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Response of *Pisum sativum* cytokinin oxidase/dehydrogenase expression and specific activity to drought stress and herbicide treatments

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Abstract

The expression of cytokinin oxidase/dehydrogenase (CKX EC: 1.5.99.12) is subject to fine regulation and it provides a rapid turnover of cytokinins, which serves as a signal for triggering developmental events during plant growth. The activity of this enzyme is believed to be responsible for the changes in cytokinin pool under adverse environmental conditions. CKX gene-specific assay to measure the expression in response to different stress treatments in the tissues of *Pisum sativum* plants was developed. Pea *CKX* genes were amplified and sequenced using primers designed from the sequences of *Medicago truncatula CKX* genes. Expression of two *P. sativum CKX* genes was assessed using relative-quantification in real time two-step RT-PCR, in leaves and roots of drought-, glufosinate- and atrazine-treated cv. Manuela pea plants. Varied CKX responses support the existence of complicated regulating mechanism of cytokinin oxidase/dehydrogenase gene expression.

Abbreviations: CKX – cytokinin oxidase/dehydrogenase; ABA – abscisic acid

Introduction

Changes in hormone levels controlled by the respective hormone systems are essential steps in acclimation of the plant to stress (Levitt 1980; Hale et al. 1986; Mok and Mok 2001). The increase or decrease in concentration of a hormone is achieved by changes in the levels of gene expression leading to synthesis of the enzymes involved in their biosynthesis, or by regulating the enzymes that degrade or deactivate the compound. Alterations in endogenous cytokinins along with changes in other plant hormones under stress conditions may be responsible for generating certain physiological reactions. The most consistent observation is that cytokinin activity decreases in response to a number of stresses (drought, salt, flooding, heat, shading, and nitrogen/phosphorous deficiency) (Hare et al. 1997) with a concomitant increase in abscisic acid (ABA) levels (Morgan 1990). Cytokinin oxidase/dehydrogenase (Hare et al. 1994; Houba-Hérin et al. 1999; Bilyeu et al. 2001) is the only known enzyme, which performs the degradation of cytokinins in plants resulting in

formation of adenine-type compounds and corresponding isopentenyl aldehyde (Galuszka et al. 2001). Under normal conditions CKX maintains the homeostasis of endogenous cytokinin levels required for plant growth and development (Kaminek et al. 1997). It is likely that the activity of this enzyme is responsible for the changes in the cytokinin pool under adverse environmental conditions since it is able to provide a means for rapid turnover of cytokinins.

Cytokinin oxidase/dehydrogenase enzymes are encoded by complex and heterogeneous gene family, which is often the case for developmentally regulated plant genes (Schmülling et al. 2003). This complexity may explain the paucity of data about the role of CKX in regulation of endogenous cytokinin levels in response to environmental stress (Li et al. 2000; Manju et al. 2001; Vaseva-Gemisheva et al 2004). The study of CKX is further complicated because of the existing tissue specificity of CKX isozymes.

Weed control is a major problem for the growth of pea crops. Glufosinate and atrazine are among the variety of herbicides that are applied to maintain weed control in agricultural practice.

Glufosinate (glufosinate-ammonium) is a broad-spectrum contact herbicide and is used to control a wide range of weeds after the crop emerges or for total vegetation control on land not used for cultivation. Glufosinate herbicides are also used to desiccate crops such as peas, beans, barley and wheat before harvest. The application of glufosinate leads to reduced glutamine and increased ammonia levels in the plant tissues. This causes photosynthesis to stop and the plant dies within a few days. Glufosinate has been found to be highly persistent in sandy soils due to lack of biodegradation (Allenking et al. 1995). Atrazine provides season-long weed control of broad leaf weeds and wild oats. It can also be tank mixed with other broadleaf and annual grass herbicides to provide a wide spectrum of weed control. It can persist in the soil longer especially under dry and cool weather conditions. It has been found that peas are sensitive to carryover of atrazine.

Drought stress is one of the environmental factors that cause a reduction in growth rates, stem elongation, leaf expansion and stomatal movements (Hsiao 1973). It significantly influences some physiological and biochemical processes that are important for the normal plant growth and development (Daie 1988; Alexieva et al. 2001). Most of these phenomena are at least partially governed by cytokinin flux. Recently Brugière et al. (2003) showed that under environmental stress conditions, CKX expression induction by ABA resulted in degradation of cytokinins and this prevented the normal plant development. Authors have demonstrated that *Zea mays* CKX1 expression was induced by drought and heat stress. It was suggested that induction of CKX transcripts by abiotic stress might be in part mediated by an increase in ABA concentration and that cytokinins and ABA treatments are additive in the control of CKX gene expression. Moreover it was suggested that CKX glucosylation pattern and its secretion might be involved in the control of CKX activity and its physiological role in plant cells and tissues (Motyka et al. 2003). These new aspects of cytokinin oxidase/dehydrogenase activity control might be very important steps of cytokinin metabolism and their role remains to be revealed in relation to CKX stress response.

The aim of this study was the development of CKX gene-specific assay to measure the expression in response to different stress treatments in the tissues of *P. sativum* plants. Assessment of CKX expression was made in leaves and roots of differently stressed cv. Manuela pea plants by real-time RT-PCR analysis. The expression of *P. sativum* actin and cytochrome b-559 alpha subunit (psbE) genes was used as control to normalise between the samples. Expression profiles showed that the two studied CKX transcripts are mainly presented in leaf tissue. Ddrought induced the expression of both genes in leaves and roots. Herbicide application exhibited diverse effects over CKX expression and activity.

Materials and methods

Seed for cv. Manuela and cv. Scinado was purchased from Sortovi Semena Ltd. (Ohrid Str. 29, Sofia, Bulgaria) and plants were grown in Shamrock professional growing medium in a green house department without supplementary heating, at the end of March/begging of April (respectively 12 h and 30 min – 13 h/11 h and 30 min – 11 h photoperiod; average temperatures: max 12.2–14.6 °C/ min 2.5–4.6 °C). Plant material (leaves from the last fully expanded bud and secondary roots from cv.

Manuela pea plants) for RNA extraction and CKX determination was collected in the early afternoon (2–3 p.m.). It was frozen in liquid nitrogen and preserved at −80 °C for not longer than 96 h till the moment of analyses.

All primers were designed using Oligo® (Wojciech Rychlik) and were purchased from Sigma-Genosys (UK).

Development of ckx-specific PCR primers sequencing

Primers to amplify CKX genes were designed using the published *Medicago truncatula* mRNA sequences coding relevant ckx products (http://www.tigr.org/tdb/tgi/mtgi/).

Sequences of primer pairs were as follows: GGYMACGGYCAYTCCATMAAC Forward/ RMGATTCKKGCTCKAGTGATGA Reverse (for amplification product named '*PCKX1*'); AAGA TTTTGGCAATAGATAC Forward/TAGATG CAAATAATCTGTCC Reverse (for amplification product named '*PCKX2*'). DNA was extracted from young pea leaves (cv. Scinado and cv. Manuela) as described elsewhere (Ellis et al. 1994).

PCR was performed for 40 cycles using 200 ng/ reaction DNA template in GeneAmp® PCR System 9700, PE Applied Biosystems with FastStart Taq DNA Polymerase kit (Roche Diagnostics Ltd.) following manufacturer's protocol in 20 µl reaction volume containing 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM Forward and 0.5 µM Reverse primers and 2 U FastStart Taq DNA Polymerase.

PCR conditions were 1 cycle denaturation step at 95 °C for 4 min; 40 cycles denaturation/annealing/elongation at 95 °C for 30 s/55 °C for 30s/72 °C for 1 min followed by 1 cycle final extension at 72 °C for 7 min. Bands of the amplification products (Figure 1) were excised and purified using QIAquick Gel Extraction Kit (QIAGEN). The products were sequenced on ABI Prism 3100 Genetic Analyzer using The Big Dye Terminator™ system (Applied Biosystems) (Table 1).

Primers for specific *CKX* genes were designed from the obtained *Pisum sativum* sequences: *PsCKX1* CAGCGTTTCAACACTTCTTC Forward/ CACGGTCACTCCATCAAC Reverse; *PsCKX2* GGTCATTCGCTTCAAGG Forward/GTGCCA AACCATACTTCAAT Reverse. They were used

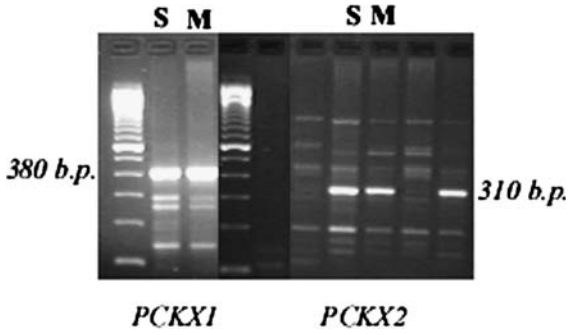

Figure 1. PCKX1 (379 bp) and PCKX2 (309 bp) products obtained after PCR amplification on total DNA from several pea cultivars [Lines 5, 6, 9, 10 were loaded with the same PCR (with primers for PCKX2 amplification) performed with DNA of different individuals from a mapping population (parent accessions JI 281 and JI 399 – from the collection of JI – Norwich) – kind gift from the team of Dr Noel Ellis – John Innes Institute, Norwich, UK.], including cv. Scinado (S) and cv. Manuela (M). Relevant bands were excised, purified and sequenced on ABI Prism 3100 Genetic Analyzer.

for the reverse transcription and the real-time two-step RT-PCR analysis. Expected product sizes were 158 bp (for *PsCKX1*) and 171 bp (for *PsCKX2*). Expression of *Pisum sativum* actin (X90378) (primers: AATCAACAATGGCAGA AGC Forward/ATCACCAACATACGCATCTT Reverse) and cytochrome b-559 alpha subunit (*psbE*) (primers: GGTTGGTTATTTGTCAG CAC Forward/ AATTCCTTGTCGGGTTTCT Reverse) was used to normalise between reactions. The amplification products of the normalizing reactions were: 182 bp (actin) and 92 bp (*psbE*).

RNA source, total RNA extraction, reverse transcription and LightCycler™ real-time two-step RT-PCR

Pea plants (cv. Manuela) were grown for 19 days until third fully expanded bud. Some of the plants were not watered for a period of four days. Others were sprayed using a small hand-spray, with 0.25 g/l atrazine (Houston 1977) or glufosinate (0.20 g/l), two days before collecting the samples. Total RNA was extracted using SV Total RNA Isolation System (Promega Corporation, Madison, USA) and the quality was checked on ethidium bromide-stained agarose gel. RNA was quantified using a spectrophotometer and stored at −80 °C.

Table 1. Pisum sativum cv. Manuela *ckx* sequences. Relevant primer pairs for the RT-PCR analysis are given in gray fields[a].

Primers	NCBI Gene Bank Accession N	Sequence	RTPCR Product
GGYMACGGYCAYTCCATMAAC *Forward* RMGATTCKK[GCTC] KA[GT] GATG[A] *Reverse*	AY444352 (379 b.p.) *PCKX1*	*ggtcacggtcactccatcaac* TTT ▶ GGCCAAGCCATGGCAGA AAAAGGACTGGTCCTCGACATGCGCGCCACCGCGGAG GAACATTTCCAACTCCTCTATCTCGAAGGATTACCATA CGTCGACGTTTCAGGAGGGGCATTATGG◀GAAGAAGT GTTGAAACGCTGCGTTTCGCAGTTTCAACTTGTTCCTAG ATCGTGGACGGATTATCTCGGGCTAACGGTGGGTGGAA CGCTCTCTAACGCCGGAGTTAGTGGGCAGACTTTCCGT TACGGTCCACAAACGGCAAAGGTAACGGAATTAGAAG TCGTTACTGGGAAAGGTGAAAGCTTANTTNATCCGAAA *a tg ctcg* ATCAAAATTCAGAGCTTTTTGC CGCT GG TC	***PsCKX1*** 158 b.p.
AAGATTTTGGCAATA[GATAC] *Forward* TAGATGCAAATAATCTGTCC *Reverse*	AY444353 (309 b.p.) *PCKX2*	*ctatg* ACGTGCTTCATCCAAATCAGTCTCTGATATTGCAGT TACTGTCACGCCCNCCNTGGAGTTGGGGTACTAGCTCGG AGTTAACGGTTACGGCTAGCNGACATGGTCATTCGCTTC AAGG▶TCAAGCTCAAGCTCATGGAGGAATTGTGATTAA TATGGAATCGCTTAAAGTTGAAGGGATTAAGGTGTATGA TGGAGAGTTTCCTTATGTGGATGTTTCAGGAGGTGATTT GTGGATAAATGTTTTGAATGAGAC◀ATTGAAGTATGGT *ggacagattatttgcatcta* TTGGCACCAAGATCTT AA	***PsCKX2*** 171 b.p.

[a]Primers to amplify CKX sequences in pea genomic DNA are given in small italic letters.

Reverse transcription (RT) of 50 ng total RNA derived from differently treated leaf and root tissue was performed with Sensiscript® RT kit (QIAGENE Ltd.) using RNasin® Ribonuclease Inhibitor (Promega Corporation, Madison, USA) and 1 μM specific forward and reverse primers (for *PsCKX1; PsCKX2*, actin and *psbE*) following the manufacturer's protocols in GeneAmp® PCR System 9700 (PE Applied Biosystems).

Real-time RT-PCR was performed using the LightCycler™ machine with LC FastStart DNA Master Kit SYBR Green I (Roche Diagnostics Ltd.) according to the manufacturer's protocol with 0.5 μl of the RT-reactions (*PsCKX1*-RT, *PsCKX2*-RT, actin-RT and *psbE*-RT reactions) using relevant 1.0 μM forward and reverse primers in final reaction volume of 20 μl.

The LightCycler™ PCR experimental run protocol began with 1 cycle initial activation step 15 min/95 °C/ramp 20 °C/s. Amplification and quantification cycle was repeated 50 times: denaturising 10 s/94 °C; annealing 5 s/55 °C for *PsCKX1* and *PsCKX2*, 60 °C for actin expression and 50 °C for cytochrome b-559 alpha subunit (*psbE*); extension 72 °C/10 s, with a single fluorescence measurement.

The run proceeded with melting curve program (65–95 °C with a heating rate of 0.1 °C/s and continuous fluorescence measurement) and ended with a cooling step to 40 °C. Crossing points (CP) for each transcript were detected using 'Fit Point Method' of the LightCycler software 3.3 (Roche Diagnostics Ltd.).

Specificity of RT-PCR products was assessed with a LightCycler™ Melting curve analysis. The two products differ in their melting temperatures: PsCKX1 – 83.87 °C and PsCKX2 – 78.80 °C.

Mathematical model

Relative quantification was made according to the mathematical model developed by Pfaffl (2001), where the relative expression ratio (*R*) of

a target gene is calculated based on its real-time PCR efficiency (E) and the crossing point (CP) deviation of an unknown sample versus a control, and it is expressed in comparison to a reference gene:

$$R = \frac{(E_{\text{target}})^{\Delta \text{CP}_{\text{target}}^{(\text{control}-\text{sample})}}}{(E_{\text{ref}})^{\Delta \text{CP}_{\text{ref}}^{(\text{control}-\text{sample})}}}$$

E_{target} is the real–time PCR efficiency of target gene transcript; E_{ref} is the real-time PCR efficiency of a relative gene transcript (actin or *psbE*); $\Delta\text{CP}_{\text{target}}$ is the CP deviation of control – sample of the target gene transcript; $\Delta\text{CP}_{\text{ref}}$ is deviation of control sample of reference gene transcript (CP is the point at which the fluorescence rises appreciably above the background fluorescence). For the calculation of R, the individual real-time PCR efficiencies and the CP deviation (ΔCP) of the investigated transcripts must be known. Real–time PCR efficiencies (E) were calculated, according to:

$$E = 10^{[-1/\text{slope}]}.$$

CP deviations of control cDNA minus sample of the target gene and reference genes were calculated according to the derived CP values.

Determination of specific CKX activity

CKX activity was measured spectrophotometrically on the basis of 3-methyl-2-butenal production (Liberos-Minotta and Tipton 1995). Approximately 0.5 g frozen material was grinded in 1.5 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 18000 g for 50 min and at 18000 g for 40 min (after additional treatment with 0.5 mM PMSF, 25 mg/ml streptomycin sulphate and 0.1% solution of bovine serum albumin). The reactions for CKX activity were carried out at 37 °C for 50 min in a final volume of 0.55 ml 100 mM imidazole buffer (pH 6.5) containing $CuCl_2$ and 0.050 mM iP.

The analysed material was obtained from at least three different individuals. Absorbance of the samples (λ = 352 nm) was measured on Shimadzu spectrophotometer UV-120–02 (Shimadzu Corporation).

Soluble protein was determined by dye binding technique (Bradford 1976) using bovine serum albumin as a protein standard. All measurements were made in triplicates and standard error was calculated with SigmaPlot for Windows Version 4.00 Software. Chemicals used were purchased from Sigma-Aldrich (Shaftesbury, UK).

Results

Pea *CKX* genes were amplified using primers designed based on the sequences of *Medicago truncatula CKX* genes. Primers were chosen from sites that exhibited over 70% homology with known mRNA sequences of *Arabidopsis CKX* genes in order to obtain amplification products consistent with coding regions of the genes. The alignment analysis of the sequences available from the public databases was performed using DNAStar™ Megalighn Program.

Two putative cytokinin oxidase/dehydrogenase sequences (called PCKX1 and PCKX2) were identified in *Pisum sativum* plants (cv. Manuela and cv. Scinado) (Figure 1) and sequenced. The sequences were submitted to NCBI GenBank. Accession numbers are PCKX1: AY444352 and PCKX2: AY444353 (Table 1). BLAST results showed that PCKX1 has high homology with *Arabidopsis thaliana* CKX5 mRNA sequence (Accession No. BT002757, identity with PCKX1 S = 75.8 E = $6e^{-11}$). PCKX2 resembled *Hordeum vulgare* cytokinin dehydrogenase 2 mRNA (Accession N: AF540382, identity shown S = 63.9, E = $2e^{-07}$). PCKX2 was found to have even higher homology with two other sequences: *Medicago truncatula* clone mth1–6 m 23 S = 216 E = $2e^{-53}$ and UniGene Cluster Os .53839 *Oryza sativa* S = 66 E = $5e^{-08}$. These sequences are still not characterised as genes responsible for the synthesis of CKX enzymes but such a possibility is very probable.

Specific CKX primer pairs were designed from the *Pisum sativum* CKX sequences and used for the reverse transcription and the real-time two-step RT-PCR analysis (Table 1). Expression of the *CKX* genes was measured in last fully expanded leaves and secondary roots of plants that had undergone different stress treatments (drought-, glufosinate- and atrazine-treated pea plants cv. Manuela) using real-time two-step RT-PCR technique (Figure 2a). In order to distinguish specific

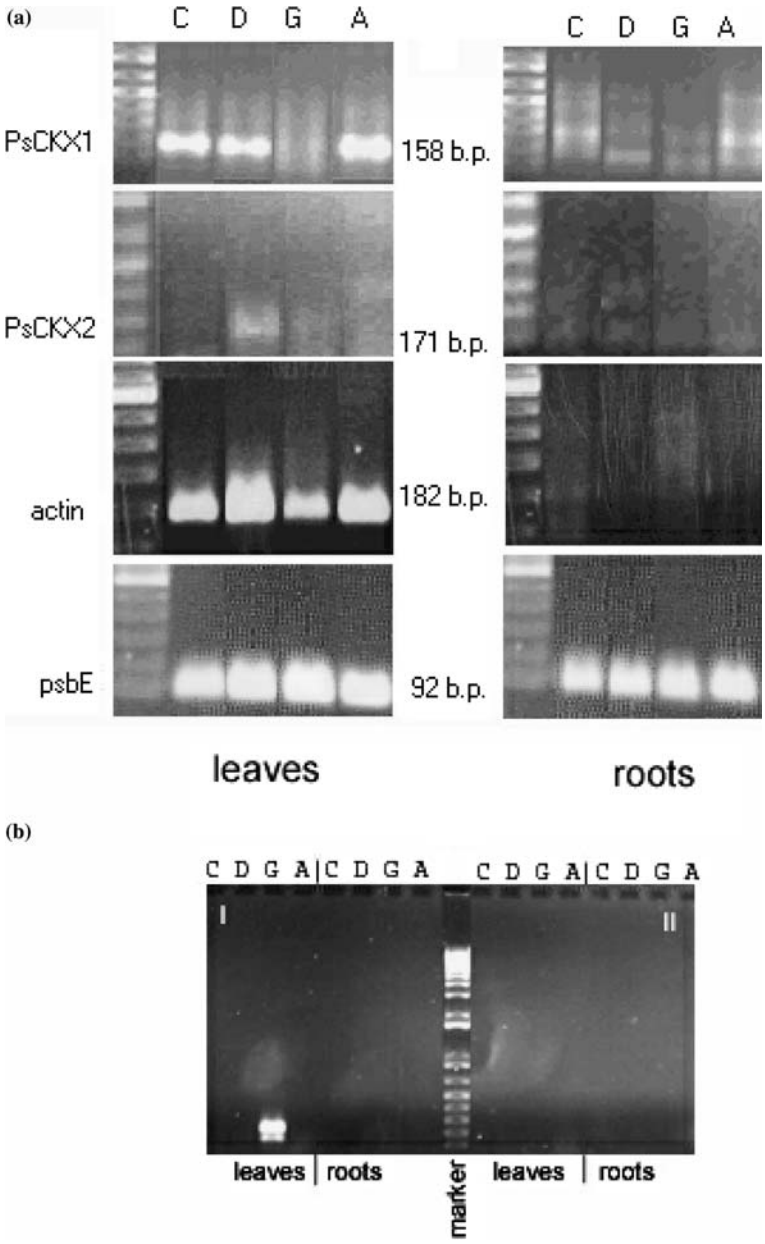

Figure 2. (a) Two-step real-time RT-PCR (PsCKX1 – size of the amplification product – 158 bp, and PsCKX2 – size of the amplification product – 171 bp) performed in LightCycler™ Machine loaded on 1.5% ethidium bromide-stained agarose gel. Total RNA was derived from the last fully expanded leaves and secondary roots of control (C), drought (D), glufosinate (G) and atrazine (A) treated 19-days old pea plants (cv. Manuela). As a normalizing standard the expression of *Pisum sativum* actin (X90378) and *psbE* genes in the same samples was used. (b)Negative control-check for DNA contamination in template. Control reaction contains relevant RNA template, actin PCR primers, and 2µl of LightCycler – DNA Master SYBR Green I (nucleotides, PCR buffer, Taq DNA polymerase, instead of reverse transcriptase, and $MgCl_2$). (I) DNA-contaminated RNA (derived from the glufosinate-treated leaves), (II) Non-contaminated RNA samples which have been used for RT-PCR analysis.

RT-PCR products from possible contamination of RNA template with DNA, a negative control was prepared (Figure 2b).

Real-time RT-PCR analysis showed that *PsCKX1* and *PsCKX2* were differently expressed in the leaf and root tissue of the control plants

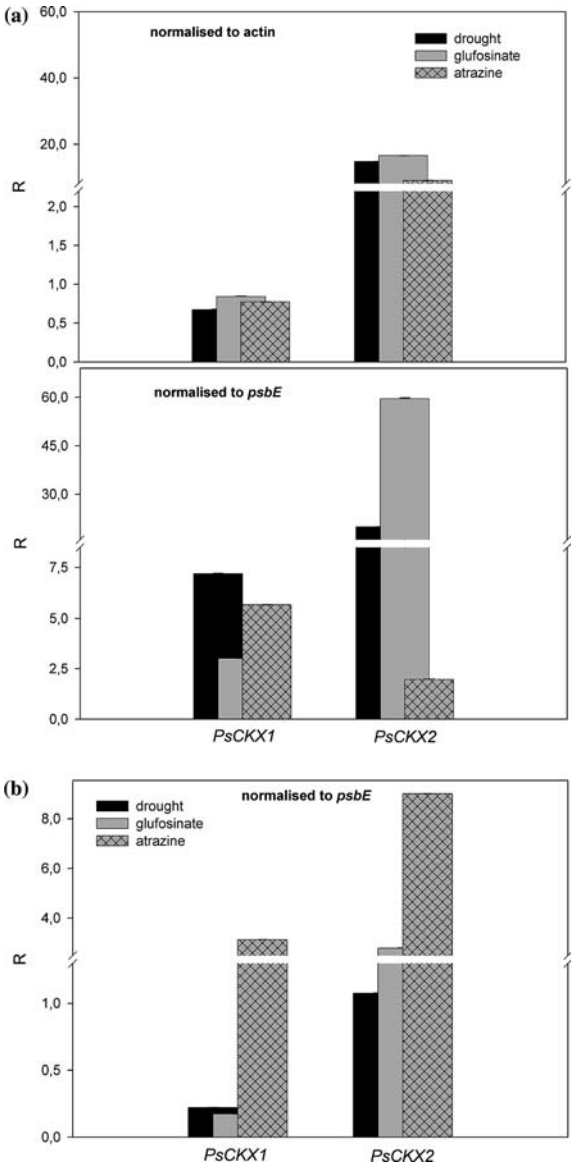

Figure 3. The relative expression ratio (*R*) of *PsCKX1* and *PsCKX2* in leaves (a) and roots (b), calculated based on its real-time PCR efficiency (*E*) and the crossing point (CP) deviation of an unknown sample (from drought-, glufosinate- and atrazine treated pea plants) versus a control (non-treated variants), and expressed in comparison to reference actin and *psbE* genes. The results presented are from one experiment and the analysis was performed in 3 separate runs with three independent samples taken from different plants. Vertical bars indicate the standard error (Std.Err.) according to SigmaPlot for Windows Version 4.00 Software.

(Figure 2a). High expression levels of *PsCKX1* and *PsCKX2* in the leaves were observed.

PsCKX1 amplification in control and atrazine-treated roots resulted in two bands with different sizes −158 bp and a bigger band at around 300-bp-position, which was not visible in leaves' RT-PCR reactions loaded on ethidium bromide-stained agarose gel. The same expression profile was observed in all root samples and the two bands were with diverse intensity in differently treated samples. Possible DNA contamination of RNA samples was excluded (Figure 2b).

Band 158 b.p. exhibited comparatively high intensity in drought and atrazine-treated roots (Figure 2a). The measured relative expression ratio (*R*) in drought-stressed roots (normalised to *psbE* expression only – Figure 3b) was comparatively low, while the highest *R* was calculated for atrazine-treated roots. Actin standards were not applicable for root samples as it is obvious from Figure 2a. Increased *PsCKX1* mRNA expression was observed in leaves of drought stressed plants (Figure 3a).

A very poor amplification of *PsCKX2* in control leaves was documented when the LightCycler reactions were loaded on ethidium bromide stained gel (Figure 2a). Lack of *PsCKX2* transcripts was documented in all root samples as well (Figure 2a). *PsCKX2* relative expression ratio (*R*) reached comparatively high levels in the leaves of water-deprived variants (Figures 2a, 3a).

Herbicide treatments exhibited diverse effects over expression of both *CKX* genes. Calculated *R* for glufosinate-treated leaves was comparatively higher than the relative expression ratio of atrazine-treated leaves (Figure 3a) while in roots higher *R* values were detected in root samples of atrazine-sprayed plants (Figure 3b).

Measured CKX activity in drought-stressed plants was inhibited in leaves and was above the control in roots. Decreased specific enzymatic activity in tissues of glufosinate-sprayed plants was established. Atrazine-treated plants did not exhibited any significant changes regarding CKX levels – in leaves enzymatic activity was slightly stimulated by the herbicide application and in roots it was decreased after treatment (Figure 4).

Discussion

Environmental stress triggers a wide range of plant responses, from altered gene expression and cellular metabolism to changes of growth rates and crop yields. Cytokinin breakdown via CKX appears to be the predominant mechanism for

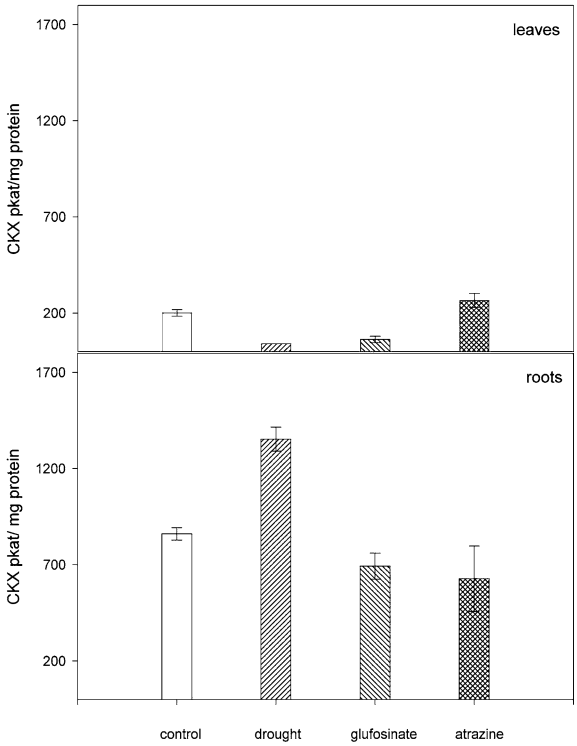

Figure 4. Specific CKX activity (pkat/mg protein) in the last fully expanded leaves and secondary roots of 19 days-old cv. Manuela plants subjected to drought, glufosinate and atrazine treatment.

cytokinin inactivation under adverse environmental conditions. The effect of drought over CKX activity has been an object of study in water-deprived sunflower plants (Manju et al. 2001) and three-fold increase of the enzyme was detected in the stressed roots. CKX activity measured in the stressed leaf tissue was negligible but still detectable compared to its complete absence in the controls. Difficulties to explain the CKX activity results (as it is well-known that the enzyme is substrate-inducible) were provoked by the decreased cytokinin contents in the roots and leaves of the water-deprived plants. Results obtained in the present study support the existence of tissue specific CKX response towards drought at least on biochemical level. Cytokinin flux under stress seems to be submitted to complicated regulatory mechanisms, which are able to maintain hormone levels under adverse conditions.

As a model species for comparative and functional legume genomics *Medicago truncatula* is a good starting point for gene expression studies in other legumes. Using CKX gene-specific primers designed on the existing sequences in TIGR *Medicago truncatula* database we were able to identify DNA sequences from putative cytokinin oxidase/dehydrogenase genes in *Pisum sativum* (which we have called *PCKX1* and *PCKX2*) and to measure their relative expression rates in the tissues of differently stressed young pea plants. Previous studies have shown that CKX is represented in relatively small quantities in plant tissues (Galuszka et al. 2001; Laskey et al. 2003). Real-time RT-PCR in a LightCycler™ using SYBR Green I fluorescence dye is a rapid and sensitive method to detect low amounts of mRNA molecules and would be suitable for precise assessment of the *CKX* transcript levels. Since the commonly used internal standards can quantitatively vary in response to various factors it has been suggested to use at least two types of housekeeping gene transcripts as internal standards (Thellin et al. 1999). We choose two pea housekeeping genes – actin (one of the most commonly used normalising standards) and the plastid gene *psbE*. The highly conserved *psbE-F-L-J* operon encodes low molecular weight, single transmembrane proteins of Photo System II (PS II) (Cushman et al. 1988; Webber et al. 1989; Anderson and Styring 1991). This four-gene transcribtion unit is transcribed and translated in the dark as well as in the light (Webber et al. 1989; Kawaguchi et al. 1992). Experimental inactivation of *psbE* and *psbF*, the cytochrome b559 α and β subunits, respectively, prevents assembly of PS II (Meurer et al. 2004). It has been confirmed that during salt induced water stress (Forsthoefel and Cushman 1994) as well as high intensity light stress (Hihara et al. 2001) the expression rates of *psbE* gene remained at almost the same level as the control one. In the present study *psbE* showed better result as a normalising standard than actin in the two-step real-time RT-PCR analysis – it was expressed at almost equal levels in differently treated variants both in leaves and roots (Figure 2a). Nevertheless both internal standards showed similar trend in the expression profiles of the studied variants in leaves (Figure 3a).

Assessment of the normalized relative expression of the two genes showed certain tissue specificity and diverse response to the different treatments (Figures 2a, 3a, b). Data suggested that certain members of cytokinin oxidase/dehydrogenase

gene family are under specific regulation and are differently influenced by various stress treatments. Both transcripts tended to be significantly induced by the treatments in a similar manner, which suggests common control mechanisms of gene expression.

It has been described that after heat stress (Cheikh and Jones 1994) and in water stressed maize plants (Setter et al. 2001), zeatin and zeatin riboside were greatly reduced and there were indications that this was due to increased CKX activity. It was unexpected to detect negligible specific CKX activity in drought stressed leaf tissues. The model system deals with relatively short period of water-depravation (four days), which could be classified as comparatively mild drought stress. Measured activity in water-stressed plants was below the control levels, which means that it had been shifted towards decreased level of cytokinin inactivation and this could be regarded as adaptation towards the new water regime. High expression rates of the two studied *CKX* transcripts in the same samples were detected. The relation between *CKX* gene expression and enzymatic activity will remain vague till the other members of cytokinin oxidase/dehydrogenase gene family and the control of their expression is revealed. Varied CKX responses towards atrazine, glufosinate and drought support the existence of complicated regulating mechanism of gene expression.

Comparatively few data are available at present for cytokinin oxidase/dehydrogenase and its role in vegetative development (Jones and Shreiber 1997; Werner et al. 2001). Most studies regarding this side of cytokinin metabolism use tissue culture (Jäger et al. 1997; Redig et al. 1997; Auer et al. 1999) or developing seeds (Brugière et al. 2003) as model systems. During active vegetation, plants exhibit fluctuating levels of CKX activity, which depend on the developmental stage (Vaseva-Gemisheva et al. 2004). This implies certain problems for investigators developing reliable model systems. Nevertheless we believe that studying the changes of cytokinin oxidase/dehydrogenase during vegetative development in plants that have been differently stressed will bring additional knowledge about the interplay between growth and stress acclimation and how they are dependent upon hormone changes.

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