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RESEARCH PAPER

Antibody-mediated modulation of cytokinins in tobacco: organ-specific changes in cytokinin homeostasis

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Abstract

Cytokinins comprise a group of phytohormones with an organ-specific mode of action. Although the mechanisms controlling the complex networks of cytokinin metabolism are partially known, the role of individual cytokinin types in the maintenance of cytokinin homeostasis remains unclear. Utilizing the overproduction of single-chain Fv antibodies selected for their ability to bind *trans*-zeatin riboside and targeted to the endoplasmic reticulum, we post-synthetically modulated cytokinin ribosides, the proposed transport forms of cytokinins. We observed asymmetric activity of cytokinin biosynthetic genes and cytokinin distribution in wild-type tobacco seedlings with higher cytokinin abundance in the root than in the shoot. Antibody-mediated modulation of cytokinin ribosides further enhanced the relative cytokinin abundance in the roots and induced cytokinin-related phenotypes in an organ-specific manner. The activity of cytokinin oxidase/dehydrogenase in the roots was strongly up-regulated in response to antibody-mediated formation of the cytokinin pool in the endoplasmic reticulum. However, we only detected a slight decrease in the root cytokinin levels. In contrast, a significant decrease of cytokinins occurred in the shoot. We suggest the roots as the main site of cytokinin biosynthesis in tobacco seedlings. Conversely, cytokinin levels in the shoot seem to depend largely on long-range transport of cytokinin ribosides from the root and their subsequent metabolic activation.

Keywords: Antibody-mediated modulation, biosynthesis, CKX, cytokinin, homeostasis, organ specificity, tobacco.

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Introduction

The plant hormones cytokinins represent a group of growth regulators responsible for numerous developmental and physiological processes during the plant life cycle. Cytokinins control gameto- and embryogenesis, meristem specification and positioning of its organizing centers, cell division and differentiation during shoot and root growth, and the onset of plant senescence (for a recent review, see Zürcher and Müller, 2016). Interestingly, cytokinins act in an organ-specific way. Positive cytokinin effects have been described in the shoot, where cytokinins stimulate shoot apical meristem (SAM) activity and size (Werner *et al.*, 2003; Kurakawa *et al.*, 2007). In contrast, cytokinins are negative regulators of the root apical meristem (RAM) size and inhibit primary root growth (Werner *et al.*, 2003; Dello Ioio *et al.*, 2007).

Chemically, natural cytokinins are adenine derivatives occurring as several (sub)types. The most important natural cytokinins are N^6-(Δ^2-isopentenyl)adenine (iP) and its hydroxylated forms *trans*-zeatin (*t*Z) and *cis*-zeatin (*c*Z). Endogenous cytokinin levels are subject to tight tissue- and organ-specific control that includes biosynthesis, degradation, and conjugation. The first step of cytokinin biosynthesis is catalyzed by the isopentenyltransferase (IPT) enzyme, which transfers an isopentenyl group from dimethylallyl diphosphate (DMAPP) to adenosine phosphates (AMP, ADP, or ATP), resulting in formation of iP ribonucleotides (also called riboside phosphates or simply cytokinin nucleotides) (Kakimoto, 2001; Takei *et al.*, 2001*a*). Moreover, some IPTs catalyze isopentenylation of tRNAs, considered as the main source of *c*Z (Miyawaki *et al.*, 2006), the highly abundant cytokinin subtype of mostly unknown function (Gajdosová *et al.*, 2011). A second important step is the root-specific hydroxylation of iP nucleotides by cytochrome P450 monooxygenases (CYP735A) to form *t*Z nucleotides (Takei *et al.*, 2004*b*; Kiba *et al.*, 2013). As a side branch, the *t*Z types are most probably converted by zeatin reductase to the dihydrozeatin (DHZ) cytokinin subtype (Gaudinova *et al.*, 2005). However, the detailed mechanism of DHZ biosynthesis is still unclear. The last step in cytokinin biosynthesis is mediated by a phosphoribohydrolase LONELY GUY (LOG) (Kurakawa *et al.*, 2007), which catalyzes conversion of cytokinin nucleotides to their free bases, recently considered as the only active cytokinin forms (Lomin *et al.*, 2015). An alternative pathway in the formation of free cytokinin bases is two-step conversion (Kakimoto, 1996). This pathway includes dephosphorylation of cytokinin nucleotides to ribosides and subsequent cleavage of the sugar moiety to release free bases. However, with the exception of enzymes mediating the first step (Kopecná *et al.*, 2013), proteins catalyzing the two-step conversion remain to be identified.

Cytokinin ribosides may serve as a storage source for cytokinin nucleotides and are also considered as the main transport form of cytokinins (Hirose *et al.*, 2008; Matsumoto-Kitano *et al.*, 2008; Kudo *et al.*, 2010). The root-borne *t*Z subtypes represent dominant cytokinins in xylem sap that regulate shoot growth (Matsumoto-Kitano *et al.*, 2008; Kiba *et al.*, 2013; Ko *et al.*, 2014). In contrast, iP types prevail in the phloem (Corbesier *et al.*, 2003; Hirose *et al.*, 2008), and through basipetal (toward the root apex, rootward) transport regulate vascular patterning in the RAM (Bishopp *et al.*, 2011).

Cytokinin types with a hydroxylated side chain (*t*Z, *c*Z, and DHZ) can be reversibly glycosylated by the enzyme UGT85A1 (Hou *et al.*, 2004). *O*-Glycosylation is one of the first reactions to exogenous cytokinin application and produces non-active cytokinins that can be re-activated by β-glucosidases (Brzobohatý *et al.*, 1993). Cytokinin *O*-glucosides could serve as a storage pool and readily available source of active free bases (Kiran *et al.*, 2012). On the other hand, cytokinin glycosylation at the N7 and N9 positions of the adenine moiety catalyzed by the enzymes UGT76C1 and UGT76C2 (Hou *et al.*, 2004; Wang *et al.*, 2011) is irreversible, and therefore *N*-glucosides represent a permanent deactivation in the cytokinin lifetime (Parker and Letham, 1973). Even though glycosylation is an important part of cytokinin homeostatic regulation, the central role in controlling the active cytokinin pool seems to be played by cytokinin oxidase/dehydrogenases (CKXs) (Werner *et al.*, 2001). CKXs cleave unsaturated isoprenoid side chains, leading to irreversible cytokinin degradation (Galuszka *et al.*, 2007; Schmülling *et al.*, 2003). Further details of the complex metabolism of cytokinins are discussed in several recent reviews (Osugi and Sakakibara, 2015; Zürcher and Müller, 2016).

Cytokinin metabolism represents a tightly interconnected network in which every intervention results in complex changes in the spectra and levels of endogenous cytokinins and cytokinin metabolites, which are often difficult to assign to observed developmental abnormalities. To date, cytokinin metabolism and homeostasis have been predominantly investigated using gain- or loss-of-function mutants. However, these approaches are often hampered by possible non-physiological responses to the misregulation of gene expression or high levels of functional redundancy in some plant gene families. Moreover, these approaches mostly do not allow study of the specific roles of individual cytokinin metabolites.

Antibody-mediated modulation (also called immunomodulation) is a molecular technique based on the ectopic production of specific recombinant antibodies, which modulate the function of a targeted molecule (for a review, see Conrad and Manteuffel, 2001). In plants, usage of single-chain variable fragments (scFvs) as well as their expression in specific cell compartments has been optimized (Conrad and Fiedler, 1998; De Jaeger *et al.*, 2000). An antibody-mediated modulatory approach has been successfully used to inhibit viral or bacterial infections in plants (Boonrod *et al.*, 2004; Yajima *et al.*, 2010). Further, antibody-mediated modulation of plant hormones has been performed to alter the hormonal status without directly interfering with its synthesis, degradation, or signaling pathways (Conrad and Manteuffel, 2001). Plant hormones such as abscisic acid (ABA) (Artsaenko *et al.*, 1995) or jasmonate (ten Hoopen *et al.*, 2007) have been modulated in tobacco. As previously described, scFv antibodies targeted to the endoplasmic reticulum (ER) bind hormone molecules and as such represent an artificial ER sink of the

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hormone, decreasing the accessibility of the non-bound hormone (Conrad and Manteuffel, 2001; Strauss *et al.*, 2001; Radchuk *et al.*, 2010). Expression of the corresponding scFv antibodies was reported to cause specific hormone-deficient phenotypes (Staroske *et al.*, 2016). The authors show that in transgenic barley seeds, antibody-mediated modulation induced up-regulation of ABA during transition phases of seed development that resulted in precocious maturation and negatively interfered with growth and development. Recently, the antibody-mediated modulation of cytokinin signaling has also been demonstrated (Zábrady *et al.*, 2014).

Here, we show the use of antibody-mediated cytokinin modulation via overproduction of cytokinin-specific scFv (αCK_scFv) to study cytokinin homeostasis in tobacco and its subsequent impact on phenotypic changes. We present our first results as a case study demonstrating the potential of the antibody-mediated modulatory approach in cytokinin research. We modulated cytokinin ribosides through formation of an artificial cytokinin sink in the ER and we describe its impact on organ-specific activation of homeostatic mechanisms and plant development.

Materials and methods

Cloning and preparation of transgenic plants

The specific αCK_scFv was selected by phage display (Winter *et al.*, 1994) performed on the Human Single Fold scFv Library A+B (de Wildt *et al.*, 2000). The *t*ZR molecule coupled with BSA was used as a target molecule for screening. The selected αCK_scFv gene coding region was amplified by PCR as a αCK_scFv c-myc fusion and inserted in the pRTRA7/3 vector to create an expression cassette for scFv retention in the ER (Artsaenko *et al.*, 1995). The final cassette consists of the *Cauliflower mosaic virus* (CaMV) 35S promoter–legumin signal peptide–αCK_scFv–c-myc tag–KDEL ER retention signal peptide–CaMV 35S terminator. Further, the αCK_scFv expression cassette was cloned in the binary pBIN19 vector and used for *Agrobacterium*-mediated gene transfer to *Nicotiana tabacum* cv. Samsun NN plants (Zambryski *et al.*, 1983). Transgenic tobacco plants overexpressing αCK_scFv were generated and selected as described previously (ten Hoopen *et al.*, 2007).

Plant material and growth conditions

For immunolocalization and protein purification, young leaves obtained from 8-week-old plants grown in soil under standard conditions in a greenhouse were used. *In vitro* growth of seedlings was generally performed in Petri dishes on 1× Murashige and Skoog (MS) medium (Duchefa) with 1% sucrose and 1% plant agar (Duchefa). Seedlings were cultivated in growth chambers (CLF Plant Climatics GmbH) under long-day conditions (16 h light/8 h dark) at 23 °C with a light intensity of 150 μM m^{-2} s^{-1} and 40% relative humidity. For western blot analysis, 3-week-old seedlings were used. For quantitave reverse transcription–real-time PCR (qRT–PCR) analysis, 8-day-old seedlings were used. For endogenous cytokinin measurements, seedlings were grown for 8 days on square plates fitted with a nylon mesh (Uhelon 120T; Silk & Progress) positioned vertically. In root elongation assays, 8-day-old seedlings were replanted from standard MS medium into 1× MS medium supplemented with 100 nM *t*ZR/6-benzylaminopurine (BAP), 1 μM *t*ZR/BAP (Duchefa, OlChemIm, respectively; dissolved in DMSO), or 0.01% DMSO as a control. The seedlings were incubated on the cytokinin-supplemented medium for 3 days. In the rosette size measurement experiment, seedlings were grown on round plates for 1 week in a vertical position and then for 2 weeks in a horizontal position on 1× MS medium supplemented with 100 nM *t*ZR/BAP (Duchefa, OlChemIm; dissolved in DMSO) and with a corresponding amount of DMSO as a control. Cultivation of etiolated hypocotyls and the hypocotyl explant assay were carried out according to the protocol described elsewhere (Pernisova *et al.*, 2009). Hypocotyl explants were cultivated on 1/2 MS medium for 33 days.

Western blotting analysis

αCK_scFv protein from 3-week-old shoots and roots of transgenic seedlings and the wild type (WT) was extracted using extraction buffer [50 mM Tris pH 7, 0.1 mM DTT, 0.5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), Sigma protease inhibitor cocktail]. The total protein extracts were separated by SDS–PAGE. One gel was stained by Coomassie G-250 stain (Bio-Rad) for 1 h and the second gel was electroblotted onto a polyvinylidene fluoride (PVDF) membrane in Towbin buffer (25 mM Tris–HCl, 150 mM glycine, 10% methanol, pH 8.3) and blocked for 1 h in blocking buffer (5% skimmed milk, Tris-buffered saline, 0.1% Tween-20). The αCK_scFv protein was detected using primary anti-c-myc antibody (Sigma) diluted 1:2000 followed by alkaline phosphatase (ALP)-conjugated secondary anti-mouse IgG antibody produced in goat (Sigma) diluted 1:5000 and by NBT/BCIP substrate (Biotech).

Indirect enzyme-linked immunosorbent assay (ELISA)

Soluble αCK_scFv protein was purified from tobacco leaves ground in liquid nitrogen as described previously (ten Hoopen *et al.*, 2007). Fractions containing scFv protein were mixed and diluted 1:1 in 2% BSA in PBST (8 mM Na_2HPO_4, 2 mM KH_2PO4, 150 mM NaCl, 0.05% Tween, pH 7.2). A microtiter plate was coated overnight at room temperature with either BSA, ABA–BSA conjugate, or different cytokinin conjugates [*t*ZR–ovalbumin (OVA), DHZR–OVA, iPR–OVA, and *c*ZR–BSA] diluted in phage-PBS buffer (100 mM NaCl, 32 mM Na_2HPO_4, 17 mM NaH_2PO_4, pH 7.2) at the following concentrations: 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, and 0.1 pg per well. Unspecific binding sites were blocked with 2% BSA in PBST for 2 h. Plant-purified αCK_scFv protein was added at a dilution of 1:5 in 1% BSA in PBST and incubated for 1 h at 25 °C. Detection using 9E10 anti-c-myc antibodies followed by anti-mouse IgG–ALP conjugate (Sigma) and the enzymatic reaction was performed as described previously (ten Hoopen *et al.*, 2007). The results were evaluated using SigmaPlot software.

Measurement of endogenous cytokinins

Quantification of cytokinin metabolites was performed according to the method described by Svačinová *et al.* (2012), including modifications described by Antoniadi *et al.* (2015). Samples (200 mg FW) were homogenized and extracted in 1 ml of modified Bieleski buffer (60% MeOH, 10% HCOOH, and 30% H_2O) together with a cocktail of stable isotope-labeled internal standards (0.25 pmol of CK bases, ribosides, and *N*-glucosides, 0.5 pmol of CK *O*-glucosides and nucleotides per sample added). The extracts were purified using two solid phase extraction columns: an octadecyl silica-based column (C18, 500 mg of sorbent, Applied Separations) and after that an Oasis MCX column (30 mg ml^{-1}, Waters). Analytes were eluted by three-step elution using a 60% (v/v) MeOH, 0.35 M NH_4OH aqueous solution and 0.35 M NH_4OH in 60% (v/v) MeOH solution. Cytokinin levels were determined by ultra-high performance liquid chromatography-electrospray tandem mass spectrometry (UPLC-MS/MS) using stable isotope-labeled internal standards as a reference (Rittenberg and Foster, 1940). Three independent biological replicates were performed.

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Cytokinin oxidase activity measurement

CKX from tobacco shoots and roots was extracted and partially purified according to the protocol described elsewhere (Motyka *et al.*, 2003) and its activity was determined by *in vitro* radioisotope assays based on conversion of [2-^{3}H]iP ([2-^{3}H]iP prepared by the Isotope Laboratory, IEB AS CR, Prague, Czech Republic) to [2-^{3}H]adenine. The assay mixture (50 µl final volume) comprised 100 mM TAPS-NaOH buffer containing 75 µM 2,6-dichloroindophenol (pH 8.5), 2 µM substrate [2-^{3}H]iP (7.4 TBq mol^{-1}), and enzyme preparation equivalent to 20 mg or 35 mg tissue fresh weight (corresponding to 0.006–0.009 or 0.002–0.005 mg protein g^{-1} FW) for shoots and roots, respectively. After incubation (18 h, 37 °C), the reaction was terminated by adding 10 µl of Na_4EDTA (200 mM) and 120 µl of 95% (v/v) ethanol, and the substrate was separated from the product of the enzyme reaction by HPLC as described elsewhere (Gaudinova *et al.*, 2005). Protein concentrations were determined according to the method of Bradford (1976) using BSA as a standard. Results represent the mean of four samples obtained from two different experiments with two replicates each.

Cross-sectioning and staining of the SAM

Eight-day-old tobacco seedlings were fixed in 4% paraformaldehyde in PBS (135 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4), dehydrated by a gradient series of ethanol and embedded in Technovit® 7100 (Kulzer) according to the product manual. Sections 2 µm thick were prepared using an ultramicrotome (Leica), stained by 0.05% Toluidine Blue solution, and analyzed by bright field microscopy.

Immunolocalization of leaf and root cross-sections

Small pieces of tobacco leaves/roots were fixed in 4% (w/v) paraformaldehyde and 0.1% (v/v) Triton X-100 in PBS, dehydrated by a graded series of ethanol, and embedded in PEG1500 according to Hause *et al.* (1996). Cross-sections 2 µm (leaves) or 20 µm (roots) thick were immunolabeled as described previously (Isayenkov *et al.*, 2005; ten Hoopen *et al.*, 2007) and analyzed. Leaf cross-sections were labeled with either anti-c-myc (clone 9E10, dilution 1:25) or HDEL antibody (Invitrogen, dilution 1:500) followed by goat anti-mouse IgG coupled to AlexaFluor488 (Invitrogen, dilution 1:500). Root cross-sections were labeled simultaneously with anti-c-myc (clone 9E10 dilution 1:25) followed by goat anti-mouse IgG coupled to AlexaFluor488 (Invitrogen, dilution 1:500) and anti-BiP antibody (Agrisera, dilution 1:500) followed by donkey anti-rabbit IgG AlexaFluor594 (dilution 1:500) and DAPI (1 µg ml^{-1}).

Microscopy and image analysis

Confocal microscopy of immunolabeled leaf cross-sections was carried out on an inverted Zeiss Observer.Z1 microscope equipped with a LSM780 confocal unit and an ×63 oil immersion objective. Cross-sections of roots were analyzed using an LSM700 (Zeiss) in multitracking mode to visualize DAPI (excitation 405 nm, emission 410–490 nm), AlexaFluor488 (excitation 488 nm, emission 490–550 nm), and AlexaFluor594 (excitation 555 nm, emission 605–700 nm) simultaneously. An Olympus BX61 microscope using differential interference contrast microscopy was used for measurements of RAM lengths and SAM analysis. The root elongation, etiolated hypocotyl length, and rosette size were measured in the ImageJ program (National Institutes of Health; http://rsb.info.nih.gov/ij). In each type of experiment, at least 20 samples of each line were evaluated in three independent biological replicates giving the same statistically significant results.

Nested ANOVA statistical analysis

Differences in RAM length between the WT, transgenic line αCK_8, and transgenic line αCK_18 together with DMSO control–cytokinin treatment (BAP; *t*ZR) were analyzed using nested design ANOVA. The predictor experimental treatment is nested in transgenic lines. $P<0.05$ was regarded as significant. Differences in total cytokinin content in root and shoot between the WT, transgenic line αCK_8, and transgenic line αCK_18 were analyzed by repeated measures ANOVA. Statistical analyses were performed with Statistica software, version 12 (StatSoft, Tulsa, OK, USA).

Identification of putative tobacco orthologs of Arabidopsis cytokinin biosynthetic genes

Putative *N. tabacum* orthologs of *AtIPT* and *AtLOG* genes were selected according to the highest identity of their putative amino acid sequence to Arabidopsis proteins (by BLAST), presence of a full domain (IPP transferase for IPT and lysine decarboxylase for LOG), and predicted cytoplasmic protein localization. Based on these criteria, *NtIPT3*-like (XP_016502996) and *NtIPT5*-like (XP_016440428) were selected as the closest homologs of *AtIPT3* (52% amino acid identity) and *AtIPT5* (55% amino acid identity), respectively. *NtLOG7*-like (XP_016495755) and *NtLOG8*-like (XP_016512140) were selected as the closest homologs of *AtLOG7* (85%) and *AtLOG8* (78%), respectively. All IPT and LOG protein sequences were downloaded from the NCBI server. Putative protein localization was analyzed with WoLF PSORT (Horton *et al.*, 2007) and ChloroP 1.1 (Emanuelsson *et al.*, 1999) online tools. Protein identity score was identified by BLAST (Boratyn *et al.*, 2012). Domain structure was identified with the Conserved Domain Database (CDD) (Marchler-Bauer *et al.*, 2017).

qRT–PCR and data analysis

Shoots and roots of 8-day-old seedlings were detached with a scalpel, collected, immediately frozen in liquid nitrogen, and analyzed separately using three independent biological replicates. Total RNA from plant tissue was isolated using the RNAqueous Small Scale Phenol-Free Total RNA Isolation Kit (Ambion) according to the manufacturer's instructions. cDNA was prepared using the RTP3 primer and Superscript III (Invitrogen) according to the manufacturer's instructions, and qRT–PCR was performed using FastStart SYBR Green Master Kit (Roche) according to the manufacturer's instructions on a Rotor-Gene 6000 (Corbett Research) instrument in four technical replicates. All expression levels were normalized to ubiquitin-conjugating enzyme E2 (NtUBC2; AB026056) as described previously (Schmidt and Delaney, 2010). RTP3 primer: 5'-CGT TCG ACG GTA CCT ACG TTT TTT TTT TTT TTT TT-3'. For relative quantification of expression of biosynthetic genes, we used the following primers. NtUBC2 (Schmidt and Delaney, 2010); ForNtIPT3 (5'- AAAGGACTTGT CAACGAGGT-3'); RevNtIPT3 (5'-TTCTCTAACTGTTGGCTT GC-3'); ForNtIPT5 (5'-AGGATTCTTCAAGTTGCCAT-3'); RevNtIPT5 (5'-AGAGTTCTTCACGACGAAAA-3'); ForNtLOG7 (5'-TGTCTGCTCCAACAGCTAAA-3'); RevNtLOG7 (5'-TTGG CAATGCACAAACTACT-3'); ForNtLOG8 (5'-ATGATAACAT GGGCACAACT-3'); and RevNtLOG8 (5'- AGCAGCAAG AACAATGTCAC-3'). For individual pairs of primers, we used the following conditions (with reaction efficiency between 0.95% and 1.05%): NtUBC2 (0.5 µM), NtLOG7 (0.3 µM), and NtLOG8, 95 °C/7 min, 40 times (95 °C/15 s+60 °C/60 s), and melt; NtIPT3 (0.3 µM), 95 °C/7 min, 40 times (95 °C/15 s+60 °C/50 s), and melt; NtIPT5 (0.5 µM), 95 °C/7 min, 40 times (95 °C/15 s+58 °C/60 s), and melt. Calculation of the relative level of gene expression was done as described (Rao *et al.*, 2013). First, all expression levels (Ct values) were normalized to the reference gene *NtUBC2* to obtain the dCt value and relative gene expression (RQ) was calculated using the formula RQ = 2 exp–(dCt). For the evaluation of statistical significance, we used the *t*-test at α=0.05, 0.01, and 0.001, in figures depicted by *, **, and ***, respectively.

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Results

Plant-produced αCK_scFv antibodies are localized in the endoplasmic reticulum

We selected an anti-cytokinin riboside scFv antibody fragment (αCK_scFv) by screening in the Human Single Fold scFv Library A+B (de Wildt *et al.*, 2000) using *t*ZR–BSA conjugate. The antibody-coding region was cloned into the cassette for c-myc-tagged protein production targeted to the ER (Artsaenko *et al.*, 1995). The sequence of αCK_scFv is accessible in the European Nucleotide Archive under the accession number LT799001. For sequence and cloning cassette description, see Materials and methods and Supplementary Fig. S1 at *JXB* online. The construct was transformed to *N. tabacum* plants. Two independent transgenic lines producing soluble αCK_scFv (αCK_8 and αCK_18) at high concentrations were selected. Immunoblot analysis using crude plant extracts confirmed accumulation of intact αCK_scFv protein at a comparable level in both shoots and roots (Supplementary Fig. S2A, B). The antibodies against the c-myc tag detected αCK_scFv protein in the ER of epidermal and mesophyll cells in leaves as well as in the majority of cell files in roots of both transgenic lines. The ER subcellular localization was confirmed by the similar localization pattern of the ER-specific markers HDEL and BiP (Supplementary Fig. S2C, D).

Plant-produced αCK_scFv binds different cytokinin ribosides in vitro

To explore the binding specificity of plant-produced αCK_scFv antibodies, αCK_scFv protein was purified from a tobacco leaf extract and tested by indirect ELISA. The OVA/BSA molecules conjugated with different cytokinin ribosides (iPR, *t*ZR, DHZR, and *c*ZR) were examined in the assay together with the ABA–BSA conjugate and BSA alone to exclude unspecific binding to the conjugated immunocarrier. The concentration curves obtained showed binding of αCK_scFv protein to all tested cytokinin riboside conjugates at picomolar levels, whereas no binding with either ABA–BSA conjugate or BSA alone was observed (Fig. 1). In summary, the indirect ELISA experiment proved that plant-produced αCK_scFv antibodies are able to recognize specifically various cytokinin ribosides.

αCK_scFv transgenic plants exhibit differentially modulated cytokinin metabolism in the shoot and root

To examine possible changes in the cytokinin metabolism network upon αCK_scFv overexpression, we analyzed the endogenous content of >20 different cytokinin types in the WT and both αCK_scFv lines. We compared data obtained from WT control plants with both αCK_scFv transgenic lines; only statistically significant changes confirmed in both transgenic lines were considered as significant for a given cytokinin type.

Overall, our data showed that cytokinin metabolism was affected differentially in the shoot and root (Fig. 2A). In the shoot of transgenic plants, the total cytokinin content was

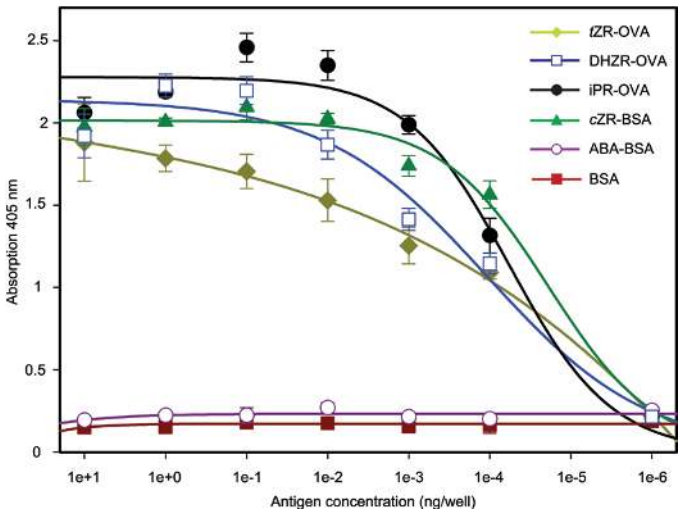

Fig. 1. Plant-produced αCK_scFv specifically recognizes cytokinin ribosides. The specificity of αCK_scFv protein toward different conjugates and BSA as negative control was tested in an indirect ELISA. Absorbance values of triplicates at 405 nm are displayed for αCK_scFv (1:10/well) and each antigen as a function of concentrations. Error bars correspond to the SD.

decreased to 26–40% when compared with the WT. In comparison, a much smaller decrease (10–12%) was observed in the root of antibody-modulated lines. Also the spectrum of cytokinin metabolites affected was broader in the shoot than in the root. In the shoot, the amounts of cytokinin nucleotides (riboside phosphates), as well as of all cytokinin ribosides, were distinctly down-regulated (Fig. 2B; Supplementary Fig. S3). Interestingly, levels of the free bases iP and *t*Z, the primary active cytokinin forms, remained unchanged, whereas those of DHZ and *c*Z were reduced. Further, we observed a decrease in the levels of some cytokinin *O*-glucosides (*t*ZROG, DHZROG, DHZOG, *c*ZOG, and *c*ZROG), the products of reversible cytokinin deactivation. However, the amount of cytokinin *N*-glucosides, considered as a pool of irreversibly inactivated cytokinins, remained unchanged, with the only exception being levels of iP7G. In contrast to all those changes observed in the shoot, the whole cytokinin metabolic pathway remained almost unchanged in the root. A significant decrease in the roots of both assayed αCK_scFv lines when compared with the WT was observable only in the case of *t*ZR, DHZR, and DHZROG (Fig. 2C; Supplementary Fig. S3).

Importantly, in line with previous data from Arabidopsis (Žd'árská *et al.*, 2013), our data demonstrated that there was an asymmetric shoot/root cytokinin distribution in tobacco (Fig. 2A; Supplementary Fig. S4). In the root of WT tobacco seedlings, we found 130% of the total cytokinins located in the shoot tissue. With the exception of DHZ cytokinin types and some of the cytokinin glucosides, the dominant root accumulation was also apparent in the case of cytokinin free bases, ribosides, and riboside phosphates (Supplementary Fig. S4). Importantly, the asymmetric distribution was even more pronounced in transgenic lines overexpressing αCK_scFv. The increase in the relative amount of cytokinins in the roots was observed for the total cytokinins: 159% and 193% of the shoot cytokinin levels were found in the roots of line

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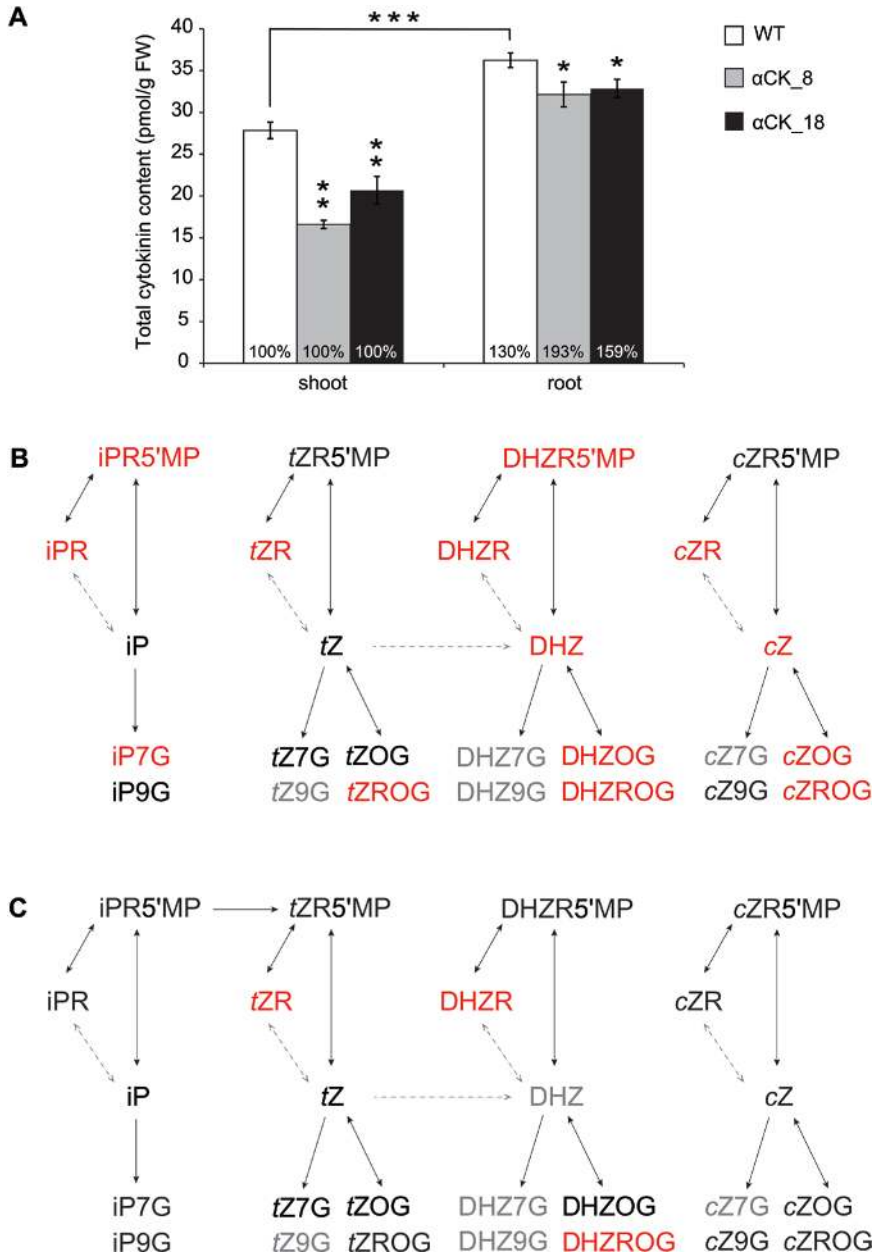

Fig. 2. Antibody-mediated modulation of cytokinins results in shoot- and root-specific changes in cytokinin homeostasis. (A) Total cytokinin content in shoots and in roots of WT and both αCK_scFv transgenic lines; all measured cytokinin types and their metabolites are included; statistical significance (*t*-test) of the difference when comparing shoot with root or transgenic lines with WT levels in three biological replicates at α=0.05, 0.01, and 0.001 is depicted by *, **, and ***, respectively. Error bars indicate the SD. The root/shoot ratio is expressed as a percentage of the shoot content in the root of the corresponding line. Total cytokinin contents differ significantly between roots and shoots [repeated measures ANOVA, $F(1,6)=190.12$, $P<0.001$] as well as between WT and transgenic lines [$F(2,6)=153.58$, $P<0.001$]. Differences in the endogenous cytokinin levels between the WT and both αCK_scFv transgenic lines in (B) shoots and (C) roots were significantly changed in both transgenic lines. Red color indicates down-regulation, black indicates unchanged levels, and gray indicated that the value was under the detection limit or unmeasured metabolites. Arrows show the direction of metabolic conversions and gray dashed lines depict suggested/experimentally not proven conversions. N^6-(Δ^2-isopentenyl) adenine (iP); *trans*-zeatin (*tZ*); dihydrozeatin (DHZ); *cis*-zeatin (*cZ*), and corresponding riboside-5'-monophosphates (-R5'MP); ribosides (-R); riboside *O*-glucosides (-ROG); *O*-glucosides (-OG); *N*-7-glucosides (-7G), and *N*-9-glucosides (-9G). Abbreviations of cytokinins are adopted and modified according to (Kaminek *et al.*, 2000). The statistical significance of interaction between total cytokinin content in roots and in shoots and transgenic lines confirmed that upon antibody-mediated cytokinin modulation, cytokinin metabolism was affected differentially in the shoot and root [$F(2,6)=5.57$, $P=0.043$].

αCK_18 and αCK_8, respectively. Furthermore, while for the free bases the root abundance was comparable with that of the WT, a stronger decrease in the shoot than in the root resulted in a relatively much higher root abundance of iP and *t*Z ribosides and riboside phosphates in both αCK_scFv lines when compared with the WT (Supplementary Fig. S4).

The shoot/root-specific changes in endogenous cytokinin content might reflect tissue-specific activation of cytokinin homeostatic mechanisms. Up-regulation of the CKX activity is considered a sensitive and immediate response to the increase in endogenous cytokinin levels (Werner *et al.*, 2006). Therefore, we measured the CKX enzyme activity

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as a potential indicator of disturbed cytokinin metabolism. Notably, similarly to the aforementioned asymmetric cytokinin distribution, in the WT plants, we observed asymmetric CKX activity approximately three times higher in the shoot than in the root (Fig. 3). Interestingly, whereas the CKX activity in the shoot of αCK_scFv lines was unchanged, it was strongly up-regulated in the roots of both αCK_scFv lines.

To conclude, the antibody-mediated modulation of cytokinin ribosides had a strong impact in the shoot, leading to down-regulation of the endogenous content of most of the cytokinin metabolites. In contrast, the only observed significant difference in root was down-regulation of cytokinin ribosides, the direct targets of αCK_scFvs. However, strong root-specific up-regulation of CKX activity suggesting disturbed cytokinin homeostasis also occurs in the root. Additionally, the cytokinin asymmetric distribution further enhanced by antibody-mediated modulation proposes the root as a cytokinin reservoir in young tobacco seedlings.

Asymmetric expression of cytokinin biosynthetic genes in the shoot and root is not affected by antibody-mediated modulation

Higher CKX activity has been shown to occur in response to increased cytokinin concentrations (Motyka and Kaminek, 1992; Motyka *et al.*, 1996, 2003). This might suggest activation of cytokinin biosynthesis in a response to antibody-mediated cytokinin modulation, as previously shown in the case of antibody-mediated modulation of ABA (Staroske *et al.*, 2016). In that scenario, the increase of CKX activity would compensate for the enhanced cytokinin biosynthesis, leading to rather minor changes in the endogenous cytokinins in the root. To test this hypothesis, we used BLAST search to identify the as yet unknown genes possibly involved in cytokinin biosynthesis in tobacco. The putative tobacco orthologs of previously characterized Arabidopsis *IPT* (*NtIPT3* and *NtIPT5*) and *LOG* genes (*NtLOG7* and *NtLOG8*) were identified via BLAST search (see the Materials and methods for more details) and their expression was quantified via qRT–PCR separately in the shoot and root.

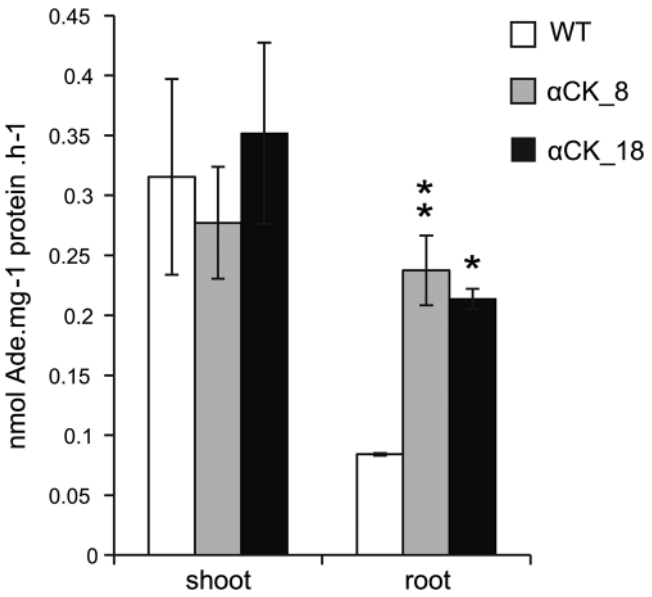

Fig. 3. Antibody-mediated modulation up-regulates CKX activity specifically in the root. CKX activity measured in the shoots and roots of WT, αCK_8, and αCK_18 lines is shown. The statistical significance (*t*-test) of differences between the WT and both αCK_8 and αCK_18 lines in two biological replicates and at α=0.05, 0.01, and 0.001 is depicted by *, **, and ***, respectively. Error bars indicate the SD.

Strong expression asymmetry in favor of the root tissue was observed in two out of four tested cytokinin biosynthetic genes (Fig. 4). In particular, RNA levels of *NtLOG7* were ~15 times higher in the root when compared with the shoot. Similar but weaker asymmetry (three times higher expression in the root than in the shoot) was found in the case of the *NtIPT3* gene. The *NtIPT5* gene also shows a trend towards higher expression in the root which is, however, statistically non-significant, and *NtLOG8* was found to be expressed equally in the shoot and root tissue. Nonetheless, we observed no changes in the expression of the selected cytokinin biosynthetic genes between the WT and either of the two αCK_scFv lines (Supplementary Fig. S5).

Taken together, *NtLOG7* and *NtIPT3* are predominantly expressed in the root tissue. However, the antibody-mediated modulation of cytokinins does not influence the expression of the assayed cytokinin biosynthetic genes.

αCK_scFv transgenic plants reveal cytokinin-related phenotypes in both the shoot and root

To evaluate the biological impact of the changes in cytokinin metabolism observed in response to antibody-mediated modulation of cytokinin ribosides, we performed phenotypic analysis of αCK_scFv transgenic plants. Cytokinins have been shown to act as positive regulators of SAM activity (Werner *et al.*, 2001, 2003; Kurakawa *et al.*, 2007). Although the majority of cytokinin metabolites were down-regulated in the shoot of αCK_scFv transgenic plants, no obvious aberrations were observed in shoot development. However, upon closer examination, cell division was increased in the SAM, particularly in its L1 layer (Fig. 5A).

In contrast, cytokinins are known to play a mostly negative regulatory role in the hypocotyl and root growth in both Arabidopsis and tobacco (Medford *et al.*, 1989; Smigocki, 1991; Hewelt *et al.*, 1994). Cytokinins control primary root growth via negative regulation of the RAM size (Werner *et al.*, 2003; Dello Ioio *et al.*, 2007; Ruzicka *et al.*, 2009). We observed that in both αCK_scFv lines, the root length was significantly shortened, whereas, paradoxically, the RAM was enlarged (Fig. 5B, C). Additionally, the αCK_scFv etiolated hypocotyls were greatly shortened (Fig. 5D) compared with WT plants.

Altogether, we observed cytokinin-related phenotypes as a response of both shoot and root to antibody-mediated cytokinin modulation.

αCK_scFv transgenic plants show differently changed sensitivity to exogenously applied cytokinins in shoot and root

The aforementioned results of the endogenous cytokinin measurements and CKX activity imply activation of

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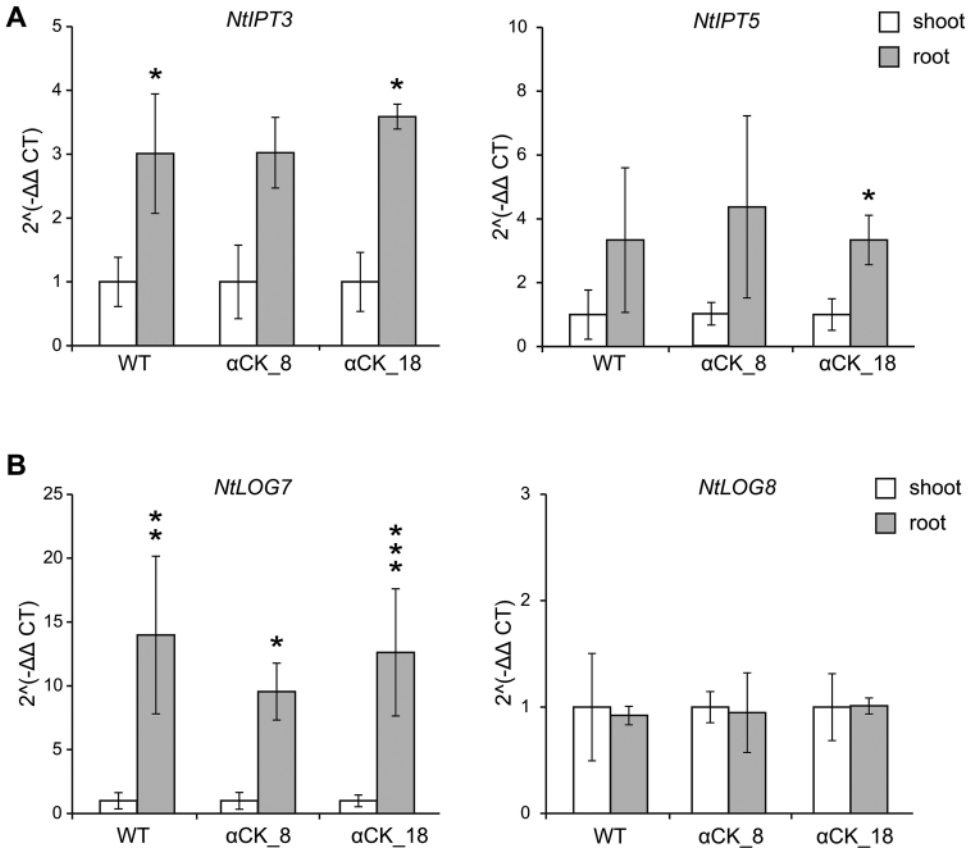

Fig. 4. Relative expression of cytokinin biosynthetic genes is asymmetric between the shoot and root. Relative expression of cytokinin biosynthetic genes *NtIPT3*, *NtIPT5*, *NtLOG7*, and *NtLOG8* double normalized to the internal control (*NtUBC2*) and the transcription level in shoots of the corresponding line. The statistical significance between shoot and root tested by *t*-test in three biological replicates at α=0.05, 0.01, and 0.001 is depicted by *, **, and ***, respectively. Error bars indicate the SD between three independent biological replicates.

mechanisms allowing maintenance of cytokinin homeostasis, which might at least partially mask the effects of disturbed endogenous levels of functional (biologically active or activatable) cytokinins on the plant phenotype. Activation of such mechanisms might result in disturbed sensitivity to exogenous cytokinins. In line with that, both αCK_scFv lines revealed changes in the response to exogenous cytokinin application when compared with the WT. We observed significant RAM shortening in the αCK_scFv lines after 100 nM *t*ZR/BAP treatment (an ~10% decrease), whereas no effect or even slight up-regulation was detectable in the case of the WT (Fig. 6A). However, the higher sensitivity of αCK_scFv lines in RAM shortening when compared with the WT was lost in the presence of 1 μM cytokinins (Fig. 6B). Further, the hypocotyl explant assay (Pernisova *et al.*, 2009) revealed that the αCK_scFv plants showed higher sensitivity regarding cytokinin-induced shoot formation and greening (Fig. 6C). The similar response (increased cytokinin sensitivity) observed in the root and etiolated hypocotyl-derived organs is in line with previous findings that the *de novo* organogenesis in hypocotyls occurs via a root-specific pathway (Sugimoto *et al.*, 2010).

In contrast, we observed lower cytokinin sensitivity of the αCK_scFv transgenic lines regarding cytokinin-mediated regulation of the rosette size. Whereas in the WT plants, a 3 week application of 100 nM BAP enlarged the rosettes by approximately three times when compared with the non-treated controls, the rosette size of αCK_scFv plants increased by just approximately twice, suggesting a disturbed cytokinin response (Fig. 6D). Similarly, the shortened etiolated hypocotyls observed in the αCK_scFv lines were not further sensitive to exogenous cytokinins mediating hypocotyl shortening in the etiolated WT (Fig. 6E).

Overall, we conclude that αCK_scFv plants show organ- and tissue-specific changes in response to exogenous cytokinins.

Discussion

Antibody-mediated formation of the cytokinin pool in the endoplasmic reticulum

Our work addresses the activation and changes in cytokinin homeostasis upon antibody-mediated modulation of cytokinin ribosides in the early stages of tobacco development. Indirect ELISA proved the specificity of αCK_scFv for cytokinin ribosides; however, its binding to free bases or other cytokinin metabolites cannot be excluded. Expression of ER-targeted αCK_scFv antibodies ensures both their better stability and higher accumulation in plant cells (Conrad and Fiedler, 1998), allowing efficient post-synthetic elimination of phytohormones. Based on previous reports using antibody-mediated modulation as a tool to control the endogenous pool of ABA (Conrad and Manteuffel, 2001; Strauss *et al.*, 2001; Staroske *et al.*, 2016),

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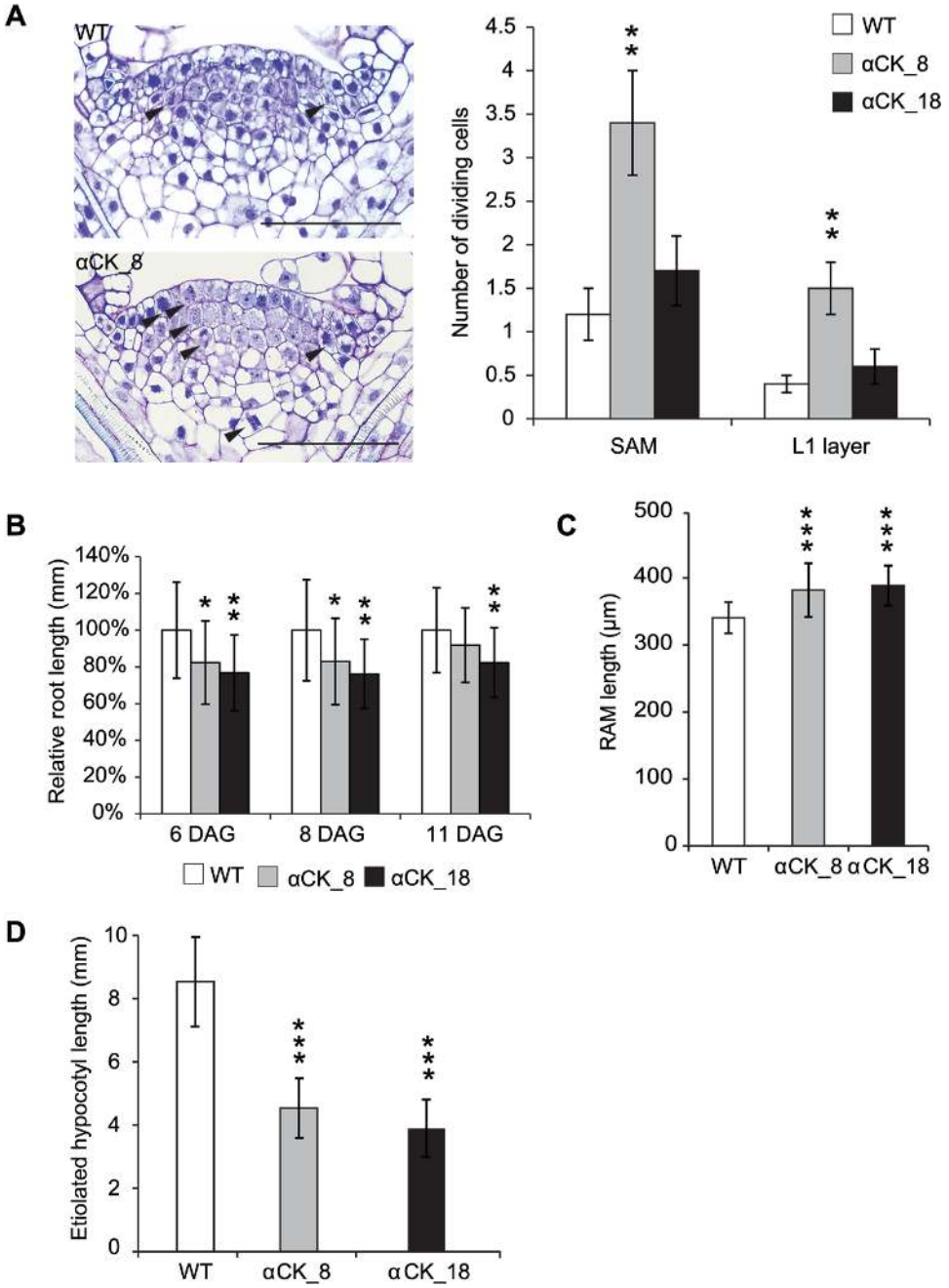

Fig. 5. αCK_scFv transgenic plants show cytokinin-related phenotypes. (A) Longitudinal sections through the SAM of 8-day-old WT and αCK_8 line plants as a representative of transgenic lines (left). Scale bars correspond to 100 μm. The arrows indicate dividing cells (only the cells with clearly apparent condensed mitotic chromosomes were scored). The number of dividing cells in the entire SAM and separately for the L1 layer is shown (right). (B) Root length of 6-, 8-, and 11-day-old seedlings (DAG: days after germination). (C) RAM length of 8-day-old seedlings. (D) Length of hypocotyls in etiolated seedlings. The statistical significance (*t*-test) of differences between the WT and both the αCK_8 and αCK_18 lines at α =0.05, 0.01, and 0.001 is depicted by *, **, and ***, respectively. All measurements were done in three biological replicates with $n \geq 15$. Error bars indicate the SD.

we hypothesize that an artificial sink of cytokinin ribosides in the ER may be formed. Unfortunately, using recently available techniques, we did not succeed in measuring cytokinin levels in the ER-enriched membrane fraction (data not shown). The ER accumulation of cytokinin ribosides might disturb their proposed role in long-range cytokinin transport (Hirose *et al.*, 2008; Kudo *et al.*, 2010; Takei *et al.*, 2001*b*; Lomin *et al.*, 2015). Furthermore, formation of the cytokinin pool in the ER might be the reason for the observed increase of CKX activity in the root, as cytokinin receptors were found to be dominantly localized in the ER (Caesar *et al.*, 2011; Wulfetange *et al.*, 2011). However, based on the general decrease in cytokinin riboside levels throughout the plant, at least partial degradation of antibody-bound cytokinins in αCK_scFv lines cannot be excluded.

Cytokinins are dominantly synthesized in the root of young seedlings

tZ types are thought to be synthesized dominantly in the root by the hydroxylation of iP types via cytochrome P450

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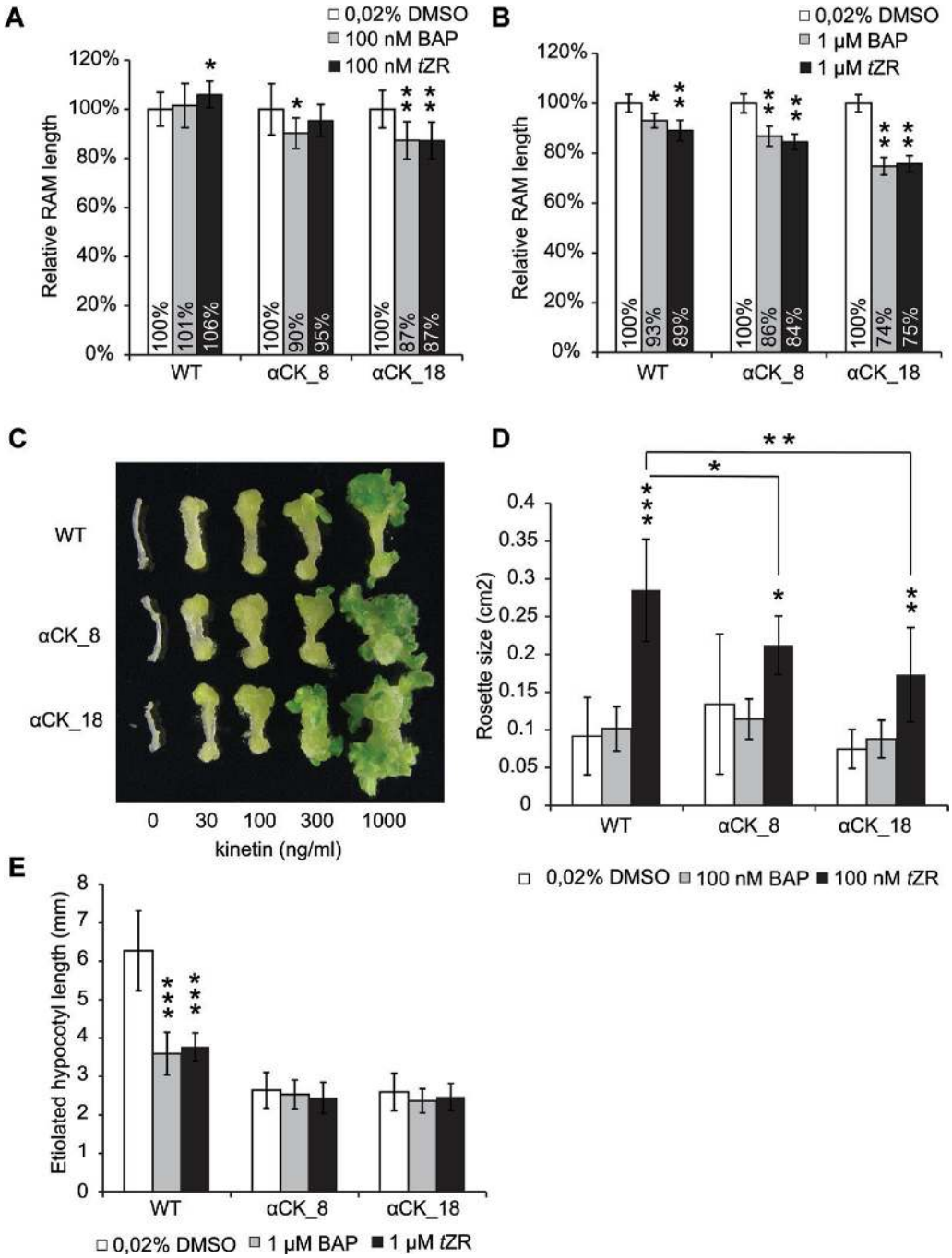

Fig. 6. αCK_scFv transgenic lines reveal changes in the sensitivity to exogenous cytokinins. (A, B) Relative size of the RAM of 8-day-old seedlings grown in the presence of BAP and *t*ZR normalized to the RAM length of the respective mock-treated controls (mean ±SD). The absolute values for WT, αCK_8, and αCK_18, respectively, were as follows: (A) 339.40 ± 24.92, 353.85 ± 27.03, and 380.04 ± 26.68 μm; RAM length is influenced by transgenic line [nested ANOVA, $F(2,308)=6.61$, $P=0.002$] as well as by experimental cytokinin treatment nested in line [$F(6,308)=18.62$, $P<0.001$]. (B) The absolute values for WT, αCK_8, and αCK_18, respectively, were 340.59 ± 23.47, 382.05 ± 40, and 388.67 ± 29.66 μm; RAM length is influenced by experimental treatment (± cytokinin) nested in line [nested ANOVA, $F(6,285)=62.06$, $P<0.001$); however, the main effect of line (WT versus αCK_8 or αCK_18) is not significant [$F(2,285)=0.25$, $P = 0.781$]. The percentage values are listed at the bottom of the corresponding bars. (C) Organogenic response of etiolated hypocotyls from WT and both transgenic lines in the presence of 100 ng ml^{-1} NAA and different concentrations of kinetin. (D) Rosette size in response to 100 nM BAP/100 nM *t*ZR application. (E) Hypocotyl length of etiolated seedlings grown in the presence of 1 μM *t*ZR and BAP. The statistical significance (*t*-test) of differences between either non-treated/treated plants or WT/transgenic line (for rosette size) at $\alpha=0.05$, 0.01, and 0.001 is depicted by *, **, and ***, respectively. All measurements were done in three biological replicates with $n\geq15$. Error bars correspond to the SD.

monooxygenases (CYP735A) (Takei *et al.*, 2004*b*) and transported to the shoot via xylem sap (Hirose *et al.*, 2008; Matsumoto-Kitano *et al.*, 2008; Takei *et al.*, 2001*b*; Kiba *et al.*, 2013; Ko *et al.*, 2014; Zhang *et al.*, 2014). Although, the iP-type cytokinins are considered to be synthetized throughout the whole plant body during the generative growth phase of Arabidopsis plants (Miyawaki *et al.*, 2004; Takei *et al.*, 2004*b*), our data and those of others show that similarly to *t*Z types, iP-type cytokinins are also predominantly localized in the root of young tobacco (this study)

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and Arabidopsis (Žďárská *et al.*, 2013) seedlings. In support of that, our qRT–PCR analysis of cytokinin biosynthetic genes revealed higher expression of *NtIPT3* and *NtLOG7* in the root. Similar (root-prevailing) expression pattern of *AtIPT3*, *AtIPT5*, and *AtIPT7* was observed in 2-week-old Arabidopsis seedlings (Takei *et al.*, 2004*b*). These findings imply that both iP- and *t*Z-ribosides could be predominantly synthetized in the root of young seedlings. On the other hand, iPR mainly occurs in phloem sap in Arabidopsis (Corbesier *et al.*, 2003; Hirose *et al.*, 2008), implying its shoot origin. The differential transport of *t*Z from the root to the shoot with the opposite flow of iP from the shoot to the root was suggested by the results of grafting experiment using quadruple *atipt1;3;5;7* mutants (Matsumoto-Kitano *et al.*, 2008). Accordingly, the phloem-mediated transport of radioactively labeled BAP and iPR from the hypocotyl to the root meristem was also demonstrated (Bishopp *et al.*, 2011). The expression of *AtIPT3* in the phloem and/or phloem companion cells was demonstrated in both the shoot and root (Miyawaki *et al.*, 2004; Takei *et al.*, 2004*a*). That might explain the dominant phloem localization also for root-synthesized iP cytokinins. Interestingly, the phloem localization of root-synthesized pyrrolizidine alkaloids and their transport from root to shoot against the typical flow in phloem was shown in *Senicio* sp. (Turgeon and Wolf, 2009). Thus, even the shootward transport of iPR via phloem cannot be excluded. Accordingly, the possible role of phloem-located iP in systemic signaling (including both shootward and rootward transport) was proposed (Hirose *et al.*, 2008; Kudo *et al.*, 2010). Finally, the developmental-specific distribution of several *AtIPT* genes was observed between the shoot and root (Takei *et al.*, 2004*a*).

Altogether, these data suggest possible dynamics of the cytokinin transport throughout the plant, which will probably reflect both physiological status and developmental context, and further studies will be necessary to find its biological relevance.

Model of organ-specific cytokinin homeostasis

Based on our data, we propose a model describing root/shoot-specific regulation maintaining homeostasis of cytokinin metabolism in young tobacco seedlings (Fig. 7). In response to antibody-mediated modulation of cytokinin ribosides, the cytokinins accumulate in the ER of the root, where most of the cytokinins are synthesized. This leads to the activation of cytokinin homeostatic regulation mechanisms including the activation of CKX activity and partial decrease of endogenous cytokinin levels in the root, associated with enlargement of RAM size. However, preservation of optimal levels of active cytokinins seems also to be crucial in the shoot, but possibly independent of the homeostatic regulation described for the root. The shoot pool of free bases seems to be refilled from riboside precursors transported from the root and/or the shoot-deposited cytokinin *O*-glucosides. In this scenario, the higher sensitivity of the shoot regarding changes in the endogenous cytokinins might be a consequence of αCK_scFv-mediated immobilization (antibody-mediated transport attenuation) of both *t*ZR and iPR in the root. As a result of

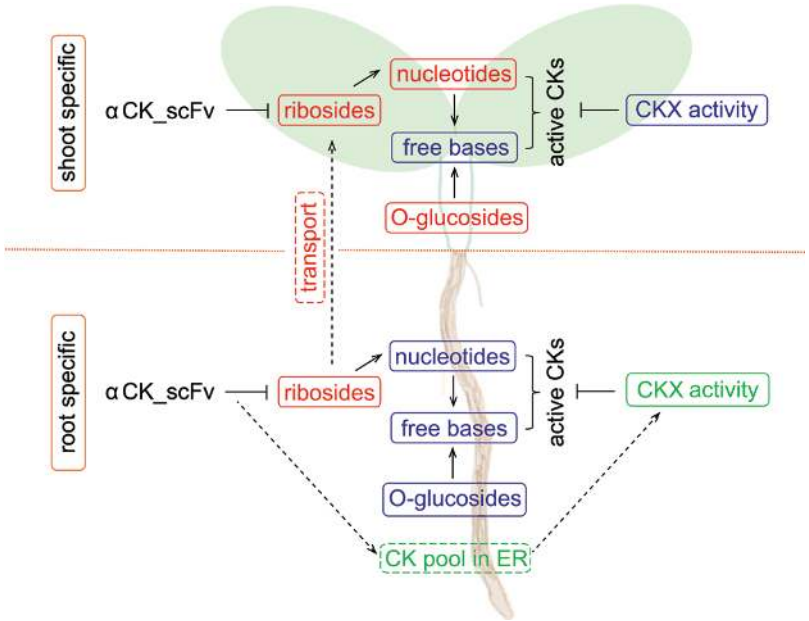

Fig. 7. Proposed model of shoot- and root-specific regulation controlling cytokinin homeostasis as identified using antibody-mediated modulation of cytokinin ribosides in tobacco. Antibody-mediated modulation of cytokinins creates an artificial cytokinin pool in the ER of the root cells, which seem to be the main source of both iP and *t*Z cytokinins in tobacco (this work) and Arabidopsis (Žďárská *et al.*, 2013). This results in up-regulation of CKX activity, possibly representing a homeostatic mechanism induced by ER-located cytokinin receptors. The antibody-mediated modulation of cytokinin ribosides disturbs their transport from the root, leading to a decrease of cytokinin ribosides as well as their phosphorylated forms (cytokinin nucleotides) in the shoot. Decreased levels of cytokinin *O*-glucosides suggest that one of the mechanisms maintaining WT-like levels of free cytokinin bases could be hydrolysis of cytokinin *O*-glucosides as a cytokinin storage form. Both experimentally obtained data (full lines) and predicted/indirectly supported mechanisms (dashed lines) are depicted. Up-regulation (green), down-regulation (red), and unchanged status (blue) of individual nodes representing metabolites/enzymatic activities/processes in αCK_scFv transgenic lines are shown in comparison with WT plants.

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complex homeostatic regulations, the αCK_scFv transgenic lines did not differ much from the WT plant phenotype, but exhibited root- and shoot-specific changes in their sensitivity to exogenous cytokinins.

The impaired iPR transport from the root might also explain the observed decrease in the levels of cytokinin nucleotides (riboside phosphates) in the shoot. This may suggest the existence of enzymes mediating riboside phosphorylation [*in vitro* evidence provided by Kopecná *et al.* (2013) and Osugi and Sakakibara (2015)]. Namely, cytokinin ribosides may act as mobile cytokinin precursors, which after their phosphorylation could be converted into free bases via LOG proteins. Maintenance of constant levels of cytokinin free bases throughout the plant suggests the existence of strong homeostatic mechanisms preserving the active cytokinin levels in both shoot and root. Activation of such homeostatic mechanisms (possibly in a tissue-specific context and thus difficult to detect using measurements of cytokinin levels in the entire shoot) could be the reason for the unexpected increase of cell division in the SAM. The decreased content of *O*-glucosides in the shoot implies that one such homeostatic mechanism could be deglycosylation of cytokinin *O*-glucosides by plant β-glucosidases, as proposed previously (Brzobohatý *et al.*, 1993; Kiran *et al.*, 2006).

Conclusions and future perspectives

The results presented here as a case study show that antibody-mediated modulation can be used to elucidate the complex cytokinin metabolism and role of organ-specific compartmentalization of individual cytokinin metabolites in plant development. The post-synthetic cytokinin antibody-mediated modulation using targeted overexpression of cytokinin riboside-recognizing antibodies shows shoot- and root-specific changes in cytokinin metabolism and cytokinin-related phenotypes. The antibody-mediated modulatory approach could be used in studies addressing further aspects of cytokinin biology, including targeted manipulations of the subcellular cytokinin distribution to study its importance in cytokinin metabolism and/or development. More sensitive analytical methods are currently being developed, and the potential progress in this field is highly encouraging. Importantly, combination of antibody-mediated modulation with genetic screening will help to identify key players involved in the organ-specific/subcellular distribution of individual cytokinin (sub)types and the control of cytokinin homeostasis. Finally, investigation of recombinant antibodies and isolation of scFvs and nanobodies (Muyldermans, 2013) more specific for individual cytokinin (sub)types and/or their metabolites, including cytokinin free bases, represents a key challenge for further exploitation of antibody-mediated modulation-based approaches.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Sequence and cloning cassette description

Fig. S2. Immunochemical characterization of αCK_scFv transgenic plants.

Fig. S3. Endogenous cytokinin levels in the shoot and root of tobacco seedlings.

Fig. S4. Shoot/root asymmetric distribution of endogenous cytokinins.

Fig. S5. Relative expression of cytokinin biosynthetic genes is similar between WT and transgenic plants.

Acknowledgements

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