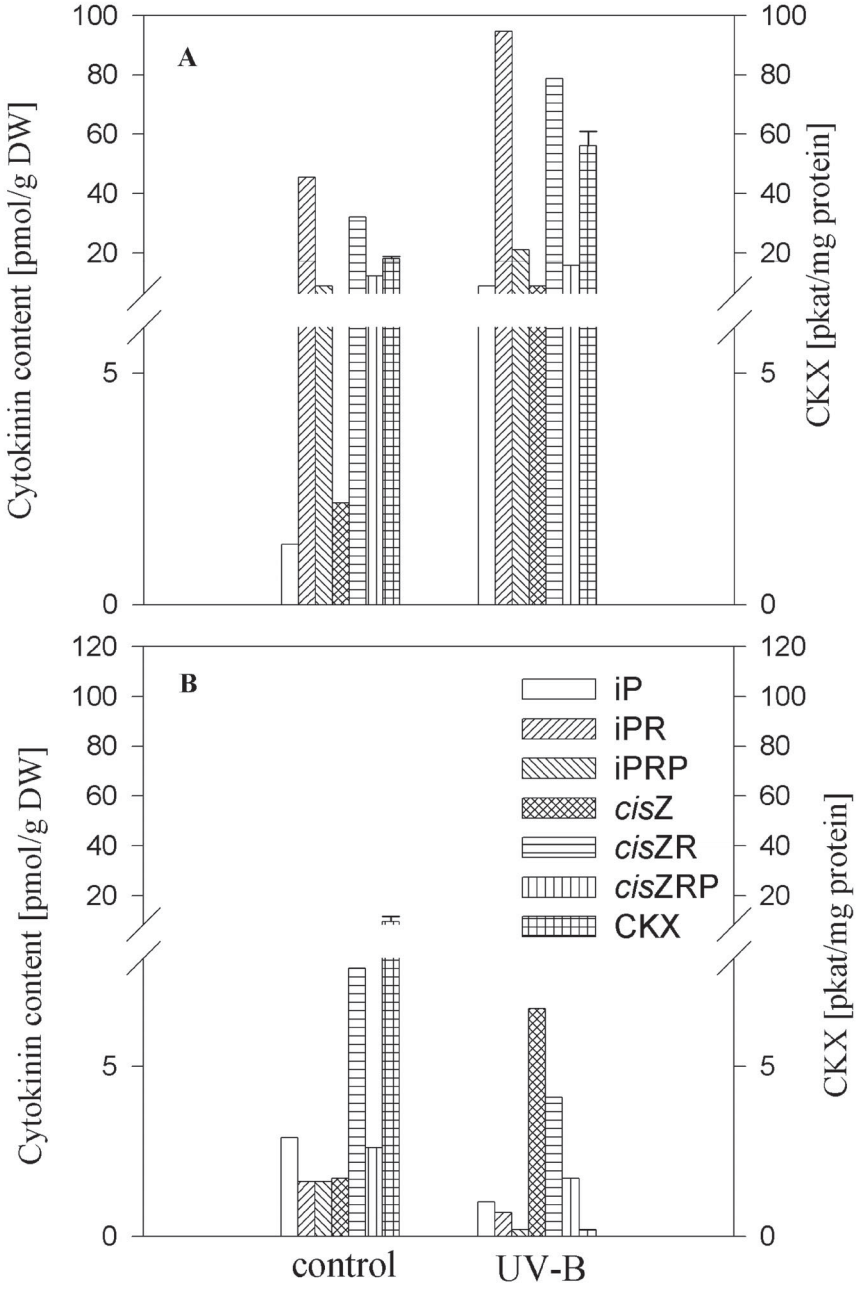

Figure 2. Changes in specific CKX activity and endogenous cytokinin content in the last fully expanded leaves (A) and secondary roots (B) of cv. "Scinado" irradiated with UV-B (λ = 302 nm).

leaves submitted to UV-B irradiation could be regarded as a prerequisite for higher adaptation potential of this variety to UV-B stress. Decreased CK titers and CKX activity detected in cv. "Manuela" leaves define this variety as a more vulnerable one to UV-B irradiation. Presumably the established diversity in CK and CKX response to UV-B light in cv. "Scinado" and cv. "Manuela" pea plants could be due to the presence of different alleles controlling the studied processes in their genomes. Results demonstrated the existence of variety specific CKX response towards stress factors.

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