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The Pea Nodulation Mutant R50 (*sym16*) Displays Altered Activity and Expression Profiles for Cytokinin Dehydrogenase

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Abstract R50 (*sym16*) is a pleiotropic mutant of pea (*Pisum sativum* L.) which develops few, pale nodules and has pale young leaves. This phenotype coincides with elevated cytokinin content in vegetative organs, especially mature shoots. Because cytokinin content is known to be tightly regulated by the catabolic action of cytokinin dehydrogenase (CKX), this study focuses on whether CKX-mediated regulation of cytokinin content is involved in the R50 phenotype. Thus, we analyzed the biochemical activity of this enzyme *in vitro* and found that R50 displayed an aberrant activity profile. During development, PsCKX activity was significantly reduced when compared to wild-type (WT); this was observed in many tissues, specifically in mature shoots and nodules where decrease in activity correlated with elevated cytokinin content. To further address this issue, a full-length cDNA corresponding to CKX1 from pea (*PsCKX1*) was obtained via RACE-PCR. Although sequencing the entire *PsCKX1* cDNA from R50 did not reveal any significant mutations that could have linked *PsCKX1* to the *sym16* mutation, relative transcript levels of *PsCKX1* and of another *PsCKX* homolog (*PsCKX2*) were compared between R50 and WT using semiquantitative reverse transcriptase PCR. Interestingly, transcription of these homologs was upregulated in the tissues of R50 displaying the most aberrant phenotype, namely, the mature shoots and nodules. We propose that the R50 phenotype is linked to elevated cytokinin content as a result of deficient PsCKX activity and that transcription of two *PsCKX* homologs is upregulated as a means to compensate for the biochemical deficiency of this enzyme in R50 mutants.

Keywords Cytokinin · Cytokinin dehydrogenase · Development · Legume · Nodulation · Pea mutant

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Introduction

Legumes form a mutually beneficial relationship with nitrogen-fixing soil bacteria; this symbiosis is characterized by the development of nodules on the infected organ. R50, a mutant of pea (*Pisum sativum* L.) carrying the recessive *sym16* mutation (Kneen and others 1994; Ellis and Poyser 2002), displays an abnormal nodulation phenotype. In R50 roots, rhizobial infection threads appear to lack directional control and loop in the cortex; they end up forming a coiled mass in individual cortical cells (Guinel and Sloetjes 2000). Few, often pale, nodules are formed; these appear oblong and flat compared to those of the wild type (WT) because of a lack of periclinal divisions in the nodule primordium (Guinel and Sloetjes 2000).

Low nodulation has often been attributed to the overproduction of, or hypersensitivity to, ethylene. Specifically, several studies have shown that nodulation can be restored to WT levels when inhibitors targeting ethylene biosynthesis or action are applied exogenously to roots of legumes. For example, inoculated R50 roots form as many nodules as the WT when they are treated with amino-vinyl-glycine, an

inhibitor of amino-cyclopropane-1-carboxylic acid (ACC) synthase (for example, Guinel and Sloetjes 2000); this enzyme catalyzes the conversion of S-adenosyl methionine to ACC, the immediate ethylene precursor, and represents the first committed step in ethylene biosynthesis. Vogel and others (1998) confirmed a putative crosstalk between ethylene and cytokinin when they demonstrated that the treatment of *Arabidopsis* with cytokinin leads to increased ethylene production; they proposed that this occurred through a post-transcriptional stimulation of the ACC synthase. Interaction between the two hormones was further evidenced in pea by Lorteau and others (2001) who showed that the synthetic cytokinin 6-benzyl-amino-purine (BAP) induced a dose-dependent increase in ethylene production. This treatment had a rippling effect on nodulation as rhizobia-infected roots treated with 15 μM BAP had few nodules that were often flat and pale. Furthermore, the infection threads in the outer cortex of the BAP-treated WT peas were highly branched and looped, lacking directional growth as they proceeded parallel to the root surface rather than toward the inner cortex (Lorteau and others 2001). This work confirmed previous work (for example, Fang and Hirsch 1998) linking cytokinin and nodulation, and preceded many recent studies (for example, Gonzalez-Rizzo and others 2006; Murray and others 2007; Tirichine and others 2007) implicating cytokinin in the nitrogen-fixing symbiosis.

The striking similarities between the nodulation phenotype of cytokinin-treated WT roots and that of untreated R50 roots prompted us to examine the cytokinin content of their roots and shoots; R50 was found to have elevated levels of cytokinin, particularly in its mature shoots (Ferguson and others 2005). At that time, we proposed that elevated cytokinin content was involved in the R50 phenotype; this hypothesis was further strengthened recently by two studies (Riefler and others 2006; Murray and others 2007). Indeed, when the phenotypic traits exhibited by the loss-of-function cytokinin receptor mutants of *Arabidopsis* are compared with those of R50, many appear to be similar: reduced chlorophyll content (Guinel and Sloetjes 2000), a partial de-etiolation phenotype (Ferguson and others 2005), increased seed size, delayed flowering time (data not shown), and abnormal root vasculature (Pepper and others 2007). The infection threads that form in the cytokinin-deficient mutant *hit1-1* of *Lotus japonicus* (Murray and others 2007) are furthermore reminiscent of those formed in inoculated R50 plants (Guinel and Sloetjes 2000).

Because R50 was found to accumulate cytokinins, we decided to look into their regulation, particularly their degradation. Cytokinin homeostasis within a cell is maintained through the catabolic action of cytokinin dehydrogenase enzymes (CKX, EC 1.5.99.12); these enzymes irreversibly inactivate isoprenoid cytokinins such as isopentenyladenine (iP; Galuszka and others 2007). Following the successful cloning of the first full-length CKX gene from maize (Houba-Hérin and others 1999; Morris and others 1999), molecular analyses have vastly increased our understanding of CKX-mediated cytokinin regulation (Werner and others 2003; Yang and others 2003; Ashikari and others 2005; Riefler and others 2006; Werner and others 2006). It is clear now that in higher plants CKX exists in small gene families of perhaps five to seven functional members (Schmülling and others 2003; Galuszka and others 2004; Massonneau and others 2004), with some cereal crops containing as many as 11 or 12 homologs. Although this situation is likely to be the case in leguminous species, there is less known regarding the size and organization of their *CKX* gene families. The *Medicago truncatula* genome project has revealed the presence of a *CKX* gene and EST sequences; however, it is not yet clear what the size of the gene family might be in this legume. Similarly, little is known about the *CKX* gene from pea, with only two partial sequences available (Vaseva-Gemisheva and others 2005a). However, in that same species, two studies on the effect of abiotic stresses on the enzyme activity have been published (Vaseva-Gemisheva and others 2004, 2005b). Recently, Todorova and others (2006) have re-examined the crosstalk between cytokinin and ethylene by testing whether a defect in CKX could explain some of the phenotypic traits of the ethylene-insensitive *eti5* mutant of *Arabidopsis*; they found that the elevated cytokinin content of the mutant was indeed due to reduced CKX activity. In this regard, it is noteworthy to mention that R50 shoots are also ethylene-insensitive (Ferguson and others 2005).

Because so many traits of this low-nodulation pea mutant link its phenotype to cytokinins and their homeostasis, we investigated the biochemical and molecular aspects of PsCKX in R50 to expand our understanding of this crucial regulatory mechanism in pea. We report here that the phenotype of the mutant is linked to lower enzymatic activity of PsCKX and altered expression of two *PsCKX* homologs. This situation is most apparent in the mature tissues and nodules, which both accumulate cytokinin and display the most aberrant phenotype compared to the WT. Our results implicate CKX in the regulation of cytokinin content during the normal development of pea and specifically provide evidence of a role for this enzyme during the unique symbiotic interaction existing between leguminous plants and nitrogen-fixing rhizobia.

Materials and Methods

Plant Material

Seeds of R50 and WT pea (*Pisum sativum* cv. Sparkle) were surface-sterilized with an 8% bleach solution for 5 min, rinsed with four consecutive 1-min washes with sterile water, and imbibed in sterile water for 12–15 h in

the dark. Five seeds were planted per pot (15.5 cm diameter, 1.7 L total volume) in sterilized vermiculite (4 mm accordion grade; V.I.L. Inc., Toronto, ON). The pots were placed in a controlled growth-room (light/dark 16 h/8 h; 23°C/18°C) with an average light intensity of 300 μE m^{-2} s^{-1}. Plants were given an aqueous nutrient solution every 4 days, the composition of which is given elsewhere (Guinel and Sloetjes 2000). Some pea seedlings were inoculated 5 days after planting (DAP) with 5 ml of a 2% solution of *R. leguminosarum* bv. *viciae* 128C53K (Liphatech Inc., Milwaukee, WI) grown in yeast-mannitol broth. Plants received a second inoculation 5 days later and were harvested 24 DAP. The inoculated plants were watered with a low-nitrogen nutrient solution, as above, but with only 0.5 mM calcium nitrate. At harvest, all tissues were flash-frozen with liquid nitrogen before being stored at −80°C until further use. When applicable, organs were separated from each other (nodules from roots, roots from shoots, and flowers from shoots) prior to freezing.

Extraction of Cytokinin Dehydrogenase

Harvested tissues from which CKX was extracted included roots and shoots of seedlings (9 DAP) and mature plants (17 DAP), fully expanded flowers from 30-day-old plants, and mature nodules from 24 day-old plants; CKX was also extracted from wheat germ to assess the efficiency of the protocol (data not shown). For all samples, tissue was pooled from no less than 12 separate individuals for each biological replicate, of which there were three. The enzyme was extracted following the protocol of Liberos-Minotta and Tipton (1995), as modified by Frébort and others (2002). To improve the quality of the extract, a protease inhibitor cocktail (Sigma P9599) was added to the slurry at a 100-fold dilution before centrifuging for 20 min at 20,000*g*. Aliquots (200 μl) of the resulting supernatant were stored at −80°C. All procedures were performed in a cold room maintained below 4°C.

Colorimetric Assay for PsCKX Specific Activity

Specific activity was determined through the use of a colorimetric assay (Liberos-Minotta and Tipton 1995), as modified by Frébort and others (2002). The reaction (0.6 ml total volume) contained 200 μl of the enzyme extract and 400 μl of a 75 mM Tris/HCl buffer (pH 8.5) containing 2,6-dichlorophenol-indophenol (0.5 mM) and iP (0.15 mM). The reaction was incubated at 37°C for 1 h. The absorbance of the Schiff's base product was measured at 352 nm on a Cary 50 UV-visible spectrophotometer. Total protein content of all extracts used for the CKX assay was assessed quantitatively by the standard Bradford assay (Bradford 1976) as well as qualitatively by SDS-PAGE analysis.

Extraction and Purification of Cytokinins

Cytokinins were extracted and separated under procedures, established by Emery and others (1998) and Ferguson and others (2005), that were designed to prevent enzyme activity that could cause cytokinin nucleotide degradation and cytokinin isomerization. Frozen tissue samples of fully expanded flowers and mature nodules (ages as above) were homogenized (Ultra-Turrax T8; IKA-Werke GmbH, Staufen, Germany) over ice in cold (−20°C) modified Bieleski extraction buffer (CH_3OH:H_2O:HCOOH [15:4:1 v:v:v]) at 20 ml g^{-1} dry weight and were extracted according to Dobrev and Kaminek (2002). One hundred nanograms of each deuterated cytokinin, [$^2H_6$]iP, [$^2H_6$][9R] iP, *trans*-[$^2H_5$] zeatin, [$^2H_3$] dihydrozeatin, trans-[$^2H_5$][9R] zeatin, [$^2H_3$][9R] dihydrozeatin, [$^2H_6$][9R-MP] iP, and [$^2H_6$][9R-MP] dihydrozeatin (OlChemIm Ltd, Olomouc, Czech Republic), were added as quantitative internal standards. The levels of *cis*-cytokinin were quantified based on the recovery of the deuterated standards of the corresponding *trans*-compounds. Pooled extract supernatants were dried *in vacuo* at 40°C with residues reconstituted in 5 ml of 1.0 M HCOOH for purification on an Oasis MCX column (Waters, Mississauga, ON) as described in Dobrev and Kaminek (2002). Eluted nucleotides were converted to nucleosides for quantification, and resultant nucleosides were further purified on a reversed-phase C18 column (AccuBOND ODS; Fisher Scientific, Mississauga, ON) as described in Emery and others (2000).

LC-(ES+)-MS/MS Conditions for Cytokinin Analysis

Purified cytokinin fractions were separated and analyzed as in Ferguson and others (2005). Once the cytokinin had been eluted, the HPLC effluent was introduced into the electrospray source (source block temperature = 80°C, desolvation temperature = 250°C) using conditions specific for each cytokinin; quantification was obtained by multiple reaction monitoring (MRM) of the mother (parent) ion and the appropriate daughter (product) ion as in Prinsen and others (1995).

RACE-PCR and Sequence Analysis

Total RNA was isolated from roots, leaves, fully expanded floral tissues, and nodules of the WT pea plant (ages as above, 0.1 g for each) using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was eluted twice with 200 μl of RNase-free water and stored at −80°C until further use. The concentration of RNA in all samples was estimated from the A_{260}. Total RNA (4 μg) from 17-DAP WT shoots was used to synthesize first-strand cDNA according to the

guidelines of the GeneRacer kit (Invitrogen, Carlsbad, CA). Gene-specific primers (GSPs) for RACE-PCR amplification of *PsCKX1* were based on a partial gene sequence (Vaseva-Gemisheva and others 2005a; accession No. AY444352). 5′ RACE-PCR was performed using a reverse GSP (5′-CGTTCCACCCACCGTTAGCCCGAGA T-3′) and the GeneRacer 5′ primer. 3′ RACE-PCR was carried out using the GeneRacer 3′ primer and a forward GSP (5′-CGGCCAAGCCATGGCAGAAAAAGGA-3′). Nested PCR was then performed for *PsCKX1* using a nested forward GSP, 5′-AAAAGGACTGGTCCTCGACA-3′, together with the GeneRacer 3′ nested primer. A hot start touchdown PCR regimen was used as specified by the manufacturer; however, an annealing temperature of 60°C was used. PCR products were isolated from 1% agarose gels following electrophoresis, cloned into the pCR4Blunt-TOPO vector (Invitrogen, Carlsbad, CA), and sequenced (MOBIX lab, McMaster University, Hamilton, ON).

Initially, a truncated product was obtained by 3′ RACE-PCR (that is, a stop codon could not be identified) and it was presumed that secondary mRNA structure had interfered with the synthesis of a full-length cDNA. Omniscript RT (Qiagen) was then used to synthesize cDNA using an initial denaturation step at 65°C and reverse transcription was performed at 42°C for 2 h, as suggested by the manufacturer for templates with a high degree of secondary structure. 3′ RACE-PCR was performed with another forward GSP (5′-CGCTCGCTCTTCCGAAGTTGACACGA A-3′) and the GeneRacer 3′ primer using the same cycling parameters as given above. The resulting cDNA was cloned and sequenced as described above. The sequence of *PsCKX1* from the R50 mutant was also determined. Total RNA was isolated from 17-DAP R50 shoots and used to prepare cDNA using OmniScript reverse transcriptase as described for WT. Specific primers were designed based on the *PsCKX1* sequence from WT plants, and these were used to amplify the corresponding sequence from R50 by PCR in three overlapping fragments. PCR products were cloned into pCR-4Blunt-TOPO vectors and sequenced.

Protein-protein BLAST comparisons (Altschul and others 1997) were performed using the deduced amino acid sequences, and an amino acid sequence alignment with other plant CKXs was generated using ClustalW (Higgins and others 1994). The *PsCKX1* sequence was further analyzed using Peptide-Mass (Wilkins and others 1997), NetNGlyc 1.0, and iPSORT (Bannai and others 2002).

Accession Numbers

All sequences utilized and/or presented in this article are available online from the national resource for molecular biology information website (http://www.ncbi.nih.gov).

Semiquantitative RT-PCR

First-strand cDNA was synthesized with the Omniscript RT kit (Qiagen) using 1 μg of RNA as template. Primers were designed to amplify a β-actin fragment [accession No. X68649, herein referred to as *PsActin*, 5′ - CAA TCG GAG CTG AGA GGT TC - 3′ (forward) and 5′ - CAG AGG ACT GCC ACA GAA CA - 3′ (reverse)], a 325-bp *PsCKX1* cDNA product [accession No. AY444352, 5′ - AA A AGG ACT GGT CGA CA - 3′ (forward) and 5′ - GCG TGC AAA AAG CTC AT - 3′ (reverse)], and a 283-bp fragment of *PsCKX2* [accession No. AY444353, 5′ - TGA CGT GCT TCA TCC AAA TC - 3′ (forward) and 5′ - CTT GGT GCC AAA CCA TAC TTC - 3′ (reverse)]. The PCR program for the linear amplification of the *PsActin*, *PsCKX1*, and *PsCKX2* products began with a 3-min denaturation at 94°C and continued for 50 cycles of 94°C for 30 s, 54°C for 1 min, and 72°C for 1 min, followed by a 6-min extension at 72°C. The linear range of amplification was determined to be 20-30, 20–40, and 25–40 cycles for the *PsActin*, *PsCKX1*, and *PsCKX2* products, respectively. This was achieved by analyzing the amount of specific product formed in the PCR reaction at a 5-cycle interval; this began at 10 cycles and continued until 50 cycles had been run. To compensate for template competition between *PsActin* primers and *PsCKX* primers in duplex PCR reactions, the optimal primer ratios for *PsCKX1:PsActin* and *PsCKX2:PsActin* were determined by conducting duplex PCR reactions containing both the *PsCKX* primers (that is, either *PsCKX1* or *PsCKX2*) and the *PsActin* primers at a wide range of concentrations (5, 8, 10, 15, and 25:1). The optimal primer ratios were found to be 15:1 and 25:1 for *PsCKX1* and *PsCKX2*, respectively. Each *PsCKX* fragment was resolved on 1.5% agarose gels, which were stained with ethidium bromide. The PCR was run according to the program outlined above and lasted for 25 cycles and 30 cycles for *PsCKX1* and *PsCKX2*, respectively. On all semiquantitative RT-PCR gels, the *PsActin* band is the upper band (544 bp), whereas the *PsCKX1* or *PsCKX2* band is the lower band (325 and 283 bp, respectively). Products were visualized and analyzed using Quantity One software (v4.6, Bio-Rad, Hercules, CA).

Statistical Analyses

Mean values and standard errors (SE) for all data sets were generated using SigmaStat (v2.03, SysStat Software Inc., Richmond, VA). The reported values for the semiquantitative RT-PCR data represent the mean of three biological replications ($n = 3$); as well, all selected images are representative of these three biological replications. The reported values for the PsCKX activity data represent the mean of three biological replicates. For each replicate,

three replications were conducted using tissue pooled from several different plants. In all cases, comparisons made between R50 and WT were done using Student's *t* tests ($P < 0.05$, $n = 3$).

Results

PsCKX Activity is Reduced in R50

In both pea lines, the enzyme was active in both roots and shoots, with the vast preponderance of activity found within the roots. In the R50 mutant, PsCKX activity in the vegetative organs was lower than that of the WT at all points analyzed (Figure 1). The most significant reductions in activity were observed in the mature shoots (7.4-fold) and the nodules of R50 (16.8-fold, Figure 1); no difference was noted between WT and mutant flowers. These results suggest that CKX displays tissue- and age-specific activity profiles during the development of WT pea and that its activity is reduced significantly in the vegetative organs and nodules of the R50 plants.

Cytokinin Homeostasis is Altered in the Pale Nodules of R50

No differences were noted in the cytokinin content of the flowers of the two pea lines (data not shown). In stark contrast, nodules of R50 displayed a number of striking abnormalities when compared with the WT, although the total cytokinin levels did not differ significantly (220 ± 21 pmol for WT and 182 ± 14 pmol for R50). First, in R50 nodules, there was an accumulation of the presumed active cytokinin forms such as the *cis*-isomer of zeatin (*cis*-Z) and its riboside (*cis*-[9R]Z) (Figure 2). Second, other active cytokinin forms that are not substrates of cytokinin dehydrogenase, such as dihydrozeatin (DHZ, especially its riboside form), were also accumulated in R50 nodules. Finally, early precursor cytokinin forms (such as *cis*-[9R]ZMP and iPMP), which are associated with active cytokinin biosynthesis, were lacking in the nodules of R50 (Figure 2).

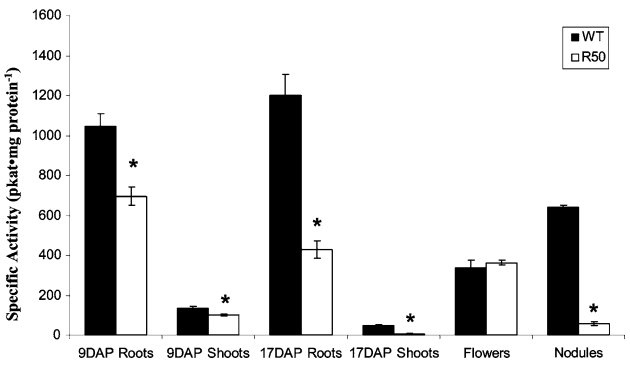

Fig. 1 *In vitro* cytokinin dehydrogenase specific activity (pkat mg protein^{-1}) from roots and shoots of seedlings (9 DAP) and mature plants (17 DAP), as well as flowers and nodules of WT (black box) and R50 (white box). An asterisk denotes significant line-related differences between R50 and WT (*t* test, $P < 0.05$, $n = 3$). Mean values ± SE are shown

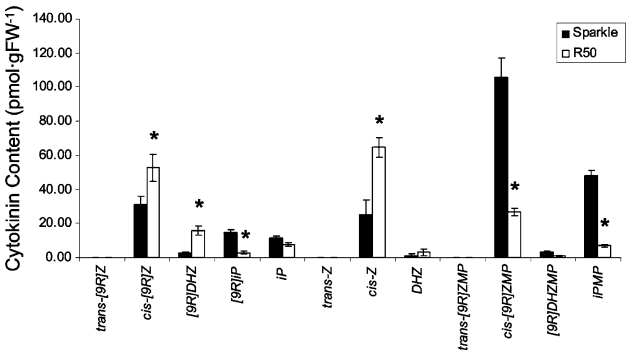

Fig. 2 Cytokinin content (pmol g FW^{-1}) of nodules isolated from WT (black bars) and R50 (white bars) plants. An asterisk denotes significant line-related differences between R50 and WT (*t* test, $P < 0.05$). Mean values ± SE are shown

Cloning of *PsCKX1*

To further investigate CKX-mediated regulation of cytokinin content in the R50 mutant line, an existing partial sequence (previously referred to as PCKX1, AY444352.1; Vaseva-Gemisheva and others 2005a) was used as a template for 5′ and 3′ RACE-PCR of the wild-type *PsCKX1* cDNA. The coding region of the full-length *PsCKX1* cDNA was determined to be 1560 bp in length (accession No. EF030477), which translates into a 519-amino-acid polypeptide (Figure 3) with a predicted mass of 58 kDa. The entire cDNA of PsCKX1 was also sequenced in the R50 (*sym16*) mutant background; a comparison of the two sequences did not allow us to notice any obvious mutations.

PsCKX1 is homologous with *CKX* genes from other model systems (*Arabidopsis thaliana*, *Zea mays*, and *Oryza sativa*) and codes for highly conserved domains such as the GHS FAD-binding motif (Figure 3). BLAST analysis indicates that there are no significant similarities between *PsCKX1* and any other known genes in the pea genome (data not shown); however, *PsCKX1* does share 93% identity over 95% of its length with a sequence found on a genomic BAC clone (accession No. AC151806.21) that was sequenced as part of the *Medicago truncatula* genome project (data not shown). This *M. truncatula* sequence corresponds to a number of ESTs [for example, EST484466 (accession No. BG582720.1), and EST391860 (accession No. AW980707.1)] that are available in the EST database, suggesting that this sequence codes for a transcriptionally active gene likely orthologous to *PsCKX1*.

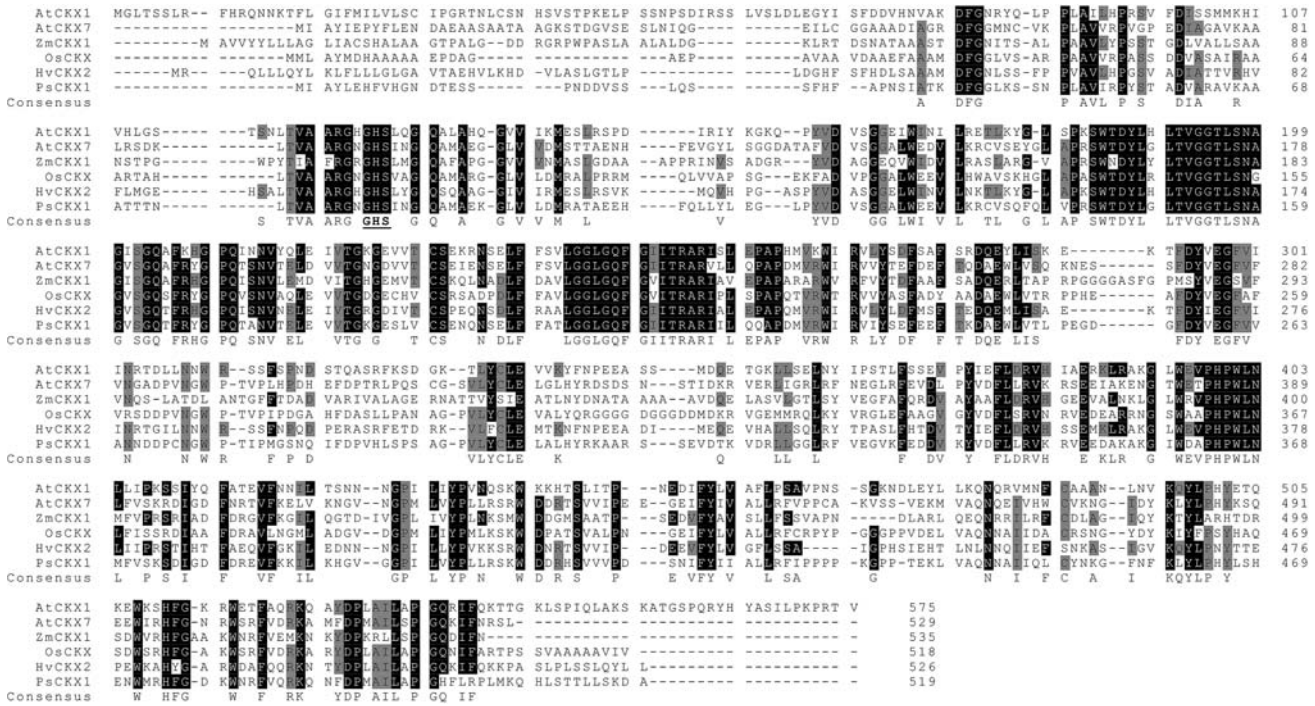

Fig. 3 Amino acid alignment of PsCKX1 (EF030477) with representative CKXs from *Arabidopsis thaliana* (AtCKX1 and AtCKX7; At2g41510 and AF303981), *Zea mays* (ZmCKX1; AF044603), *Oryza sativa* (putative OsCKX; AP004707), *Hordeum vulgare* (HvCKX2; AF540382), and the consensus. Amino acids highlighted in black are greater than 80% conserved, those in gray are greater than 60% conserved (percent homologies are based on an alignment of 7 AtCKXs, 3 ZmCKXs, 6 OsCKXs, 2 HvCKXs, and PsCKX1). The FAD-binding motif (GHS) is underlined

A BLAST search using the predicted amino acid sequence of PsCKX1 revealed that the most closely related protein (62% identity) is AtCKX7 (previously AtCKX5; accession No. AF303981) from *Arabidopsis*. Also on the list of closely related proteins were a putative cytokinin dehydrogenase from *Oryza sativa* (56% identity) and a FAD-linked oxidase from *M. truncatula* (48% identity; accession No. ABE85203). The amino acid sequence analyses predict that PsCKX1 has three potential N-glycosylation sites (12-NDTE, 74-NLTV, and 174-NVTE) but lacks an N-terminal signal sequence, a mitochondrial presequence, or a chloroplast transit peptide. The protein is therefore expected to be cytosolic.

Rigorous attempts were also made to clone a full-length cDNA for PsCKX2 (previously referred to as PCKX2, accession No. AY444353; Vaseva-Gemisheva and others 2005a) but without success. The available partial sequence (309 bp) did not provide adequate resources for cDNA amplification by means of RACE-PCR or genomic amplification utilizing a Genome-Walking strategy; thus it is not included in these comparisons.

Expression Analyses of Two *PsCKX* Homologs

The expression profiles of both *PsCKX1* and *PsCKX2* in the R50 mutant line differed significantly from those of the WT (Figures 4 and 5). The expression of both *PsCKX* transcripts was always lower in the young (9 DAP) roots of R50 than in those of the WT. Conversely, *PsCKX1* and *PsCKX2* transcript levels were more elevated in mature (17 DAP) roots and shoots of R50 than in those of WT. *PsCKX* expression was also analyzed in the flowers but no differences were noted between the two pea lines for either of the two PsCKX homologs analyzed (Figures 4 and 5). Most intriguing was the observation that the pale nodules of R50 expressed both *PsCKX1* and *PsCKX2* at a much higher level than those of WT (Figures 4 and 5).

Discussion

This report provides analyses on the biochemical and molecular aspects of cytokinin dehydrogenase in the low-nodulating mutant R50 (*sym16*), a pea mutant that accumulates cytokinin. R50 displays lower CKX activity than WT at several distinct developmental stages; this deficiency correlates well with cytokinin accumulation in its mature shoots (Ferguson and others 2005) as well as in its nodules. After having cloned the first full-length *PsCKX* cDNA (*PsCKX1*, accession No. EF030477) that displays characteristic features of known CKXs, we analyzed the expression of this gene as well as that of

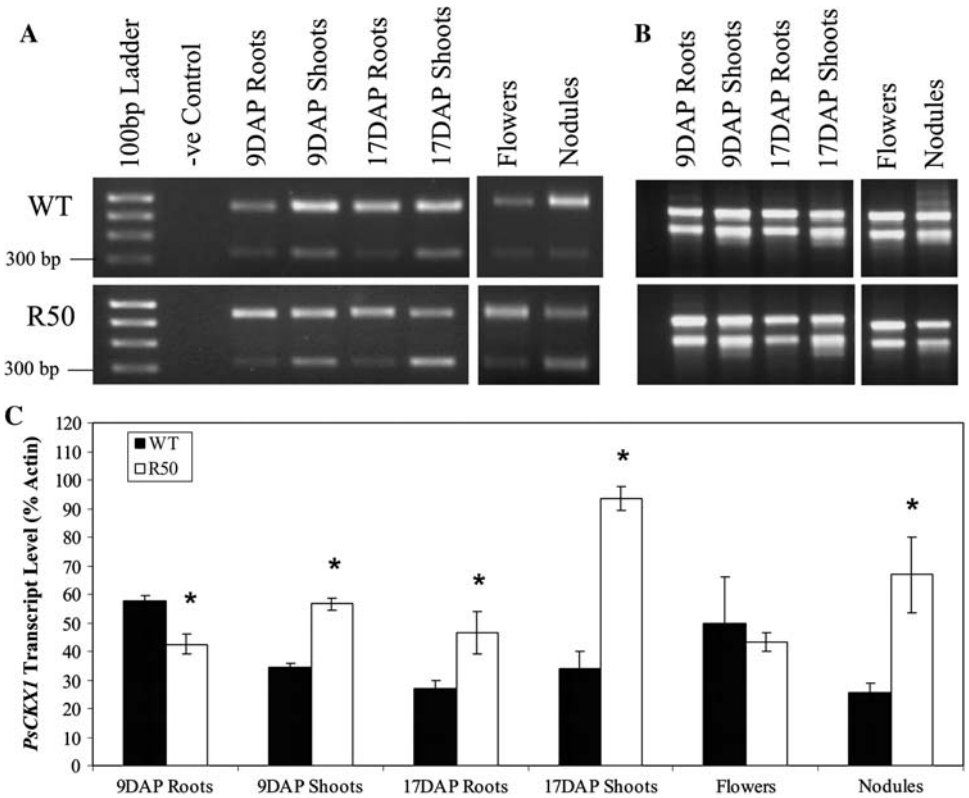

Fig. 4 Semiquantitative RT-PCR of *PsCKX1* transcripts from roots and shoots of seedlings (9 DAP) and mature plants (17 DAP), as well as flowers and nodules of both WT and R50. (**A**) RT-PCR for *PsCKX1* from WT (upper panel) and R50 (lower panel). The *PsCKX1* product (lower band, 325 bp) was amplified simultaneously with the *PsActin* control (upper band, 544 bp) in a duplex PCR reaction. (**B**) Positive controls of the total RNA used for RT-PCR in the corresponding tissues shown in A. (**C**) Quantitation of product intensities for WT (black bars) and R50 (white bars). Transcript levels for the *PsCKX1* product are displayed as a percent of the *PsActin* control as determined by intensity-based quantification of each product using Quantity One software (v4.6, Bio-Rad). An asterisk denotes significant line-related differences between R50 and WT (*t* test, $P < 0.05$, $n = 3$). Mean values ± SE are shown

another member of the *PSCKX* gene family (*PsCKX2*) by RT-PCR. We found a direct correspondence between *PsCKX* expression and biochemical activity in some tissues of both pea lines. For example, low expression of *PsCKX1* and *PsCKX2* in young roots of R50 correlates well with decreased PsCKX activity and slightly elevated cytokinin content compared to WT plants. As well, in flowers of R50 and WT, the expression of both homologs is comparable and this is in agreement with both the specific activity of PsCKX and the cytokinin content within these tissues. Most notable, however, is the elevated transcript profiles of both *PsCKX1* and *PsCKX2* in organs of R50 that display the most significant phenotype (that is, mature shoots and nodules). The specific activity of PsCKX in these organs is always reduced and cytokinins are correspondingly accumulated. Plausible mechanisms that may shed light onto how abnormal cytokinin catabolism might affect the expression of *PsCKX* homologs and ultimately cytokinin accumulation in the R50 mutant are discussed below.

Domain Characteristics of the PsCKX1 Protein

The present study provides the first report on the cloning of a full-length *CKX* cDNA from a legume species (herein denoted as *PsCKX1*), the translational product of which has a predicted molecular weight of 58 kDa and is structurally similar to many CKX proteins from higher plant systems (see Schmülling and others 2003 for review). The majority of CKX proteins are targeted to the apoplast via an N-terminal signal peptide (Schmülling and others 2003; Galuszka and others 2005); however, because it lacks this signal, PsCKX1 is predicted to be a cytoplasmic protein. This seems reasonable because of all the *Arabidopsis* isoforms, PsCKX1 shares the greatest identity with AtCKX7, which also lacks a signal peptide and is likely cytoplasmic (Schmülling and others 2003). Because it appears not to be targeted to the apoplast, it is probably not glycosylated, although three putative glycosylation sites were found. This would be in agreement with a recent study performed on pea; indeed, Gaudinová and others (2005) suggested that

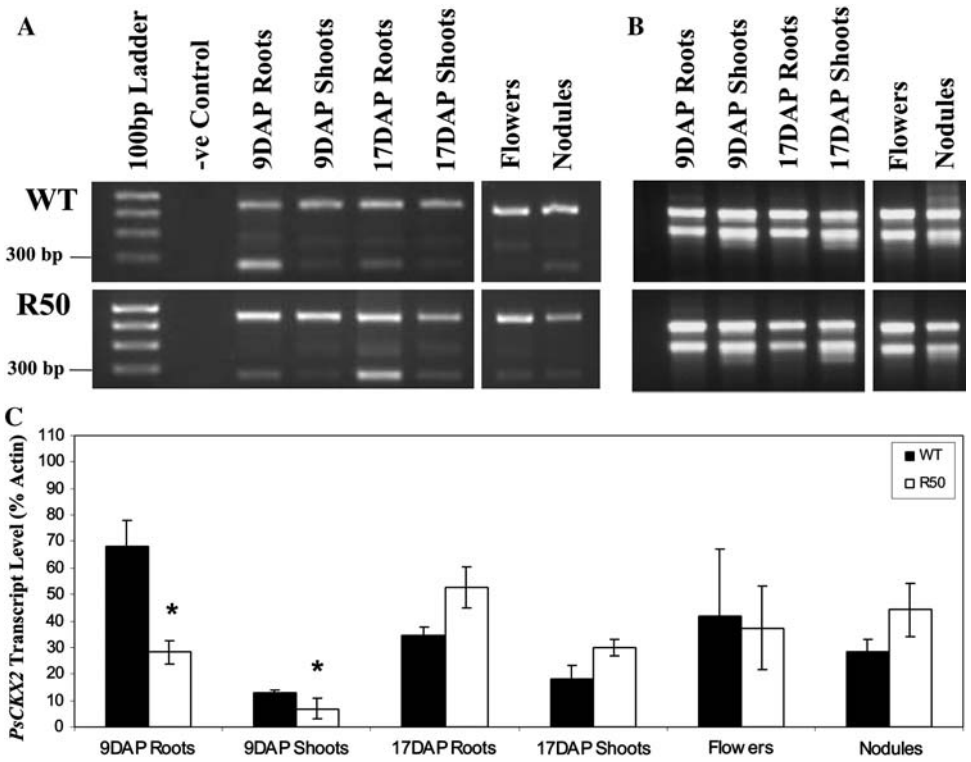

Fig. 5 Semiquantitative RT-PCR of *PsCKX2* transcripts from roots and shoots of seedlings (9 DAP) and mature plants (17 DAP), as well as flowers and nodules of both WT and R50. (**A**) RT-PCR for *PsCKX2* from WT (upper panel) and R50 (lower panel). The *PsCKX1* product (lower band, 283 bp) was amplified simultaneously with the *PsActin* control (upper band, 544 bp) in a duplex PCR reaction. (**B**) Positive controls of the total RNA used for RT-PCR in the corresponding tissues shown in A. (**C**) Relative levels of *PsCKX2* transcript for WT (black bars) and R50 (white bars). Transcript levels for the *PsCKX2* product are displayed as a percent of the *PsActin* control as determined by intensity-based quantification of each product using Quantity One software (v4.6, Bio-Rad). An asterisk denotes significant line-related differences between R50 and WT (*t* test, $P < 0.05$, $n = 3$). Mean values ± SE are shown

CKX in pea leaves was either a nonglycosylated isoform or an isoform with a very low degree of glycosylation. They based their conclusion on a biochemical study in which they demonstrated that the enzyme had a relatively high pH optimum and could not bind to lectin in a column.

The crystalline structure of ZmCKX1 has revealed the importance of a short GHS motif found within the FAD-binding domain of all CKX proteins (Malito and others 2004). In ZmCKX1, it is found at positions 104–106 (Houba-Hérin and others 1999), in AtCKX7 at positions 95–97 (Schmülling and others 2003), and in PsCKX1 at positions 82–84 (Figure 3). The histidine residue within this motif (corresponding to His83 of PsCKX1) was confirmed to be the actual site of attachment of the FAD cofactor (Malito and others 2004). Furthermore, aspartic acid 169 (D169) and glutamic acid 288 (E288) are conserved in all plant species analyzed thus far (Schmülling and others 2003), including the pea homolog reported here (D146 and E259 of PsCKX1). These residues may be involved in the oxidative cleavage of the cytokinin substrate (Malito and others 2004). The presence of these highly conserved motifs support the prediction that PsCKX1 is active and has a similar function to that of known CKX proteins of other species.

CKX and the R50 Phenotype

It is widely accepted that cytokinin homeostasis during normal plant growth and development is largely regulated by the activity of CKX (Kamínek and others 1997). Furthermore, recent studies have shown that cytokinin content is tightly regulated by CKX enzymes and that changes in CKX activity are mirrored by changes in cytokinin content. For example, cytokinin content is reduced in transgenic plants where *CKX* genes are overexpressed (Werner and others 2003) and exogenous application of cytokinin is known to induce CKX activity (Motyka and others 2003). It seems plausible that an autoregulatory feedback loop exists between cytokinin content and the specific activity of CKX; it would serve to maintain cytokinin homeostasis *in planta*. Thus, as cytokinin content increases within the cell, so would the expression of *CKX* genes; this would result in higher overall activity of CKX enzymes to quench the rising hormone levels.

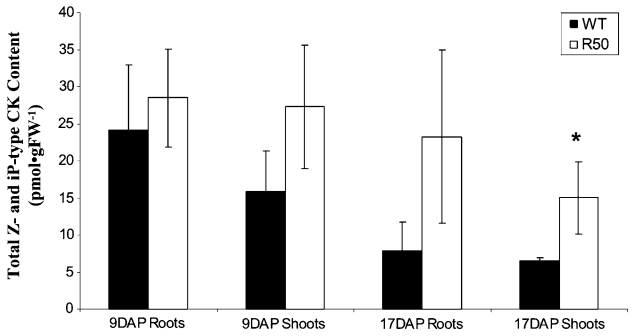

Fig. 6 Total Z- and iP-type cytokinins (pmol g FW^{-1}) from roots and shoots of seedlings (9 DAP) and mature plants (17 DAP) of WT (black bars) and R50 (white bars). An asterisk denotes significant line-related differences between R50 and WT (*t* test, $P < 0.05$, $n = 3$). Mean values ± SE are shown (adapted from Ferguson and others 2005)

Not surprisingly, the activity of CKX enzymes is often compared to cytokinin content (for example, Werner and others 2003), and for the purpose of this discussion, Figure 6, which displays the total content of all cytokinins [free-base, nucleosides, and nucleotides] found in WT and R50, has been drawn from Ferguson and others (2005). This figure allows for a better comparison of cytokinin content and PsCKX activity. The requirement for PsCKX during normal growth and development is highlighted by the deficient-activity profile of the nodulation mutant R50. In the mature roots and shoots of R50 where PsCKX activity is most deficient (Figure 1), R50 accumulates cytokinins, most of which are phosphorylated forms (Ferguson and others 2005). Although free-base and ribosyl cytokinin forms are widely accepted to be CKX substrates, it appears that phosphorylated Z- and iP-type may also be cleaved by this enzyme (Galuszka and others 2007). In support of this was the recent observation that transgenic rice plants with reduced expression of *OsCKX2* accumulate only phosphorylated cytokinins and not free-base or ribosyl forms (Ashikari and others 2005); moreover, *Arabidopsis* plants overexpressing *AtCKX* homologs are severely deficient in phosphorylated cytokinins (Werner and others 2003). We therefore concur with Gaudinová and others (2005) that PsCKX regulates cytokinin content during the development of WT pea and we propose that cytokinin accumulation in the nodulation mutant R50 is caused, at least in part, by reduced PsCKX activity.

Cytokinin Homeostasis and Nodulation in R50

The most intriguing result of this study relates to the organs of R50 which exhibit the most abnormal phenotypes (that is, the shoots and the nodules); here, we have linked the accumulation of cytokinins to high *PsCKX* transcript levels and to decreased activity of the enzyme. It is unclear at this time why the induction of *PsCKX* expression in R50 does not result in increased activity; indeed, sequencing of *PsCKX1* in the R50 background yielded no obvious indication of mutation compared to the WT. However, this phenomenon is likely linked to the yet unidentified *sym16* mutation. If one considers that an autoregulatory feedback loop exists that coordinates cytokinin content through CKX activity, then the elevated cytokinin content in selected tissues displaying obvious phenotypic traits should trigger the expression of both *PsCKX1* and *PsCKX2* as a means of attenuating the accumulating cytokinin pool. Yet, in R50, this effort is to no avail as CKX specific activity within these tissues remains low.

This becomes particularly interesting in the context of the R50 low-nodulation phenotype. It seems this phenotype may be linked to altered cytokinin homeostasis because the cytokinin profile of nodules isolated from R50 mutants displays a number of features characteristic of dysfunctional cytokinin homeostasis. The cytokinin profile we find for the WT pea nodules is similar to early studies by Syõno and Torrey (1976) and Syõno and others (1977) who reported that zeatin was the predominant cytokinin of WT pea nodules. Our results unequivocally demonstrate that the *cis*-zeatin isomers (*cis*-Z and *cis*-[9R]Z) are one of the most active forms of cytokinins in the nodule. No *trans*-zeatin (*trans*-Z) or its riboside (*trans*-[9R]Z) is detectable in WT nodules; this is intriguing as *trans*-Z has long been thought to be the predominantly active zeatin isomer. As well, R50 nodules did not contain any *trans*-Z but did accumulate significant amounts of *cis*-Z and *cis*-[9R]Z. They also possess greater quantities of the dihydrozeatin cytokinin forms; these metabolites are generally resistant to breakdown by CKX and are considered as storage forms (Mok and Mok 2001). Their prevalence in R50 nodules may be a result of the mutant trying to inactivate some of the endogenous active cytokinin pool as part of a response to elevated cytokinin content. Finally, it is apparent that phosphorylated cytokinins (such as *cis*-[9R]ZMP and iPMP) are greatly reduced in R50 nodules. Because these forms are the precursors of active cytokinins and this biosynthetic pathway is regulated by the endogenous level of cytokinin within the cell, the decrease in their abundance is further evidence for the existence of a negative feedback loop (that is, high levels of active forms inhibit further synthesis). In nodules of R50, where active cytokinin forms are highly accumulated, the amount of precursor cytokinins is likely reduced as a result of this feedback.

Recent reports on cytokinin receptor mutants of the two model legumes have strongly implicated cytokinin homeostasis and perception as a key regulatory mechanism for nodule organogenesis (Gonzalez-Rizzo and others

2006; Murray and others 2007; Tirichine and others 2007), and, as mentioned previously, some phenotypic traits are shared between the mutants described in those reports and R50. A definitive role for the regulation of cytokinin content specifically by CKX during the nitrogen-fixing symbiosis was recently illustrated by Lohar and others (2004). In their system, altered cytokinin homeostasis by overexpression of *CKX* genes from *Arabidopsis* and maize resulted in reduced nodulation of *Lotus japonicus*. Their findings match what we observed in R50, that is, elevated CKX transcription and reduced nodulation. Unfortunately, we cannot extend the comparison to either PsCKX activity or cytokinin content because Lohar and others (2004) did not report them in their transgenic plants. Nonetheless, their findings and ours suggest that cytokinin homeostasis as mediated by CKX is an essential regulatory mechanism during the development of the nitrogen-fixing symbiosis.

In summary, although it remains difficult at present to pinpoint the exact mechanism(s) underlying the regulation of cytokinin catabolism in pea until other members of the *PsCKX* gene family are identified in this species, we propose that an autoregulatory feedback loop exists whereby CKX is activated by elevated levels of cytokinin in the WT. This feedback loop is evident also in some tissues of the low-nodulation mutant R50. Taken together, our findings suggest that PsCKX activity is under transcriptional control in those R50 organs where a good agreement exists between transcript level and biochemical activity of the *PsCKX* homologs. In these instances, reduced expression of *PsCKX* genes (such as in young roots) correlated well with a reduction in the measured amount of PsCKX activity in those tissues. However, in mature (17 DAP) tissues as well as in the nodules of the mutant there exists a unique scenario whereby the transcript levels of *PsCKX* homologs are elevated, yet the measurable PsCKX activity is highly reduced and cytokinins are accumulated (Ferguson and others 2005 and this study). Furthermore, these aberrant profiles are seen in tissues of R50 which display the most significant phenotypic traits (that is, pale shoots and ineffective nodules). We therefore suggest that the altered cytokinin homeostasis is a key factor in the manifestation of the R50 mutant phenotype.

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References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Ashikari M, Sakakibara H, Lin S, Yamamoto T, Takashi T, Nishimura A, Angeles ER, Qian Q, Kitano H, Matsuoka M (2005) Cytokinin oxidase regulates rice grain production. Science 309:741–745

Bannai H, Tamada Y, Maruyama O, Nakai K, Miyano S (2002) Extensive feature detection of N-terminal protein sorting signals. Bioinformatics 18:298–305

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Dobrev PI, Kaminek M (2002) Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. J Chromatogr 950: 21–29

Ellis THN, Poyser SJ (2002) An integrated and comparative view of pea genetic and cytogenetic maps. New Phytol 153:17–25

Emery RJN, Leport L, Barton JE, Turner NC, Atkins CA (1998) *cis*-isomers of cytokinins predominate *Cicer arietinum* throughout their development. Plant Physiol 117:1515–1523

Emery RJN, Ma Q, Atkins CA (2000) The forms and sources of cytokinins in developing *Lupinus albus* seeds and fruits. Plant Physiol 123:1593–1604

Fang Y, Hirsch AM (1998) Studying early nodulin gene *ENOD40* expression and induction by nodulation factor and cytokinin in transgenic alfalfa. Plant Physiol 116:53–68

Ferguson BJ, Wiebe EM, Emery RJN, Guinel FC (2005) Cytokinin accumulation and an altered ethylene response mediate the pleiotropic phenotype of the pea nodulation mutant R50 (*sym 16*). Can J Bot 83:989–1000

Frébort I, Šebela M, Galuszka P, Werner T (2002) Cytokinin oxidase/dehydrogenase assay: Optimized procedures and applications. Anal Biochem 306:1–7

Galuszka P, Frébortová J, Werner T, Yamada M, Strnad M, Schmülling T, Frébort I (2004) Cytokinin oxidase/dehydrogenase genes in barley and wheat: cloning and heterologous expression. Eur J Biochem 271:3990–4002

Galuszka P, Frébortová J, Luhová L, Bilyeu KD, English JT, Frébort I (2005) Tissue localization of cytokinin dehydrogenase in maize: Possible involvement of quinone species generated from plant phenolics by other enzymatic systems in the catalytic reaction. Plant Cell Physiol 46:716–728

Galuszka P, Popelková H, Werner T, Frébortová J, Pospíšilová H, Mik V, Köllmer I, Schmülling T, Frébort I (2007) Biochemical characterization of cytokinin oxidases/dehydrogenases from *Arabidopsis thaliana* expressed in *Nicotiana tabacum* L. J Plant Growth Regul 26:255–267

Gaudinová A, Dobrev PI, Šolcová B, Novák O, Strnad M, Friedecký D, Motyka V (2005) The involvement of cytokinin oxidase/dehydrogenase and zeatin reductase in regulation of cytokinin levels in pea (*Pisum sativum* L.) leaves. J Plant Growth Regul 24:188–200

Gonzalez-Rizzo S, Crespi M, Frugier F (2006) The *Medicago truncatula* CRE1 cytokinin receptor regulates lateral root development and early symbiotic interaction with *Sinorhizobium meliloti*. Plant Cell 18:2680–2693

Guinel FC, Sloetjes LL (2000) Ethylene is involved in the nodulation phenotype of *Pisum sativum* R50 (*sym16*), a pleiotropic mutant that nodulates poorly and has pale green leaves. J Exp Bot 51:885–894

Higgins DG, Thompson JD, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Houba-Hérin N, Pethe C, d'Alayer J, Laloue M (1999) Cytokinin oxidase from *Zea mays*: purification, cDNA cloning and expression in moss protoplasts. Plant J 17:615–626

Kamínek M, Motyka V, Vaňkova R (1997) Regulation of cytokinin content in plant cells. Physiol Plant 101:689–700

Kneen BE, Weeden NF, LaRue TA (1994) Non-nodulating mutants of *Pisum sativum* (L.) cv. Sparkle. J Hered 85:129–133

Liberos-Minotta CA, Tipton PA (1995) A colorimetric assay for cytokinin oxidase. Anal Biochem 231:339–341

Lohar DP, Schaff JE, Laskey JG, Kieber JJ, Bilyeu KD, Bird DM (2004) Cytokinins play opposite roles in lateral root formation, and nematode and Rhizobial symbioses. Plant J 38:203–214

Lorteau MA, Ferguson BJ, Guinel FC (2001) Effects of cytokinin on ethylene production and nodulation in pea (*Pisum sativum*) cv. Sparkle. Physiol Plant 112:421–428

Malito E, Coda A, Bilyeu KD, Fraaije MW, Mattevi A (2004) Structures of Michaelis and product complexes of plant cytokinin dehydrogenase: implications for flavoenzyme catalysis. J Mol Biol 341:1237–1249

Massonneau A, Houba-Hérin N, Pethe C, Madzak C, Falque M, Mercy M, Kopecny D, Majira A, Rogowsky P, Laloue M (2004) Maize cytokinin oxidase genes: differential expression and cloning of two new cDNAs. J Exp Bot 55:2549–2557

Mok DWS, Mok MC (2001) Cytokinin metabolism and action. Annu Rev Plant Physiol Plant Mol Biol 52:89–118

Morris RO, Bilyeu KD, Laskey JG, Cheikh NN (1999) Isolation of a gene encoding a glycosylated cytokinin oxidase from maize. Biochem Biophys Res Commun 255:328–333

Motyka V, Vaňkovň R, Čapková V, Petrášek J, Kamínek M, Schmülling T (2003) Cytokinin-induced up-regulation of cytokinin oxidase activity in tobacco includes changes in enzyme glycosylation and secretion. Physiol Plant 117:11–21

Murray JD, Karas BJ, Sato S, Tabata S, Amyot L, Szczyglowski K (2007) A cytokinin perception mutant colonized by *Rhizobium* in the absence of nodule organogenesis. Science 315:101–104

Pepper AN, Morse AP, Guinel FC (2007) Abnormal root and nodule vasculature in R50 (*sym16*), a pea nodulation mutant which accumulates cytokinins. Ann Bot 99:765–776

Prinsen E, Redig P, Van Dongen W, Esmans EL, Van Onckelen HA (1995) Quantitative analysis of cytokinins by electrospray tandem mass spectrometry. Rapid Commun Mass Spectr 9:948–953

Riefler M, Novak O, Strnad M, Schmülling T (2006) *Arabidopsis* cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell 18:40–54

Schmülling T, Werner T, Riefler M, Krupkova E, Manns YB (2003) Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, Arabidopsis, and other species. J Plant Res 116:241–252

Syõno K, Torrey JG (1976) Identification of cytokinins of root nodules of the garden pea, *Pisum sativum* L. Plant Physiol 57:602–606

Syõno K, Newcomb W, Torrey JG (1976) Cytokinin production in relation to the development of pea root nodules. Can J Bot 54:2155–2162

Tirichine L, Sandal N, Madsen LH, Radutoiu S, Albrektsen AS, Sato S, Asamizu E, Tabata S, Stougaard J (2007) A gain-of-function mutation in a cytokinin receptor triggers spontaneous root nodule organogenesis. Science 315:104–107

Todorova D, Vaseva-Gemisheva I, Petrov P, Stoynova-Bakalova E, Alexieva V, Karanov E, Smith A, Hall M (2006) Cytokinin oxidase/dehydrogenase (CKX) activity in wild and ethylene-insensitive mutant *eti5* type of *Arabidopsis thaliana* (L.) Heynh plants and the effect of cytokinin N^1-(2-chloro-4-pyridyl)-N^2-phenylurea on enzymatic activity and leaf morphology. Acta Physiol Plant 28:613–617

Vaseva-Gemisheva IV, Lee D, Alexieva V, Karanov E (2004) Cytokinin oxidase/dehydrogenase in *Pisum sativum* plants during vegetative development. Influence of UV-B irradiation and high temperature on enzymatic activity. Plant Growth Regul 42:1–5

Vaseva-Gemisheva I, Lee D, Karanov E (2005a) Response of *Pisum sativum* cytokinin oxidase/dehydrogenase expression and specific activity to drought stress and herbicide treatments. Plant Growth Regul 46:199–208

Vaseva-Gemisheva I, Lee D, Karanov E (2005b) Antagonistic effects of triazolo[4,5d]pyrimidine and pyridylurea derivatives on cytokinin-induced cytokinin oxidase/dehydrogenase activity in young pea plants. Plant Growth Regul 46:193–197

Vogel JP, Woeste KE, Theologis A, Kieber JJ (1998) Recessive and dominant mutations in the ethylene biosynthetic gene *ACS5* of *Arabidopsis* confer cytokinin insensitivity and ethylene overproduction, respectively. Proc Natl Acad Sci U S A 95:4766–4771

Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, Schmülling T (2003) Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell 15:2532–2550

Werner T, Köllmer I, Bartrina I, Holst K, Schmülling T (2006) New insights into the biology of cytokinin degradation. Plant Biol 8:371–381

Wilkins MR, Lindskog I, Gasteiger E, Bairoch A, Sanchez JC, Hochstrasser DF, Appel RD (1997) Detailed peptide characterisation using PEPTIDEMASS - a World-Wide Web accessible tool. Electrophoresis 18:403–408

Yang S, Yu H, Goh CJ (2003) Functional characterization of a cytokinin oxidase gene *DsCKX1* in *Dendrobium* orchid. Plant Mol Biol 51:237–248