J Plant Growth Regul (2012) 31:460–470
DOI 10.1007/s00344-011-9255-3

# Cytokinin Profiles of *AtCKX2*-Overexpressing Potato Plants and the Impact of Altered Cytokinin Homeostasis on Tuberization In Vitro

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Received: 12 September 2011 / Accepted: 12 December 2011 / Published online: 25 January 2012


**Abstract** Genes encoding cytokinin oxidase/dehydrogenase (CKX) enzymes have been used lately to study cytokinin homeostasis in a variety of plant species. In this study *AtCKX2*-overexpressing potato plants were engineered and grown in vitro as a model system to investigate the effects of altered cytokinin levels on tuber formation and tuber size. Protein extracts from shoots and roots of transformed potato plants exhibited higher CKX activity compared to control plants. Total endogenous cytokinin levels were generally not decreased in *AtCKX2* overexpressors. However, levels of bioactive cytokinins were markedly lowered, which was accompanied by increased levels of *O*- and *N*-glucosides in some transgenic lines. The *AtCKX2*-overexpressing plants displayed reduced shoot growth but other symptoms of the "cytokinin deficiency syndrome" were not recorded. The transgenic plants were able to produce tubers in noninducing conditions. In inducing conditions they developed larger tubers than control. Tubers were also formed on a greater portion of the analyzed *AtCKX2* plants, but with a lower number of tubers per plant compared to control. Taken together, our data suggest that cytokinins cannot be regarded simply as positive or negative regulators of tuberization, at least in vitro. Interactions with other plant hormones that play an important role in control of tuberization, such as gibberellins, should be further studied in detail.

**Keywords** Bioactive cytokinins · Cytokinin oxidase/dehydrogenase (CKX) · In vitro · Potato · Transgenic · Tuberization

## Introduction

The importance of potato (*Solanum tuberosum* L.) as a food crop arises from its potential to develop tubers, which is not a

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common feature among most plant species. Potato tubers develop from stolons, modified lateral shoots characterized by diagravitropic growth and reduced leaves. Under a specific set of environmental conditions (referred to as "tuber-inducing conditions"), stolons change their growth pattern from longitudinal to radial and start accumulating high amounts of starch (Vreugdenhil and Struik 1989).

Tuber-inducing conditions are known to include low temperatures, short photoperiods, high light intensity, and high sucrose versus nitrogen supply in vitro (Jackson 1999). It has been suggested that environmental signals are transduced into tuberization signals by the plant hormonal system (Vreugdenhil and Struik 1989). To date, it is clear that gibberellins are the key plant hormones involved in tuberization signaling, likely to be mediating the transduction of all environmental tuber-inducing conditions listed above as negative regulators of tuberization (Jackson 1999). However, the precise identity of other plant hormones implicated in these processes still remains unclear (Jackson 1999; Fernie and Willmitzer 2001; Hannapel and others 2004; Vinterhalter and others 2008).

Malkawi and others (2007) identified changes in the endogenous hormone content of potato plants transferred from tuber-noninducing to -inducing conditions. Only gibberellins, jasmonic acid, and cytokinins were significantly influenced by the tuber-inducing conditions. Nevertheless, in vitro application of other plant hormones, notably auxins, has also been reported to affect potato tuberization in various cultivars (Romanov and others 2000; Dragićević and others 2008).

Cytokinins are plant hormones that play an essential role in plant growth and development. They control a wide variety of developmental processes in plants (Mok and Mok 2001; Schmülling 2002; Werner and Schmülling 2009). Being implicated in the creation of metabolic sinks (Roitsch and Ehneß 2000), cytokinins have always raised interest for tuberization research. A number of studies have confirmed the implication of a role for cytokinins in tuberization in vitro, either when exogenously applied (Hussey and Stacey 1984; Zhang and others 2005) or endogenously increased by transgenic approaches (Macháčková and others 1997; Ninković and others 1999), or both (Gális and others 1995; Romanov and others 2000). Interactions between cytokinins and other hormones in control of tuberization have also been studied in vitro, for example, interactions with auxins (Romanov and others 2000), jasmonates (Sarkar and others 2006), and growth retardants (Dragićević and others 2004). In general, cytokinins were found to favor tuberization under tuber-inducing conditions in vitro, especially increasing the number of tubers produced (Romanov and others 2000), either when exogenously added or when their biosynthesis was enhanced by transgenic expression of isopentenyltransferase (*ipt*).

Cytokinin homeostasis, among other processes, is determined by regulation of both cytokinin biosynthesis and catabolism, that is, by two main enzymes: isopentenyltransferase (IPT, EC 2.5.1.27) and cytokinin oxidase/dehydrogenase (CKX, EC 1.4.3.18/1.5.99.12), respectively. The latter catalyzes irreversible cytokinin degradation by selectively cleaving unsaturated isoprenoid side chains. CKX is a flavoprotein containing a covalently bound flavin adenine dinucleotide (FAD) molecule as a cofactor (Schmülling and others 2003; Popelková and others 2004). In various species of higher plants, CKX proteins are encoded by small gene families with a varying number of members (Schmülling and others 2003; Werner and others 2006). In *Arabidopsis thaliana*, seven *CKX* genes have been identified and cloned (Bilyeu and others 2001; Werner and others 2001, 2003). Genes encoding CKX have been used as a novel tool to study cytokinin homeostasis by transformation of homologous or heterologous plants. Thus, the impacts of overexpression of *CKX* genes from *A. thaliana* (*AtCKX*) on cytokinin metabolism, endogenous cytokinin profile and content, and overall plant morphology have been studied in tobacco (Werner and others 2001), *Arabidopsis* (Werner and others 2003), the moss *Physcomitrella patens* (von Schwartzenberg and others 2007), and *Centaurium erythraea* (Trifunović and others, unpublished results). Recently, potato (cv. Solara) transformation with *AtCKX1* has been reported in a study focused on interactions between cytokinins and gibberellins in tuber sprouting (Hartmann and others 2011). Overexpression of *AtCKX* genes in both tobacco and *Arabidopsis* consistently showed a remarkable impact on the phenotype of transgenic plants, referred to as the "cytokinin deficiency syndrome": retarded shoot growth, decreased apical dominance, reduced leaf size, delayed flowering and/or reduced number of flowers, delayed leaf senescence, enhanced root growth, decreased shoot meristem size and activity, and increased root meristem size and activity. These symptoms were more intensely pronounced in *AtCKX1* than in *AtCKX2* transgenic plants of both tobacco and *Arabidopsis*, although overexpression of *AtCKX2* had a stronger impact on increase of CKX activity of plant tissue extracts and decrease of cytokinin content than overexpression of *AtCKX1* (Werner and others 2001, 2003). Reduced shoot growth, altered leaf morphology, and prolonged tuber dormancy were reported in *AtCKX1*-overexpressing potato plants (Hartmann and others 2011).

Cytokinin profiles of potato have not yet been studied in detail, nor has the impact of lowered cytokinin levels on tuberization. Therefore, the aim of this study was to determine endogenous cytokinin profiles and content in shoots and roots of *AtCKX2*-overexpressing potato (cv. Désirée) plants grown in vitro. We further investigated whether and how the hormonal changes caused by the

*AtCKX2* overexpression influence tuberization in tuber-inducing and noninducing conditions.

## Materials and Methods

### Plant Material

Elite potato (*S. tuberosum* L. cv. Désirée) tubers were obtained from PKB Agroeconomic Institute, Belgrade, Serbia. The clone PKB3, confirmed by ELISA testing as virus-free (Potato Research Center Guča, Serbia), was used to establish shoot cultures from sprouts, as described by Dragićević and others (2008). These cultures were propagated in vitro by monthly culturing of single-node stem cuttings (SNCs). Both transgenic and control potato shoot cultures were grown on basal medium (BM), that is, MS medium (Murashige and Skoog 1962) solidified with 0.7% agar and supplemented with 3% sucrose, 100 mg $l^{-1}$ myo-inositol and vitamins (Linsmaier and Skoog 1965), without plant growth regulators. Shoot cultures were grown in a growing room at 25 ± 2°C and a 16-h/8-h photoperiod ("Tesla" white fluorescent lamps, 65 W, 4,500 K; light flux of 47 μmol $m^{-2}$ $s^{-1}$).

### Chemicals

Unless stated otherwise, all chemicals used in this study were produced by Sigma (St. Louis, MO, USA). Radiolabeled cytokinins [2-$^3$H]$N^6$-($\Delta^2$-isopentenyl)adenine ([2-$^3$H]iP), [2-$^3$H]*trans*-zeatin ([2-$^3$H]*tZ*), and [2-$^3$H]*cis*-zeatin ([2-$^3$H]*cZ*) were synthesized in the Isotope Laboratory, Institute of Experimental Botany AS CR (Prague, Czech Republic) according to Hanuš and others (2000).

### Transformation and Plant Regeneration

Transgenic shoots were obtained using the bacterial strain *Agrobacterium tumefaciens* (GV3101) carrying the plasmid pBinHTX with the *AtCKX2* gene under the control of the 35S promoter (Werner and others 2001). Bacteria were cultured at 28°C on LB medium (Bertani 1951) supplemented with 50 mg $l^{-1}$ kanamycin, 50 mg $l^{-1}$ rifampicin, and 25 mg $l^{-1}$ gentamicin.

Leaves excised from 30-day-old in vitro propagated plants were used for transformation as described by Cingel and others (2010). Briefly, leaf segments (~10 $mm^2$) were incubated for 5–10 min in overnight-grown bacterial suspension (~$10^8$ bacterial cells/ml), blotted dry on a filter paper, and cultured on callus induction medium (CIM; MS supplemented with 3% sucrose, 2 mg $l^{-1}$ BA, and 0.2 mg $l^{-1}$ NAA). Leaf segments not subjected to cocultivation with bacteria were used as control explants. After 72 h of cocultivation, the explants were washed with sterile water containing 1 g $l^{-1}$ cefotaxime (Jugoremedija, Zrenjanin, Serbia), dried on filter paper, and transferred onto CIM supplemented with 500 mg $l^{-1}$ cefotaxime and 50 mg $l^{-1}$ hygromycin B ($Hyg^+$ CIM). Control explants were transferred to the same medium, but also to medium not containing hygromycin ($Hyg^-$ CIM). After 4 weeks, the explants were transferred to shoot induction medium (SIM; MS supplemented with 1.5% sucrose, 2 mg $l^{-1}$ BA, and 5 mg $l^{-1}$ $GA_3$) plus 500 mg $l^{-1}$ cefotaxime and 50 mg $l^{-1}$ hygromycin B ($Hyg^+$ SIM), and were cultured until shoots regenerated. Control explants were transferred to both $Hyg^+$ SIM and $Hyg^-$ SIM. Every 2 weeks the explants were transferred to fresh medium and incubated under the same temperature and light conditions as described above.

Individual shoots 10–20 mm long (one shoot per explant) were excised and transferred to BM supplemented with 500 mg $l^{-1}$ cefotaxime and 50 mg $l^{-1}$ hygromycin for rooting. Viable, rooted plantlets were selected for further analyses and propagated every 4 weeks by culturing SNCs. Six months after transformation both cefotaxime and hygromycin were omitted from the culture media.

### qRT-PCR Analysis

Total RNA was isolated from shoot and root tissues of control and *AtCKX2* transgenic 30-day-old in vitro-grown potato plants, using the protocol of Gasic and others (2004), and treated with DNAse I (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was carried out using a GeneAmp® Gold RNA PCR Kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), with oligo-dT primers. Reaction conditions were set up according to the manufacturer's protocol. cDNA was used for qRT-PCR with an ABI PRISM 7000 Sequence Detection System and SYBR® Green I (Maxima™ SYBR Green/ROX qRT-PCR Master Mix, Fermentas), according to the manufacturer's instructions. Individual 25-μl PCR reactions contained 12.5 μl of the qRT-PCR Master Mix, cDNA corresponding to 100 ng RNA, and 0.3 μM of forward and reverse primers each for a single gene amplification. Constitutive expression of the potato actin gene (*PoAc58*, GenBank accession No. X55749) was confirmed in parallel. Reactions were carried out in triplicate and repeated twice. The following primers were used: *AtCKX2* forward primer: 5′-GGG AAC TTC CTC ATC CTT GGC-3′, reverse primer: 5′-GCG AGT CCC GAA GCT GAT TT-3′; potato actin forward primer: 5′-TGT TGG ACT CTG GTG ATG GTG-3′, reverse primer: 5′-AGT AAC CAC GCT CAG TGA GGA-3′. Primer specificity was confirmed by BLAST, RT-PCR products, and melting curve analysis. Reaction conditions included initial denaturation (95°C for 10 min); followed by 40

cycles of denaturation at 95°C for 15 s, annealing at 57°C for 15 s, and extension at 72°C for 30 s; final extension (72°C for 10 min); and melting curve analysis. Absolute quantification was carried out using the pBinHTX-*AtCKX2* plasmid digested with *Bam*HI (Fermentas) as a standard in serial dilution.

### Measurement of CKX Activity

The CKX was extracted and partially purified from shoots and roots of 30-day-old in vitro-grown plants according to Motyka and others (2003), and its activity and substrate specificity were determined by in vitro assays based on the conversion of [2-$^3$H]-labeled cytokinins ([2-$^3$H]iP, [2-$^3$H]*t*Z, and [2-$^3$H]*c*Z) to [2-$^3$H]adenine. The assay mixture (50 µl final volume) was composed of 100 mM *N*-tris(hydroxymethyl)methyl-3-amineпropanesulfonic acid (TAPS)-NaOH buffer with 75 µM 2,6-dichloroindophenol (pH 8.5), 2 µM labeled cytokinin (7.4 TBq mol$^{-1}$ each), and enzyme preparation (equivalent to 2.5–35 mg fresh weight [FW] tissue). Separation of the substrate from the product of the enzyme reaction was achieved by HPLC as described by Gaudinová and others (2005). Protein concentrations were determined according to the method of Bradford (1976) using bovine serum albumin as a standard. For CKX determination, three biological samples per control and each of the transgenic lines were used and every determination was repeated twice. Results are presented as mean ± SE. Statistical significance of differences between the means was determined by the Mann–Whitney *U* test ($P < 0.05$).

### Phytohormone Analysis

Endogenous cytokinins were extracted by methanol/formic acid/water (15/1/4 v/v/v) from shoot and root tissues of 30-day-old in vitro-grown plants (1 g FW), homogenized in liquid nitrogen, and purified using the dual-mode solid-phase extraction method (Dobrev and Kamínek 2002). Cytokinin ribotides were determined as corresponding ribosides following their dephosphorylation by alkaline phosphatase. Detection and quantification were carried out using HPLC/MS (Finnigan, San José, CA, USA) operated in the positive-ion full-scan MS/MS mode using a multi-level calibration graph with [$^2$H]-labeled cytokinins as internal standards. Detection limits of different cytokinins were between 0.5 and 1.0 pmol/sample. Determination of cytokinins in three biological samples per control and each of the transgenic lines was repeated twice. Results are presented as mean ± SE. Statistical significance of differences between the means for bioactive cytokinin forms was determined by a Mann–Whitney *U* test ($P < 0.05$).

### Determination of Morphological and Tuberization Parameters

Shoot cultures derived from SNCs were grown on the same medium and at the same temperature as the starting material, either under long days of a 16-h photoperiod (LD) or in continuous darkness (CD). Morphological parameters (shoot length, number of lateral branches, and number of roots) and tuberization parameters (percent of shoots with tubers, average number of tubers per shoot, and average tuber diameter) were determined in 30-day-old in vitro-grown plants. Data presented in the tables and figures are means ± SE of three independent experiments (at least 30 replicates per experiment). Statistical significance of differences between the means was determined by ANOVA and LSD tests ($P < 0.05$).

## Results

### Transformation and Regeneration

To investigate the cytokinin profiles of CKX-over-expressing potato plants and the effects of altered cytokinin levels on tuberization in vitro, the *AtCKX2* gene driven by a 35S promoter was used. At least 70 independently regenerated lines were obtained from explants subjected to transformation. Presented here are the results obtained with three *AtCKX2* lines (*AtCKX2*-39, *AtCKX2*-48, and *AtCKX2*-51) exhibiting the highest *CKX* expression levels, as evidenced by qRT-PCR analysis and/or CKX activity. The transgenic nature of the three selected lines was confirmed by PCR and Southern analyses (data not shown).

qRT-PCR analysis showed higher levels of *AtCKX2* transcript in the shoots than in the roots of the in vitro-grown transgenic plants (Fig. 1). The amount of *AtCKX* transcript in transgenic lines varied from 230 to 58,000

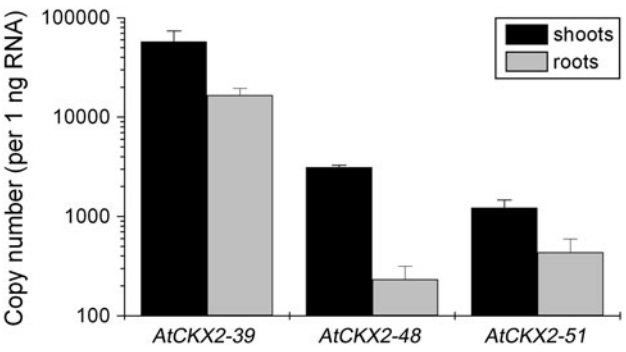


**Fig. 1** qRT-PCR analysis of *AtCKX2* expression in shoots and roots of 30-day-old in vitro-grown transgenic potato. Amounts of *AtCKX* transcripts are presented as number of copies per 1 ng total RNA. Data represent mean ± standard deviation ($n = 3$)

copies per 1 ng RNA, whereas no specific amplification of the *AtCKX2* transgene was detected in control plants.

## CKX Activity

Shoot and root tissue extracts of control and transgenic plants were analyzed for the levels of CKX activity using [2-$^3$H]iP, [2-$^3$H]*t*Z, and [2-$^3$H]*c*Z as substrates. For both shoots and roots of all examined plants, [2-$^3$H]iP was found to be the preferred substrate of CKX (data not shown).

The CKX activity in shoot and root extracts of *AtCKX2*-overexpressing lines is presented as the conversion rate of the preferred substrate [2-$^3$H]iP to [2-$^3$H]adenine (Fig. 2). Significantly increased CKX activity was observed in shoots of all three transgenic lines, with line *AtCKX2*-51 showing the highest shoot CKX activity (14.3-fold of control). A significant increase in root CKX activity was revealed only in the line *AtCKX2*-51 (6.7-fold of control).

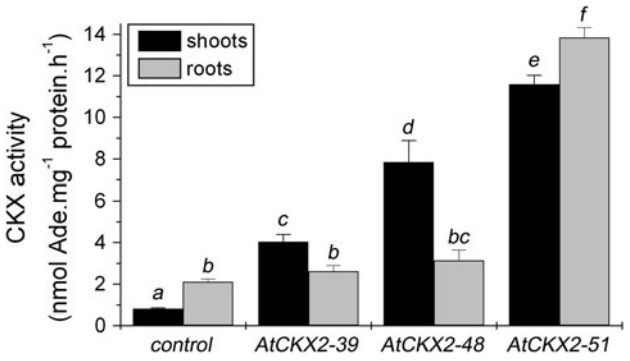


**Fig. 2** CKX activity of protein extracts from shoots and roots of 30-day-old in vitro-grown *AtCKX*-overexpressing potato plants, measured as conversion rate of [2-$^3$H]iP to [2-$^3$H]adenine. Data represent mean ± standard error ($n = 3$). Means marked with the *same letter* were not significantly different according to Mann–Whitney *U* test ($P < 0.05$)

In addition, transgenic lines *AtCKX2*-39 and *AtCKX2*-48 exhibited higher CKX activity in shoots than in roots, whereas the opposite proportion was found in the control and the *AtCKX2*-51 line.

## Cytokinin Profile and Content of *AtCKX2*-Overexpressing Potato Plants

Cytokinin profiles are presented based on the cytokinin conjugation status and physiological function, as proposed by Dwivedi and others (2010) (Table 1; Fig. 3). Cytokinin analysis of control potato plants revealed higher total amounts of endogenous cytokinins in roots compared to shoots (Table 1). The most abundant cytokinin forms in control plants were irreversibly inactive *N*-glucosides exceeding 90% of total cytokinins in shoots and 80% in roots. A difference in cytokinin profiles between control shoots and roots was also observed: bioactive cytokinins were the least abundant derivatives in roots, whereas storage forms of cytokinins were the least abundant in shoots.

As demonstrated in Table 1, bioactive cytokinins (*t*Z, *c*Z, iP, DHZ and corresponding ribosides) comprised only a small portion of total cytokinin content in both shoots (ca. 2%) and roots (ca. 3%) of nontransformed, control potato plants.

Endogenous cytokinin profiles of different *AtCKX2*-overexpressing lines are presented on the basis of the same classification criteria as in control plants (Fig. 3). Total cytokinin content was significantly decreased only in the shoots of *AtCKX2*-39 potato plants. On the contrary, considerably higher total cytokinin levels were observed in shoots of *AtCKX2*-51 plants (Fig. 3a) and in roots of *AtCKX2*-39 plants (Fig. 3b). Elevation of total cytokinin content was mostly due to the increased levels of storage forms and irreversibly inactive (or weakly active) forms of cytokinins (Fig. 3a, b).

**Table 1** Endogenous cytokinin levels of 30-day-old control potato plants, presented on the basis of conjugation status and physiological function

| Cytokinins | Shoots | | Roots | |
|---|---|---|---|---|
| | Total amount (pmol g$^{-1}$ FW) | % of Total cytokinins | Total amount (pmol g$^{-1}$ FW) | % of Total cytokinins |
| Bioactive forms | 11.2 ± 1.0 | 2.4 ± 0.2 | 26.2 ± 1.3 | 3.1 ± 0.2 |
| Storage forms | 1.9 ± 0.3 | 0.4 ± 0.1 | 90.7 ± 1.8 | 10.7 ± 0.2 |
| Irreversibly inactive forms | 437.8 ± 7.7 | 93.7 ± 0.5 | 698.9 ± 15.4 | 82.0 ± 0.4 |
| Cytokinin phosphates | 16.2 ± 1.2 | 3.5 ± 0.3 | 36.1 ± 1.3 | 4.2 ± 0.2 |
| Total cytokinins | 467.1 ± 5.8 | 100.0 ± 0.0 | 851.9 ± 16.4 | 100.0 ± 0.0 |

Bioactive forms of cytokinins: nucleobases and ribosides, that is, *t*Z, *t*ZR, *c*Z, *c*ZR, DHZ, DHZR, iP, and iPR. Storage forms: *O*-glucosides, that is, *t*ZOG, *t*ZROG, *c*ZOG, *c*ZROG, DHZOG, and DHZROG. Irreversibly inactive or weakly active forms of cytokinins: *N*-glucosides, that is, *t*Z7G, *t*Z9G, *c*Z7G, *c*Z9G, DHZ7G, DHZ9G, iP7G, and iP9G. Cytokinin phosphates: *t*ZRMP, *c*ZRMP, DHZRMP, and iPRMP. Values are presented as means ± SE ($n = 3$)

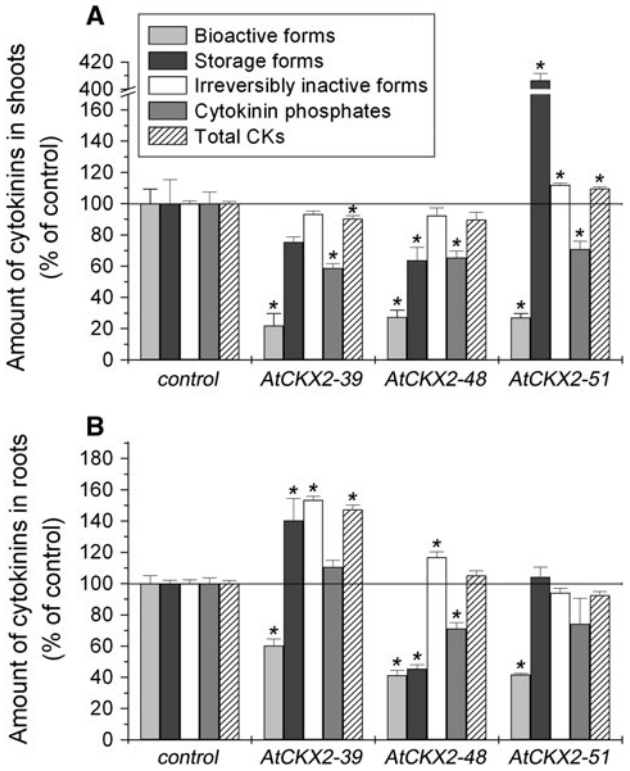


**Fig. 3** Cytokinin content and profile in shoots (**a**) and roots (**b**) of 30-day-old in vitro-grown *AtCKX*-overexpressing potato plants. Cytokinins are divided into four groups, including bioactive forms (nucleobases and ribosides), storage forms (*O*-glucosides), irreversibly inactive or weakly active forms (*N*-glucosides), and cytokinin phosphates, based on their conjugation status and biological activity. Amounts of each form of cytokinin as well as of total cytokinins are presented as percentage of mean control values. *Horizontal lines* mark the amounts corresponding to mean control values. Data represent means ± standard errors ($n = 3$). Statistically significant differences from corresponding control values (Mann–Whitney *U* test, $P < 0.05$) are marked by *asterisks*

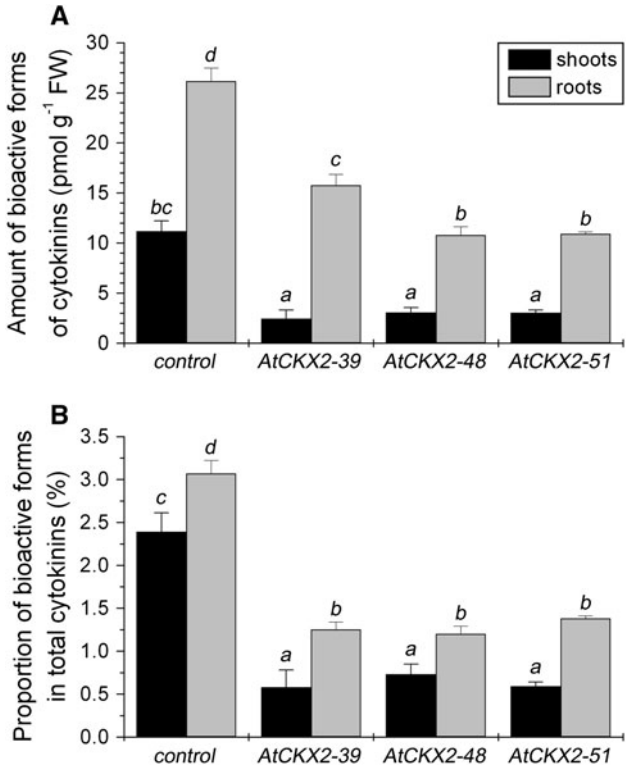


**Fig. 4** Amount of bioactive cytokinins (**a**) and proportion of bioactive forms of cytokinins (**b**), expressed as percentage of total cytokinins, in 30-day-old in vitro-grown control and *AtCKX*-overexpressing potato plants. Bioactive forms of cytokinins are nucleobases (*t*Z, *c*Z, iP, and DHZ) and their ribosides (*t*ZR, *c*ZR, iPR, and DHZR). Data represent mean ± standard error ($n = 3$). Means marked with the *same letter* were not significantly different according to Mann–Whitney *U* test ($P < 0.05$)

## Bioactive Cytokinin Levels in *AtCKX2*-Overexpressing Potato Plants

The most remarkable feature of the *AtCKX2*-overexpressing potato lines was that regardless of their total cytokinin content, the amount of bioactive cytokinins was significantly lowered compared to control (Figs. 3a, b, 4a). The decrease was observed in both shoots and roots of all transgenic lines. Although the shoots of all three transgenic lines contained similar amounts of bioactive cytokinins, in roots the content of bioactive cytokinin forms was significantly higher in *AtCKX2*-39 than in two other transgenic lines. In both control and transgenic lines, significantly higher levels of bioactive cytokinins were found in roots than in shoots (Fig. 4a).

The proportion of bioactive forms in total cytokinins was also significantly reduced in all investigated *AtCKX2*-overexpressing lines compared to the control (Fig. 4b). This proportion did not differ among individual transgenic lines and it was always higher in roots than in shoots.

## Morphological Traits

*AtCKX2*-overexpressing potato plants grown in vitro displayed only moderate morphological differences compared to control plants. Most transgenic plants developed significantly shorter shoots than the control ones. No obvious difference in lateral branching and root system development between control and transgenic plants was observed (Table 2).

## Tuberization Parameters

As a consequence of altered cytokinin homeostasis, changes in tuber formation were observed in *AtCKX2*-transgenic plants grown in vitro (Fig. 5).

Control plants grown in non-inducing (LD) conditions, as expected, did not yield tubers after 30 days in vitro, which was also the case with the *AtCKX2*-39 transgenic

**Table 2** Some important morphological traits of 30-day-old *AtCKX*-transgenic and control potato plants grown in vitro in LD conditions

| Morphological trait | Control | *AtCKX2*- | | |
|---|---|---|---|---|
| | | 39 | 48 | 51 |
| Shoot length (mm) | 76.3 ± 1.3 | 71.5 ± 4.2 | 41.9 ± 2.9* | 24.4 ± 2.7* |
| Number of lateral branches | 2.4 ± 0.4 | 2.3 ± 0.3 | 2.4 ± 0.3 | 2.0 ± 0.3 |
| Number of roots | 3.1 ± 0.2 | 3.7 ± 0.5 | 3.0 ± 0.4 | 2.8 ± 0.3 |

Values are given as mean ± SE of three independent experiments (at least 30 replicates each). Values significantly different from control (ANOVA and LSD test, $P < 0.05$) are marked with an *asterisk* (*)

line (Fig. 5a). On the contrary, tubers appeared in *AtCKX2*-48 (33%) and *AtCKX2*-51 (6%) shoots. Most of these tubers were formed at the apical part of stolons: 52% in *AtCKX2*-48 and 100% in *AtCKX2*-51 (data not shown). In inducing conditions (CD), 42% of control plants developed tubers (Fig. 5a), whereas in transgenic lines tuber formation was significantly enhanced only in *AtCKX2*-51 plants (88%). In both control and transgenic plants, the tubers appeared in a variety of positions (data not shown).

Under noninducing LD conditions, *AtCKX2*-51 plants yielded single tubers, giving an average number of 1.0 tuber per shoot (Fig. 5b). *AtCKX2*-48 plants were occasionally able to develop more than one tuber per shoot, resulting in a significantly higher number of 1.2 tubers per shoot. Under inducing CD conditions, control plants yielded an average 1.2 tubers per shoot, whereas *AtCKX2*-39 and *AtCKX2*-51 plants developed significantly fewer tubers (Fig. 5b). In *AtCKX2* plants that yielded tubers in both LD and CD (*AtCKX2*-48 and *AtCKX2*-51), the number of tubers per shoot in both light regimes did not differ significantly (Fig. 5b).

In LD conditions, the *AtCKX2*-48 plants developed significantly larger tubers (2.3 mm in diameter) than *AtCKX2*-51 plants (1.2 mm) (Fig. 5c). In CD, control plants formed tubers of 2.5 mm on average. Transgenic *AtCKX2*-39 and *AtCKX2*-51 plants produced significantly larger tubers (3.5 and 3.8 mm, respectively) compared to control and *AtCKX2*-48 plants. In the *AtCKX2*-51 line, tubers developed in LD and CD differed significantly in size (Fig. 5c).

Tubers originating from both control and *AtCKX2*-overexpressing plants grown in vitro were able to sprout after a dormancy period and give rise to the plantlets that shared the morphological features of the lines from which they were derived. They were likewise also able to develop stolons and tubers (data not shown).

## Discussion

In this study, *AtCKX2*-overexpressing potato plants were generated and grown in vitro as a model system for investigating whether and how altered endogenous cytokinin levels affect the tuberization process. Our results can be considered complementary with those recently published for soil-grown *AtCKX1*-overexpressing potato plants (Hartmann and others 2011), although different cultivars were used in the two studies.

Despite much evidence for the role of cytokinins as positive regulators of potato tuberization in vitro, their exact role in this process has not yet been elucidated (Jackson 1999). Until recently, evidence about the implication of cytokinins in potato tuberization relied on model systems exposed to increased levels of cytokinins, either by exogenous application (Hussey and Stacey 1984; Romanov and others 2000; Dragićević and others 2004) or by overexpression of genes involved in cytokinin biosynthesis (Macháčková and others 1997; Ninković and others 1999; Romanov and others 2000). Hartmann and others (2011) obtained *AtCKX1*-overexpressing potato (cv. Solara) plants, which were characterized by reduced tuber yield and a lower number of tubers per plant.

In our work, the CKX activity was remarkably enhanced in all *AtCKX2* transgenic lines. The *AtCKX2*-transformed potato plants showed an increase in CKX activity (4.9–14.3-fold of control values; Fig. 2), similar to the enhancement previously reported for *AtCKX2*-overexpressing tobacco (2.6–10.4-fold of control values; Werner and others 2001) and *Arabidopsis* (3–10-fold of control values; Werner and others 2003). The CKX activity of transgenic plants differed between shoots and roots. The expression of the *AtCKX2* transgene in shoots exceeded that in roots (Fig. 1), which in most of the transgenic lines led to an elevation of CKX activity in the shoots only (Fig. 2).

Despite significant variations in CKX activity, all *AtCKX2*-overexpressing lines showed a similar decrease in the amount of bioactive cytokinins (Fig. 4a), indicating the importance of cytokinin homeostatic mechanisms, which did not allow a decrease of bioactive cytokinins below a certain minimum level. The reduction of bioactive cytokinin levels reported here (22–27% of control levels in the shoots and 41–60% in the roots) coincided with the decrease described previously in tobacco, where the levels of iP, *tZ*, and their ribosides in transgenic plants varied

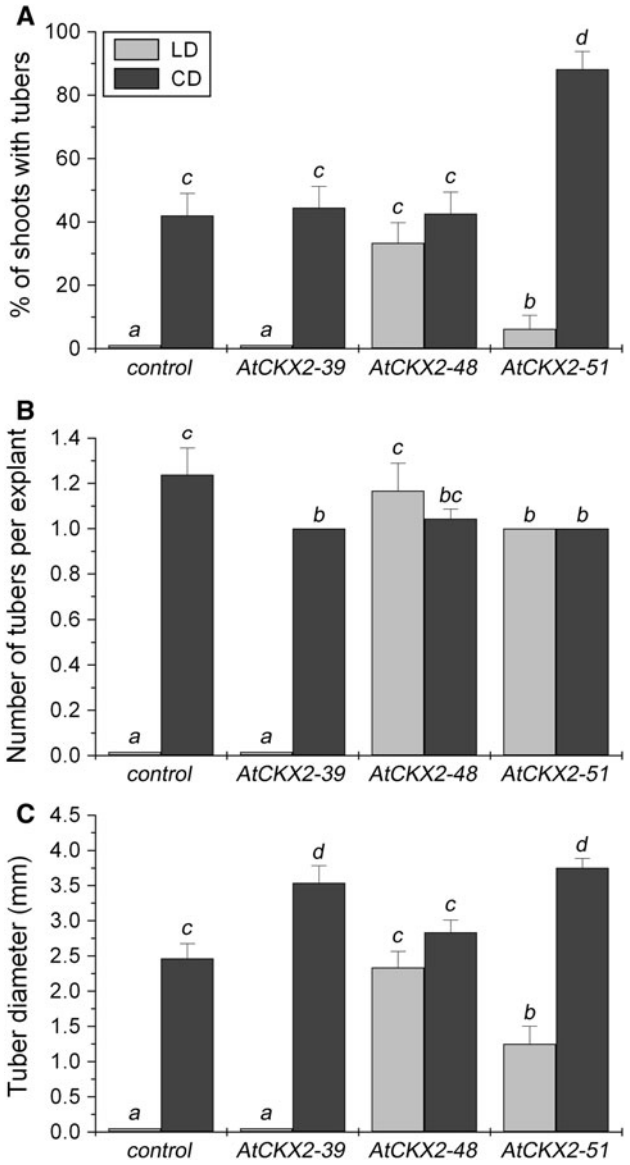


**Fig. 5** Tuber formation in *AtCKX*-overexpressing potato plants. The figures represent the percentage of shoots that developed tubers after 30 days of growth in vitro (**a**), the average number of tubers per shoot, calculated for the shoots that developed tubers (**b**), and the average tuber diameter (**c**). *LD* long day (16-h photoperiod), *CD* continuous darkness. Data represent mean ± standard error of three independent experiments with at least 30 replicates each. Means marked with the *same letter* were not significantly different according to ANOVA and LSD test ($P < 0.05$)

between 23 and 92% of control values (Werner and others 2001). The pronounced depletion of bioactive cytokinins in the shoots of transgenic plants corresponds with the significant elevation of CKX activity in all *AtCKX2* shoots. On the other hand, a reduced amount of bioactive cytokinins was found also in the roots, although the CKX activity was not always significantly enhanced in these organs, indicating the importance of cytokinin root-to-shoot transport and the relevance of cytokinin homeostasis in the shoot for whole-plant homeostasis.

In control plants, a huge prevalence of irreversibly inactive or weakly active forms (*N*-glucosides) together with a low occurrence of bioactive forms of cytokinins in both shoot and root tissues (Table 1) suggests that potato actually utilizes a very small portion of its total cytokinins. Furthermore, storage forms of cytokinins are present almost exclusively in the root tissue, pointing out the importance of roots as cytokinin storage sites in potato.

Cytokinin profiles of *AtCKX2*-overexpressing lines differed from controls (Fig. 3). However, overexpression of *AtCKX2* did not considerably contribute to lowered concentrations of total cytokinins in transgenic potato. Interestingly, the endogenous levels of bioactive cytokinins (nucleobases and ribosides), as well as their proportion in total cytokinins, were consistently decreased in *AtCKX*-overexpressing potato plants (Fig. 4a, b). This indicates the potential existence of control feedback mechanisms in *AtCKX2*-overexpressing potato plants, which aim to compensate for a constant loss of bioactive cytokinins, possibly by promoting de novo biosynthesis, which could account for diverse changes in total endogenous cytokinins. However, these mechanisms were largely unable to restore the level of bioactive cytokinins. Thus, although the supposedly enhanced cytokinin biosynthesis contributed to an elevation of total cytokinin levels, this was due to an increase in storage forms or in irreversibly inactive forms of cytokinins (Fig. 3). Furthermore, microarray analysis of tubers of *AtCKX1*-overexpressing potato revealed increased amounts of zeatin-*O*-xylosyltransferase mRNA (Hartmann and others 2011), suggesting that enhanced production of cytokinin storage forms might be common to *AtCKX*-overexpressing potato. Therefore, the higher amount of cytokinin conjugates is likely to be an indirect consequence of impaired cytokinin metabolism.

*AtCKX2*-overexpressing plants obtained in this study displayed great morphological similarity to the control potato plants grown in vitro. However, they showed reduced shoot growth compared to the control plants. Short shoots were also observed in *AtCKX*-overexpressing tobacco and *Arabidopsis* plants as a part of the "cytokinin deficiency syndrome," along with incomplete apical dominance in the shoot and increased root mass. These symptoms were more pronounced in *AtCKX1*- than in *AtCKX2*-overexpressing tobacco and *Arabidopsis* (Werner and others 2001, 2003). Reduced shoot growth was also reported for soil-grown *AtCKX1*-overexpressing potato plants obtained by Hartmann and others (2011). Morphological differences between control and *AtCKX2* potato plants in our experiments were not as obvious as those observed in the above-mentioned studies. The less pronounced effects of *AtCKX2* in the present work might be particularly ascribed to the in vitro

conditions. Comparing in vivo- and in vitro-grown potato, Konstantinova and others (1999) showed that the growth responses of plants to various photoperiodic treatments were more pronounced in the in vivo conditions. Comparison between in vitro- and soil-grown potato plants revealed a simultaneous occurrence of similar as well as different tuberization characteristics of various transgenic lines (Romanov and others 1998). The in vitro culturing might promote effects that are not necessarily related to those observed in plants grown in soil.

The tuberization process in potato consists of several steps characterized by different changes at morphological, histological, and biochemical levels (Xu and others 1998; Viola and others 2001). In general, the same sequence of events during tuberization occurs in vitro as well as ex vitro. The main difference between in vitro and ex vitro culturing is that in vitro tubers can develop as swellings along entire shoots, not only at the tip of stolons, and predominantly above the culture medium. During our experimental period of 30 days on a growth regulator-free control medium in vitro, nontransformed potato shoots did not develop tubers in noninductive conditions (LD). Because Désirée is a facultative short-day cultivar, tubers may occur even under noninductive conditions after about 6 weeks (Dragićević and others 2008).

Previous studies (Macháčková and others 1997) on *ipt*-transgenic potato plants showed that cytokinin overproducers exhibited enhanced tuberization when grown in vitro. As cytokinins seem to be positive regulators of potato tuberization, this process would be expected to be inhibited in cytokinin-deficient potato plants. In that sense, the results concerning tuberization in *AtCKX2*-overexpressing potato plants obtained in our study are rather ambiguous.

In our attempt to elucidate possible implications of the lowered bioactive cytokinin content on the tuberization process, three parameters were investigated, each reflecting different steps of this process: (1) number of shoots with tubers refers to induction/initiation of tubers, (2) number of tubers per shoot indicates assimilate partitioning, and (3) tuber diameter reflects tuber enlargement and starch accumulation. Cytokinins play a prominent role in all of these tuberization steps. They are involved in tuber induction (Hussey and Stacey 1984) as well as in the tuber setting and enlargement (Obata-Sasamoto and Suzuki 1979). In addition, it was shown that cytokinins promote cell divisions in *Arabidopsis* and tobacco (Riou-Khamlichi and others 1999) and are thus probably involved in cell proliferation that occurs in the early stages of tuber growth. Moreover, cytokinins are also known to regulate the source/sink relations, including nutrient translocation from autotrophic tissues to storage organs (Roitsch and Ehneß 2000).

In this work, tuber induction/initiation was found to be enhanced under inducing conditions (CD) in the *AtCKX2*-51 line, which exhibited the highest CKX activity. Moreover, two *AtCKX2* lines with high CKX activities (*AtCKX2*-48 and *AtCKX2*-51) were able to form tubers under noninductive conditions (LD) and thus to overcome the suppressing effect of light on tuberization (Fig. 5a). *AtCKX2* potato plants rarely formed more than one tuber per shoot, indicating low competition for sugars (Fig. 5b). Finally, increased tuber size in the majority of *AtCKX2*-overexpressing lines, in both LD and CD (Fig. 5c), could possibly be attributed to the greater sink strength of the formed tubers. In general, the expected suppression of tuber induction/initiation and decline in tuber enlargement were not demonstrated here in *AtCKX2*-overexpressing lines. As the levels of bioactive cytokinins did not considerably differ among individual *AtCKX2*-overexpressing lines, the effects described above cannot be attributed to cytokinins only, but rather to their interactions with light and/or other phytohormones.

Continuous darkness represents a strong tuber-inducing factor. In this study, the *AtCKX2*-transgenic plants grown in CD conditions developed fewer tubers per explant (Fig. 5b), but tubers were larger than in control plants (Fig. 5c). Such a reverse relationship between tuber size and number of tubers per plant is a common feature of metabolic sinks competing for photoassimilates (Fernie and Willmitzer 2001). A reduced number of tubers per plant has also been reported for *AtCKX1*-overexpressing soil-grown potato plants (Hartmann and others 2011).

The most intriguing result concerning *AtCKX2*-overexpressing plants was promotion of tuber formation in noninductive conditions (LD). Two *AtCKX2*-overexpressing lines developed tubers in LD (Fig. 5a), indicating that cytokinin deficiency overrules the inhibitory consequences of LD treatment for tuber induction. It was previously shown that the *phyB*-1 mutant of *Arabidopsis* exhibits altered cytokinin responses in the process of hypocotyl elongation, implying a connection, possibly indirect, between light and cytokinins (Su and Howell 1995). Concerning the tuber induction process in potato, the effects of cytokinins and light seem to be contradictory, and exogenously applied BA to potato grown in vitro does not overcome the inhibition of tuber formation caused by light (Dragićević and others 2004). In the present study, remarkably reduced levels of bioactive cytokinins in *AtCKX2*-overexpressing in vitro-grown potato plants with severely increased CKX activity overruled the inhibitory effect of light on tuberization.

In conclusion, overexpression of the *AtCKX2* gene in transgenic potato did not necessarily result in a decrease of total cytokinin content, but it altered the plant cytokinin profile, leading, inter alia, to a decrease in the levels of bioactive cytokinins. The "cytokinin deficiency syndrome" described previously in *AtCKX*-overexpressing tobacco and

*Arabidopsis* was not observed, although mild symptoms occasionally occurred in some of the *AtCKX2*-over-expressing potato lines. Changes in cytokinin metabolism affected tuberization in both CD and LD conditions. The absence of clear enhancement or unequivocal suppression of tuberization in *AtCKX2*-overexpressing potato lines with a severely reduced content of bioactive forms of cytokinins could be explained by in vitro conditions or by interactions with other factors affecting tuberization, especially with endogenous gibberellins and auxins. Further research to investigate those interactions in more detail and to compare tuberization in *AtCKX2*-overexpressing lines is in progress using other cytokinin-deficient (*AtCKX1*) as well as cytokinin-overproducing (*IPT*) potato lines to identify details of the signaling pathways in which cytokinins are involved in the tuberization process, in both tuber-inducing and -non-inducing conditions.

**Acknowledgments** We kindly thank Dr. Thomas Schmülling and Dr. Tomáš Werner from Frei Universität Berlin (Germany) for providing us with the bacterial strains *A. tumefaciens* GV3101/pBinHTX-*AtCKX1* and *A. tumefaciens* GV3101/pBinHTX-*AtCKX2*. Dr. Jiří Malbeck is acknowledged for his valuable contribution to HPLC/MS/MS analyses of cytokinins and Marie Korecká for excellent technical assistance. We thank also Dr. Branka Uzelac for editing the English language. The research presented here was funded by the Ministry of Education and Science of the Republic of Serbia (grant No. ON173015), the Czech Science Foundation (P506/11/0774), and the Ministry of Education, Youth and Sports of the Czech Republic (LC 06034).

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