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Multifaceted activity of cytokinin in leaf development shapes its size and structure in Arabidopsis

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SUMMARY

Phytohormone cytokinin has been shown to affect many aspects of plant development ranging from the regulation of the shoot apical meristem to leaf senescence. However some studies reported contradictory effects of cytokinin on leaf physiology. Thus, cytokinin treatments cause both chlorosis and increased greening, and both decrease and increase in cell size. To elucidate this multifaceted role of cytokinin in leaf development, we have employed a system of temporal control over the cytokinin pool and investigated the consequences of modulated cytokinin levels in the third leaf of Arabidopsis. We show that at the cell proliferation phase, cytokinin is needed to maintain cell proliferation by blocking the transition to cell expansion and the onset of photosynthesis. Transcriptome profiling revealed regulation by cytokinin of a gene suit previously shown to affect cell proliferation and expansion, and thereby a molecular mechanism by which cytokinin modulates a molecular network underlying the cellular responses. During the cell expansion phase, cytokinin stimulates cell expansion and differentiation. Consequently, a cytokinin excess at the cell expansion phase results in an increased leaf and rosette size fueled by higher cell expansion rate, yielding higher shoot biomass. Proteome profiling revealed the stimulation of primary metabolism by cytokinin, in line with an increased sugar content that is expected to increase turgor pressure, representing the driving force of cell expansion. Thus, the developmental timing of cytokinin content fluctuations, together with a tight control of primary metabolism, is a key factor mediating transitions from cell proliferation to cell expansion in leaves.

INTRODUCTION

Cytokinins (CKs) are plant hormones involved in the control of diverse developmental processes, including the determination of the final size and function of plant organs such as leaves (Kieber and Schaller, 2014; Zürcher *et al.*, 2016). Leaf development takes place at the periphery of the shoot apical meristem (SAM), where stem cells self-renew and produce daughter cells that differentiate and give rise to different organ structures such as leaf primordia (Kalve *et al.*, 2014), acquiring abaxial and adaxial leaf-polarity. The conversion of a leaf primordium into a mature leaf results from strict coordination of cell proliferation, cell expansion and cell differentiation which may partly overlap (Donnelly *et al.*, 1999; Breuninger and Lenhard, 2010; Gonzalez *et al.*, 2012). Cell proliferation

generates relatively constant and small new cells throughout the entire primordium (Gonzalez *et al.*, 2012). The subsequent transition from cell proliferation to cell expansion and differentiation forms the puzzle-shaped pavement cells with highly interdigitated lobes, guard cells, vascular tissue and trichomes (Kalve *et al.*, 2014; Gonzalez *et al.*, 2012).

Previous study on CK-deficient plants has shown that the influence of CKs on morphogenesis is achieved through the cell cycle regulation (Werner *et al.*, 2001). Moreover, the CK activity has been demonstrated to control the cell proliferation in meristems, with opposite roles in root and shoot meristems (Werner *et al.*, 2003). CKs are perceived by cells already at nanomolar range via a phosphorelay similar to bacterial two-component response systems (Heyl *et al.*, 2007; Kieber and Schaller, 2014). The interaction with a specific receptor is a crucial step leading to conversion of the signal into the specific CK response (Spíchal, 2012). Currently, CK receptor family in Arabidopsis includes Arabidopsis histidine kinases AHK2, AHK3 and AHK4 (CRE1/WOL) which share a cyclase/histidine kinase associated sensing extracellular domain (Romanov *et al.*, 2006; Heyl *et al.*, 2007). The receptor activation triggers the phosphorylation of Arabidopsis histidine phosphotransfer proteins (AHPs) that further transport the signal into the nucleus by phosphorylation of type-B Arabidopsis response regulators (ARRs) (Hwang and Sheen, 2001; Hutchison *et al.*, 2006; Dortay *et al.*, 2008). The Arabidopsis genome contains eleven type-B ARRs which act as transcription factors inducing primary CK response genes including type-A ARRs. The type-A ARRs function as negative feedback regulators of CK signaling by attenuating activity of type-B ARRs (D'Agostino *et al.*, 2000; To *et al.*, 2004; Argyros *et al.*, 2008; Kiba *et al.*, 2002; Taniguchi *et al.*, 2007; Kim *et al.*, 2006; Hwang *et al.*, 2012; Mason *et al.*, 2004; Rashotte *et al.*, 2006; Sakai *et al.*, 2000). Though subsequent meta-analysis of the microarray data associated with CK responses have defined a robust set of CK-regulated genes and helped to identify processes and pathways affected by CK (Bhargava *et al.*, 2013), little is known about the impact of CK on molecular mechanisms controlling either the onset of the transition from cell proliferation to cell expansion in an early developing leaf or the differentiation of fully expanded leaf.

Our previous transcriptome profiling of Arabidopsis leaf 3 pointed to a number of molecular events underlying the aforementioned transition (Andriankaja *et al.*, 2012), e.g. the concomitant increased expression of genes involved in chloroplast retrograde signaling, which highlights the importance of chloroplast differentiation for the onset of cell expansion in line with previous investigations (Nott *et al.*, 2006). To shed light on CK involvement in early leaf development, we re-analyzed the transcriptomic data sets with focus on transcripts involved in CK metabolism and signaling (Table S7). The analysis has revealed a significant decrease in the transcript level of the CK-activating gene *Lonely Guy* (*LOG3)* and several less pronounced alterations in other CK related transcripts during the transition from cell proliferation to cell expansion suggesting a role for CK dynamics in controlling this process.

To test this prediction, we first analyzed dynamics of CK pool and signaling in course of the transition from cell proliferation to cell expansion and differentiation. Next, we employed transgenic Arabidopsis plants harboring dexamethasone (DEX)-inducible agrobacterial isopentenyl transferase (*ipt*) and barley cytokinin oxidase/dehydrogenase (*HvCKX2*) genes (Craft *et al.*, 2005; Samalova *et al.*, 2005; Černý *et al.*, 2013) to modulate CK levels in developmental windows corresponding to the cell proliferation and expansion phases and examined the consequences of CK excess or deficiency for leaf 3 development. Leaf 3 was chosen as its development has been analyzed in depth on cellular, genetics and transcriptomics level (Andriankaja *et al.*, 2012; Gonzalez *et al.* 2012; Nelissen *et al.*, 2016) which facilitates experimental design and interpretation of data obtained. This research revealed a multifaceted CK activity at distinct phases of leaf development that shapes leaf size and structure, and identified CK-responsive genes and proteins that remained unrevealed in previous profiling studies of CK action in plants.

RESULTS

CK Pool and Signaling Dynamics in Course of Arabidopsis Leaf 3 Development

First, we have analyzed relevance of the alterations in CK related transcripts (Table S7) to dynamics of CK pool and signaling in course of the transition from cell proliferation to cell expansion and differentiation which corresponds to 9 – 13 days after stratification (DAS) in *in vitro* cultivated Arabidopsis wild type Col-0 (Andriankaja *et al.*, 2012). At 9 DAS, the CK pool was dominated by *trans*-zeatin (*t*Z) which peaked on 10 DAS. At 11 DAS, *t*Z dropped to a level similar to $N^6(\Delta^2$-isopentenyl) adenine (iP) and the two CK bases remained close to these levels till 13 DAS. The remaining CK bases *cis*-zeatin (*c*Z) and dihydrozeatin (DHZ) stayed below detection limit throughout the investigated period (Figure 1A, Table S11). To assess CK signaling output, we have employed a synthetic sensor TCSn:GFP (Zürcher *et al*., 2013). Intensity of GFP fluorescence was high at 9 and 10 DAS and then dropped on 11 DAS and remained low till 13 DAS. A negative correlation between CK signaling output and chloroplast biogenesis assessed by chlorophyll fluorescence was observed (Figure 1B). Spatial-temporal pattern of GFP fluorescence indicated high ubiquitous activity of CK signaling over the entire leaf 3 area at 9 and 10 DAS. At 10 DAS, GFP fluorescence appears also in emerging stomata cells and veins. Then the GFP fluorescence stays associated with the stomata cells and veins only. At 13 DAS, the GFP signal drops somewhat in the stomata cells and cannot be seen veins (Figure 1C).

Both Increased and Decreased CK Levels during the Proliferation Phase of Leaf Development Reduce the Mature Rosette Size

Having proven the CK pool and signaling dynamics in course of the transition from cell proliferation to cell expansion and differentiation in leaf 3, we next set up experiments to test its significance in regulation of leaf development. To this end, changes in CK levels were induced at two specific developmental stages, i.e. the stage when most cells are dividing and the cell proliferation zone covers the whole leaf, just prior to the onset of cell expansion, and the stage when most cells are expanding and the cell proliferation zone is reduced to the leaf base (Figure 2A), which corresponds to 8 and 14 DAS, respectively (Andriankaja *et al.*, 2012). A dexamethasone (DEX)-inducible system was used to increase and decrease CK contents through induction of a CK-biosynthetic *ipt* (*CaMV35S*>GR>*ipt*; hereafter designed *ipt*) or a CK-degrading *HvCKX2* (*CaMV35S*>GR>*HvCKX2*; hereafter designed *HvCKX2*) gene, respectively (Craft *et al.*, 2005; Samalova *et al.*, 2005; Černý *et al.*, 2013). The above mentioned studies have shown that both transgenic systems are fully saturated after 24h of DEX treatment. Thereto, *ipt* and *HvCKX2* plants were cultivated on nylon meshes, which enabled the transfer at the designated time points to a growth medium supplemented with DEX at concentrations of 1.0 µM, 2.5 µM and 5.0 µM. DEX treatment at 8 DAS resulted in smaller rosettes at 21 DAS for both transgenic lines compared to the mock-treated controls or DEX-treated wild-type plants (Figures 2B and 2C). Phenotype inspection suggested that a DEX concentration of 2.5 µM is close to saturation regarding the biological effects of CK increase or decrease (Figure S1). Therefore, treatment with 2.5 µM DEX was employed for subsequent analyses, unless specified otherwise.

CK Affects Both Cell Proliferation and Cell Expansion during the Proliferation Phase

To analyze the cellular basis of the reduced rosette size observed in *ipt* and *HvCKX2* plants activated at 8 DAS, the average leaf area, cell area and number of cells per leaf were determined at 21 DAS (Figure 2). The leaf area was significantly reduced by 83.3% and 73.3% in response to CK excess or deficiency, respectively (Figure 2C). Abaxial pavement cell analysis revealed that both a significant reduction in cell area (by 69%) and a significant, although more moderate, decrease in cell number (by 37%) were responsible for the reduction in leaf area observed in activated *ipt* plants (Figure 2D and 2E). In contrast, a significantly strong reduction in cell number (by 91%) resulted in a smaller leaf 3 in activated *HvCKX2* plants, which could not be compensated by the significantly increased cell area (by 34%). Further, the reduced size of the pavement cells in activated *ipt* plants was accompanied by a severe reduction in their lobing (Figure 2B and 3A). Together, these observations indicate that CK excess at the cell proliferation phase interferes mainly with cell differentiation and expansion, whereas CK deficiency leads to reduced cell proliferation and stimulation of cell expansion.

Increased CK Levels Cause Opposite Effects on Chlorophyll Accumulation and Photosynthesis Depending on the Leaf Developmental Stage

Ipt plants activated on 8 DAS developed consistently pale green leaves when compared to the wild-type control and activated *HvCKX2* plants (Figure S1). In contrast, when activated on 14 DAS, leaves of *ipt* plants were dark green when compared to the wild type and activated *HvCKX2* (Figure 3B to 3D, Figure S1 and S2). CK has been reported to stimulate chlorophyll metabolism and expression of chloroplast-related genes, to enhance the abundance of proteins involved in photosynthesis and to stimulate chloroplast biogenesis (Lochmanová *et al.*, 2008; Brenner *et al.*, 2005; Černý *et al.*, 2011). However, various lines of evidence indicate that chloroplast biogenesis is preferentially coupled to the cell expansion phase of leaf development (Andriankaja *et al.*, 2012). Therefore, we examined the effect of CK on chlorophyll accumulation, chloroplast biogenesis and photosynthetic yield in relation to the leaf developmental stage in more detail. To visualize the shape of living cells together with chlorophyll *a* auto-fluorescence, we used the well-characterized *proCaMV35S::GFP-TUA5* line (hereafter designated *GFP-TUA5*) (Shaw *et al.*, 2003) crossed with our transgenic lines. In addition to highly limited cell expansion and lobe formation, pavement cells of *ipt* plants activated at 8 DAS and imaged at 21 DAS contained very little chloroplasts, whereas the chloroplast number in activated *HvCKX2* plants was comparable to parental *GFP-TUA5* plants (Figure 3A). Consistently, chlorophyll *a* auto-fluorescence in activated *ipt* plants was negligible compared to both activated *HvCKX2* and *GFP-TUA5* plants. Next, *ipt* and *HvCKX2* plants were activated on 8, 9, 10, 11, 12, 13 or 14 DAS, and chlorophyll fluorescence of leaf 3 was analyzed at 21 DAS. *Ipt* activation in the early stages of leaf development, when all cells or their majority are proliferating, resulted in a dramatically lower photosynthetic yield (Figure 3B) and chlorophyll content (by 79.0%; Figure 3C and 3D) compared to the wild type. However, in *ipt* plants activated at 14 DAS, when all cells had started to expand and differentiate, the chlorophyll content was significantly higher (by 23.5%). Activation of *HvCKX2* on 8 DAS or 14 DAS affected neither the photosynthetic yield, nor chlorophyll content (Figure 3B-D). Thus, CK does not seem to inhibit processes connected with photosynthesis *per se*. Rather, CK excess blocks leaf cells at the end of their proliferation phase when they possess little competence to progress with differentiation, including chloroplast development. Stimulation of chlorophyll accumulation, chloroplast biogenesis and photosynthetic yield by increased CK contents can be only achieved once a developmental block of chloroplast biogenesis is lifted at or immediately following transition from cell proliferation to expansion.

Leaf 3 Transcriptome in Response to Modulation of CK Levels during the Cell Proliferation Phase

To uncover the molecular events underlying the effects of modulated CK levels on early leaf development, transcriptome profiling of leaf 3 microdissected at 9 DAS was performed using RNA sequencing (RNA-Seq). In a pilot RT-qPCR experiment, a 3-h DEX treatment was found sufficient to achieve not only full activation of *ipt* and *HvCKX2*, but also clear statistically significant modulations of steady-state levels of almost all type-A *ARR* transcripts used as indicators of endogenous CK levels (Figure S3A and S3B). Interestingly, *HvCKX2* activation caused significant fluctuations in the type-A *ARR* gene expression, which can be explained as a homeostatic reaction to reduced levels of CKs by activation of the CK biosynthetic machinery (Černý *et al.*, 2013). Therefore, a 3 h DEX treatment was employed in the RNA-Seq analysis. The final number of significantly regulated gene profiles (FDR < 0.05) was 129 and 64 for *HvCKX2*- and *ipt*-expressing plants, respectively (Figure 4, Table S1-S3). In plants with decreased CK contents, the total number of up- and down-regulated gene profiles was 47 and 82, respectively, whereas 14 genes were up-regulated and 50 down-regulated in CK-overproducing plants (Figure 4A, lower half). Thus, negative gene regulations are prevalent in response to modulation of CK levels in developing leaves. The overlap between both sets of regulated genes contained 48 genes with a similar response in *ipt* and *HvCKX2* (7 and 41 simultaneously up-regulated and down-regulated genes, respectively), and none that would have an opposite response in these two sets. GO enrichment analysis (Maere *et al.*, 2005) provided an insight into the functional categories of the 145 CK-responsive genes (Figure 4B and 4C, Table S4 and S5). The top enriched categories involved secondary metabolism, mainly of sulfur and flavonoids. Other GO categories affected by CK imbalance were cell wall proteins, regulation of transcription, and hormone metabolism, such as jasmonate metabolism. The RNA-Seq analysis also revealed a high number of CK-responsive genes involved in the response to abscisic acid (such as *ERD6*, *COR47*), which has been reported as an important part in plant responses to environmental stress (Foster and Chua, 1999; Yamada *et al.*, 2010).

Next, above data sets were compared with the set of genes found differentially regulated during the transition from cell proliferation to expansion in leaf 3 (Andriankaja *et al.*, 2012). The overlap of the three data sets reveals that a number of the genes down-regulated by CK deficiency is highly expressed at the proliferation stage under standard conditions and subsequently decrease during the transition to cell expansion (e.g. *ANT*, *JGL*, *BHLH2*, *LSH10*, *CYP78A5* (*KLUH*), *GATA17*), while genes that are up-regulated by CK deficiency are normally induced during this transition (e.g. *COL6*, *ESM1*, *PRP2*, *PRP4*, *TGG1*, *TGG2*) (Andriankaja *et al.*, 2012). Thus, CK deficiency during the cell proliferation phase triggers expression patterns specific for the transition from cell proliferation to cell expansion and therefore has a potential to release cells from the cell proliferation to the cell expansion phase, which is in line with the cellular analysis reported above.

Further, the sets of CK-responsive genes identified in this work were compared to results of the meta-analysis of CK-responsive genes found in a variety of CK-treated samples (Bhargava *et al.*, 2013). Interestingly, their overlap includes almost exclusively genes involved in CK signaling (*AHK4*, *ARR4*, *ARR7*, *ARR9*, *ARR15*) and metabolism (*AtCKX5*), and only a few genes not directly related to these two categories, e.g. *FANTASTIC FOUR 3* (*FAF3*). Thus, the remaining differentially expressed genes represent an as yet unrecognized subset of CK-responsive genes specific for early leaf development (Table S10).

In order to verify the accuracy of the RNA-Seq analysis, RT-qPCR was performed to confirm the expression of selected transcripts involved in CK signaling and growth regulation such as *cytochrome P450 KLUH* (*KLU*) and AP2-like ethylene-responsive transcription factor *ANT* (Horstman *et al.*, 2014). Our RT-qPCR analysis revealed similar expression trends as seen in the RNA-Seq analysis (Figure 4D, Table S3).

Ipt Activation Following Cell Proliferation Phase Results in Stimulation of Cell Expansion and Differentiation Leading to a Dramatic Increase in Leaf Size and Biomass

To investigate the effects of *ipt* and *HvCKX2* activation at distinct phases of rosette growth on Arabidopsis development, an in-soil experiment was designed. Plants were grown under standard conditions, and DEX was applied in a concentration range of 1 to 10 µM by watering a single dose at 0, 8 or 14 DAS. The effects of *ipt* and *HvCKX2* activation were first analyzed at 21 DAS. In *ipt* plants (Figure S4A), the response to DEX treatment at 8 DAS was found intricately dose-dependent. 1 µM DEX applied at 8 DAS caused a statistically significant increase in rosette area and biomass (Figure S4C and S4D). A response close to saturation was observed for DEX applied at a concentration of 10 µM, which caused a dramatic reduction in rosette area and biomass when applied at 0 and 8 DAS. However, DEX application at 14 DAS resulted in increases in rosette area and biomass starting from a concentration of 2.5 µM, which were statistically significant for 5 and 10 µM DEX. Thus, a single dose of 10 µM DEX applied at 14 DAS resulted in an increase of leaf area by ~31% and of biomass by ~53% compared to the wild type (Figure 5A and 5D-5F). Leaf series analysis revealed that the increases were due to growth stimulation in leaves 3, 4, 5 and 6, whose areas increased by 40%, 27%, 35% and 18%, respectively, compared to the wild type (Figure 5B and 5C). Activation of *HvCKX2* caused reductions in rosette area and biomass in all combinations of activation times and DEX concentrations (Figure S4B), which were statistically significant for concentrations of 5 and 10 µM and which were due to a decreased size of almost all leaves forming the rosette at 21 DAS (e. g. leaves 3, 4, 5 and 6, whose areas reduced by 41%, 56%, 49% and 59%, respectively, compared to the wild type; Figure 5B and 5C). At 37 DAS, *HvCKX2* plants activated by 10 µM DEX at 14 DAS produced shorter stems, fewer leaves and fewer flowers/buds by 58%, 28% and 53%, respectively, compared to the wild type (Figure S5A-S5D). In contrast to the reduced shoot growth, their root

growth was found enhanced when examined at 21 DAS (Figure S5E) in line with previous reports (Werner *et al.*, 2003). When *ipt* plants activated by 10 µM DEX at 14 DAS were examined at 37 DAS, only small (statistically not significant) increases were found for total number of leaves and flowers/buds, and stem length remained virtually unaltered compared to the wild type. The root growth examined at 21 DAS was slightly inhibited compared to mock-treated *ipt* plants and unaltered compared to the wild type (Figure S5E) which is consistent with decreasing root sensitivity to inhibition by CK excess in course of plant development (Kuderová et al., 2008). Flowering time was not affected by *ipt* and *HvCKX2* activation at 14 DAS.

CK Pool of Leaf 3 Following *ipt* and *HvCKX2* Activation

Given the fact that the area of leaf 3 was the most and consistently affected following *ipt* activation in in-soil grown Arabidopsis at 14 DAS, leaf 3 was chosen to quantify the extent to which the CK pool was altered following *ipt* and *HvCKX2* activation (Figure 6). Plants were grown in soil under standard conditions, *ipt* and *HvCKX2* expression was activated by 10 µM DEX at 14 DAS and leaf 3 was dissected for the determination of different CK levels at 21 DAS. In activated *ipt* plants, free CK bases *t*Z (the most effective natural CK in activating CK receptors of Arabidopsis) (Spíchal *et al.*, 2004) and DHZ were increased 5.3- and 7.6-fold, respectively, compared to the mock treatment (Figure 6A and 6B). iP content was not changed significantly (Figure 6C), which is consistent with the fact that the Agrobacterium isopentenyltransferase encoded by the construct used in this study specifically produces *t*Z (Lochmanová *et al.*, 2008; Craft *et al.*, 2005; Samalova *et al.*, 2005; Hradilová *et al.*, 2007). More than 10-fold increases in *trans*-zeatin riboside monophosphate (*t*ZRMP) and *trans*-zeatin riboside (*t*ZR) indicate that *ipt* remains activated at least one week following DEX administration (Figure 6A). Agrobacterium *ipt* produces *t*ZRMP directly from AMP and 1-hydroxy-2-methyl-2(*E*)-butenyl 4-diphosphate in plastids (Sakakibara *et al.*, 2005), and *t*ZR is an intermediate in a two-step conversion of *t*ZRMP to *t*Z (Kudo *et al.*, 2010). In activated *HvCKX2* plants, a dramatic drop in *t*Z and its much less active isomer *c*Z, and all their metabolites was found (Figure 6A and 6D). Free DHZ and dihydrozeatin-*O*-glucoside (DHZOG) levels were somewhat increased (Figure 6B), which is in line with resistance of DHZ to CKX action due to the absence of the double bond in the isoprenoid side chain (Armstrong, 1994; Hare and van Staden, 1994). Surprisingly, free iP contents remained almost unaffected in *HvCKX2* plants (Figure 6C). Thus, *ipt* and *HvCKX2* activation resulted in significant CK excess or deficiency, respectively, as expected based on our previous work (Černý *et al.*, 2013).

Cell Expansion Is the Cellular Cause of Leaf Enlargement in Activated *ipt* Plants

To elucidate the cause of leaf enlargement in in-soil grown *ipt* plants activated at 14 DAS, a cellular analysis was carried out in leaf 3 dissected at 21 DAS (Figure 7). At 14 DAS, almost all cells of leaf 3 have already transitioned from the proliferation to the expansion phase (Figure 2A). The cellular analysis of pavement cells from the abaxial side of leaf 3 revealed that the increase in leaf area in activated *ipt* plants was solely due to the stimulation of cell expansion (by 41% compared to the wild type), since the cell number was not significantly affected (Figure 7A and 7B). CK excess did not significantly affect the stomatal index (Figure 7C). The cellular analysis was also performed to reveal the cause of reduction of leaf size in activated *HvCKX2* plants. Here, the main cause of reduced leaf area was a lower cell number (by 46%), and the reduction in cell expansion (by 15.8%; Figure 7A and 7B). The effect of CK deficiency on the cell size is even higher than could be judged from the average cell area. This is clearly revealed by plotting a distribution of cell areas (Figure S6) and taking into account that most small cells originating from the amplifying divisions of meristemoid cells during the cell expansion phase in the wild type and CK overproducing plants are absent in the CK deficient plants. Thus, the small cells prevailing in leaf 3 of CK deficient plants apparently represent pavement cells generated during the cell proliferation phase which failed to expand in course of the cell proliferation phase due to CK deficiency. CK deficiency resulted also in the reduction of the stomatal index by 14 % compared to the wild type (Figure 7C).

To reveal how CK pool alterations regulate the endocycle, together with expansion and differentiation of Arabidopsis pavement cells, we performed a ploidy analysis of leaf 3 dissected at 21 DAS using flow cytometry. The decreased cell area of *HvCKX2* plants activated at 14 DAS was found to coincide with a 43% increase in the portion of 4C cells and an 81% decrease in the portion of 16C cells, compared with the wild type (Figure 7D). This led to a decrease in the endoreduplication index of CK-deficient plants by 28% compared to the wild type. Contrarily, the overexpression of *ipt* did not affect endoreduplication (Figure 7E).

Proteome Profiling Reveals the CK Impact on Carbohydrate Metabolism and Proteosynthesis

To get an insight into alterations of molecular networks underlying the increase and decrease, respectively, of cell size caused by *ipt* and *HvCKX2* activation at 14 DAS, plants were cultivated at standard conditions in soil, treated with 10 µM DEX at 14 DAS, and leaf 3 was dissected at 21 DAS and subjected to proteome profiling. In total, we quantified the relative abundances of 7,932 peptides representing 2,289 proteins. The LC-MS shotgun proteomic analysis yielded 96 proteins with a significant ($P < 0.05$; Student's *t*-test) and at least 1.4-fold absolute ratio between wild-type and DEX-inducible lines in three biological replicates. In detail, 65 and five proteins were identified to be up- and down-regulated, respectively, in response to *ipt* activation, and analysis of activated *HvCKX2* plants revealed 28 and 26 up- and down-regulated proteins, respectively (Figure 8A, Table S8). Many

of the regulated proteins responded to both *ipt* and *HvCKX2* activation, with 14 responding in the same directions and 14 responding in opposite directions. GO category enrichment (Bonferroni-corrected $P < 0.05$) is presented in Figure 8B and detailed in Table S9. Overall, the largest number of proteins up-regulated by CK excess was involved in carbohydrate metabolism (e.g. fructose-bisphosphate aldolase 8), amino acid metabolism (e.g. aspartate aminotransferase), the antioxidant system (monodehydroascorbate reductase) and energy processes (e.g. citrate synthase 4, isocitrate dehydrogenase 5, photosystem II protein D1, Figure 8B) (Bevan *et al.*, 1998). Only five proteins were identified to be down-regulated by CK excess, which were mainly involved in translation (e.g. 50S ribosomal protein L3-1). CK deficiency had a predominantly negative impact on the abundance of proteins related to primary metabolism (e.g. glycine dehydrogenase 2), secondary metabolism (e.g. glutamate–glyoxylate aminotransferase) and translation (e.g. 50S ribosomal protein L4, 40S ribosomal protein S2-3; for details, see Table S9). It has to be noted that the presented data do not necessarily correspond to the cell expansion only. During the cell expansion phase, the cell expansion of already existing pavement cells is concomitant with stomata formation which includes amplifying divisions of meristomatoid cells. The divisions seem largely unaffected by CK excess and inhibited by CK deficiency (see above). Nevertheless, the nature of the proteome profiling data indicates that they are related mainly to the cell expansion.

To validate the positive impact of CK excess on sugar metabolism, main soluble sugars (glucose, Glc; fructose, Fru; sucrose, Suc) and starch were quantified in leaf 3 in the same experimental setup as for the proteome profiling. CK excess led to a significant increase in Fru (by 72%) and total soluble sugar content (i.e. the sum of Glc+Fru+Suc) compared to the wild type (Figure 8C and 8D). On the contrary, CK-deficient plants contained significantly less sugars, i.e. Glc reduced by 46%, Fru by 56 % and starch by 36%, compared to the wild type (Figure 8C and 8D).

DISCUSSION

CK Controls the Transition from Cell Division to Cell Expansion

The development of a leaf is a gradual process composed of cell division, cell growth and cell fate specification (Gonzalez *et al.*, 2012; Nelissen *et al.*, 2016). Re-examination of data sets generated in our previous transcriptome profiling of transition from cell proliferation to cell expansion of leaf 3 prompted us to investigate a role for CK dynamics in regulation of the transition (Andriankaja *et al.*, 2012). To this end, we have first analyzed CK pool and signaling output during the transition. At the cell proliferation phase, CK pool was high and dominated by *t*Z which peaked at 10 DAS concomitantly with a burst of vein and stomata formation, and dropped sharply towards the cell expansion phase. CK signaling output monitored by the TCSn:GFP sensor paralleled CK pool by being high and ubiquitous during the cell proliferation phase and decreased towards the cell expansion phase. Further, during the cell expansion phase, the TCSn:GFP output appeared restricted to stomata

and veins, and finally to stomata only. The data are consistent with our prediction based on the transcriptom profiling (Andriankaja *et al.*, 2012), and in line with previous reports which have shown that CK together with auxin is necessary to induce vascular tissues (Aloni, 1995), CK depletion results in reduction of vasculature (Werner ate al., 2003) and a number of stomata per leaf area can be increased by CK treatment (Farber et al., 2016). Next, we have generated CK excess or deficiency at the proliferation phase of leaf 3 by activating the CK biosynthetic gene *ipt* or the CK-degrading gene *HvCKX2*, respectively, at 9 DAS and examined possible changes in leaf morphology and underlying cellular processes. Both CK excess and deficiency in vitro resulted in a reduction in leaf size that, however, originated from different regulations of cellular processes. CK excess does not allow the cells to proceed beyond the cell proliferation phase and, thereby, the reduced leaf size results from a block in the transition from cell proliferation to cell expansion. In contrast, CK deficiency causes suppression of cell division and premature transition from cell proliferation to expansion as previously reported (Holst *et al.*, 2011). Consequently, the leaves are smaller because of a dramatic drop in cell number. This reduction in cell number is counteracted by an increasing cell size, however, this is clearly not sufficient to fully restore the final leaf size. Previously, CK has been found to stimulate leaf cell division and to delay the exit from the proliferation phase by an enhanced expression of mitotic *CYCD3* and *CYCD3;2* genes during G_1/S transition (Riou-Khamlichi *et al.*, 1999; Soni *et al.*, 1995; Dewitte *et al.*, 2007). A reduced leaf size was reported in Arabidopsis plants constitutively overexpressing *AtCKXs* (Werner *et al.*, 2003).

Interconnection between CK-Regulated Onset of Cell Expansion and Biogenesis of the Photosynthetic Machinery

CK is generally accepted as a plant hormone stimulating chlorophyll biosynthesis and etioplast–chloroplast transition via the activation of chloroplast-related genes and proteins (Lochmanová *et al.*, 2008; Yaronskaya *et al.*, 2006; Hedtke *et al.*, 2012; Cortleven and Valcke, 2012; Riefler *et al.*, 2006; Werner *et al.*, 2008; Cortleven *et al.*, 2016). Here, we report distinct effects of CK excess on chloroplast biogenesis and photosynthesis. When induced in the cell proliferation phase, CK excess resulted in leaves with only a few chloroplasts and dramatically impaired photosynthetic activity. In the cell expansion phase, CK excess stimulated chlorophyll biosynthesis, which is often seen as an indicator of chloroplast biogenesis (Cortleven and Schmülling, 2015). Previously, interplay has been reported between the cellular leaf developmental processes and plastid differentiation into photosynthetically active chloroplasts. Hence, onset of photosynthesis has been suggested to be tightly linked to cell expansion, which is preceded by increased expression of genes involved in the tetrapyrrole biosynthetic pathway (Andriankaja *et al.*, 2012). Thus, cells locked by CK excess in their proliferation stage remain incompetent to stimulation of chloroplast biogenesis by CK. Upon the

transition to cell proliferation, CK stimulates chloroplast biogenesis in line with previous reports (Cortleven and Schmülling, 2015; Cortleven *et al.*, 2016).

Transcriptional Regulation of CK-Mediated Transition from Cell Proliferation to Cell Expansion

To get an insight into the dynamics of the molecular network underlying the cell responses to CK excess or deficiency in proliferating cells, we performed transcriptome profiling on leaf 3 dissected from *ipt* and *HvCKX2* plants 3 h after activation at 9 DAS, when this leaf is normally fully composed of proliferating cells. CK deficiency resulted in the down-regulation of several type-A *ARR*s (including *ARR4*, *ARR7*, *ARR9* and *ARR15*), coinciding with enhanced cell expansion and regulation of cell morphogenesis (Li *et al.*, 2013). Further, a comparison with transcripts differentially expressed during the transition from cell proliferation to cell expansion reported previously (Andriankaja *et al.*, 2012) revealed that a number of genes that are highly expressed in early proliferating leaves is down-regulated in response to CK deficiency, while those genes highly expressed during the transition to cell expansion are stimulated. Therefore, the observed decrease of CK content at the end of the cell proliferation phase is crucial for the transition to cell expansion.

Cell wall remodeling and biosynthesis are crucial for cell expansion. GO enrichment analysis of our data sets revealed several CK-responsive genes involved in cell wall assembly (Hawkesford and De Kok, 2006; Takahashi *et al.*, 2011; Maruyama-Nakashita *et al.*, 2004). The growth of plant cells is in tight relation with cell wall loosening, which can be mediated by expansins. Recently, interplay between CK and expansin in root development was reported (Pacifici et al., 2018). These authors suggested that ARR1 stimulates EXPA1-driven cell wall loosening and consequent cell elongation in Arabidopsis roots. Here, CK deficiency up-regulated *expansin-A5* (*EXPA5*). *EXPA5* overexpression resulted in longer roots in Arabidopsis (Xu et al., 2014) which resembles a root phenotype in CK-deficient plants. In leaf 3, *EXPA5* up-regulation was found already three hours of *HvCKX2* activation indicating that EXPA5 might represent a key molecular player involved in cell expansion caused by CK deficiency also in leaf development. Another up-regulated gene connected with cell wall organization and altered CK levels was *PRP2* (encoding a proline-rich protein) involved in specifying cell-type-specific wall structures (Fowler *et al.*, 1999). Cell growth is also connected with very-long-chain fatty acid (VLCFA) biosynthesis involving *CUT1*, the mutation of which leads to smaller cells (Qin *et al.*, 2007). It has been shown that the VLCFA synthesis in the epidermis of Arabidopsis is required to suppress CK biosynthesis in the vasculature and thus cell overproliferation therein (Nobusawa *et al.*, 2013). We show that *CUT1* is up-regulated by CK-deficiency. Thus, VLCFA biosynthesis may provide a non-autonomous signal coordinating cell proliferation and/or expansion in epidermis and lower cell layers in response to altered CK status of epidermis.

CK is known to positively regulate cell division in growing leaves. The levels of CK oscillate during the cell cycle, showing sharp peaks during the entry into mitosis and at the end of the S-phase (Hartig and Beck, 2005; Redig *et al.*, 1996). Thus, CK is supposed to control the G2/M and G1/S transitions in the plant cell cycle (Schaller *et al.*, 2014). The cell cycle is controlled by functionally conserved proteins, mainly cyclins and cyclin-dependent kinases (CDKs) (Francis, 2007). In the shoot, CK interacts with components of cell cycle, such as CYCD3 (Dewitte *et al.*, 2007; Zhang *et al.*, 2005). However, none of the major cell cycle regulators was found differentially regulated after 3 h of *ipt* and *HvCKX2* activation. Hence, we suggest that CK does not affect the transcription of the cell cycle regulators directly, but rather controls the transcription of genes encoding growth regulators, like *KLU* and *AINTEGUMENTA* (*ANT)*. *KLU* encodes the *Arabidopsis* cytochrome P450 KLUH which is known to positively regulate organ growth by stimulating cell proliferation (Anastasiou *et al.*, 2007) and was found to be down-regulated by CK deficiency in our work. Therefore, CK-deficient plants cannot reach a cell proliferation rate sufficient to stimulate standard organ growth. *ANT* encodes the AP2-like ethylene-responsive transcription factor ANT (Elliott, 1996; Nole-Wilson and Krizek, 2000), the absence of which is connected with precocious termination of cell proliferation (Mizukami and Fischer, 2000). CKs were found to regulate the expression of ANT and CYCD3 that further independently regulate the cell division during secondary growth of Arabidopsis roots (Randall *et al.*, 2015). We found that CK deficiency leads to a reduction in *ANT* transcript levels in the early proliferating leaf 3, which could lead to the loss of the cellular ability to maintain division competence and, consequently, could induce an early exit from cell proliferation, as predicted in Werner et al., 2003. Moreover, we show that modulation of CK levels leads to decreased *bHLH2* expression in the early proliferating leaf 3. bHLH2 is one of the *ENHANCER OF GLABRA* (EGL1) transcription factors that have been shown to play an important role during epidermal cell fate specification, suggesting that modulation of bHLH2 levels is one of the ways through which CK affects cell differentiation during leaf development (Bernhardt *et al.*, 2003).

CK Excess in the Cell Expansion Phase Enhances the Cell Expansion Rate by Stimulating Primary Metabolism

As leaf development progresses, leaf proliferation is gradually replaced by expansion and differentiation as the main processes driving leaf growth to reach its final size and shape (Gonzalez *et al.*, 2012; Vanhaeren *et al.*, 2016). To investigate the effect of CK on cell expansion during leaf development, we induced CK excess or deficiency at 14 DAS when almost all pavement cells of leaf 3 are actively expanding (Clauw *et al.*, 2015). In this scenario, CK excess stimulated cell expansion, leading to plants with larger leaves composed of bigger cells. This is consistent with previous reports (Efroni *et al.*, 2008; Efroni *et al.*, 2013), showing a positive effect of CK on the final leaf size in Arabidopsis plants sprayed with benzyl adenine after the first two leaves had appeared. Importantly,

we also show that biomass increases simultaneously with the leaf size. In contrast, CK deficiency reduced both cell number and cell size, and this was associated with a reduction of the average number of endocycles undergone by a typical nucleus. Endoreduplication is a cell cycle variant of multicellular eukaryotes in which the genome is replicated in the absence of mitosis (De Veylder *et al.*, 2011). A correlation between DNA ploidy level and cell size was found in Arabidopsis epidermal cells (Melaragno *et al.*, 1993), and it has been postulated that endoreduplication can be found in many cell types, especially in those undergoing differentiation and expansion (Joubès and Chevalier, 2000). However, several recent studies argue that, since many mutants with enlarged cells did not show a significant alteration of the nuclear DNA content, endoreduplication might be only one of the components that determine the size of the plant cell (De Veylder *et al.*, 2011; Dissmeyer *et al.*, 2009; Dissmeyer *et al.*, 2007). Further, we show that CK deficiency resulted in a reduced stomatal index, representing the number of stomata as a fraction of the total number of cells (Nadeau and Sack, 2002). Stomata are the final product of meristemoid division and thus less stomata reflects lower division rates of meristemoid cells (Peterson *et al.*, 2010). However, meristemoids also produce many pavement cells before terminally differentiating, and in this way contribute to pavement cell number. Thus, CK deficiency lowers the cell expansion rate and endoreduplication levels, and interferes with meristemoid division.

Proteome profiling of leaf 3 dissected from plants with CK excess or deficiency generated during the cell expansion phase revealed primary metabolism stimulation by CK excess, mainly the carbohydrate metabolism and energy-associated processes such as photosynthesis. Therefore, we quantified the content of major soluble sugars and starch and found significant alterations in response to CK excess or deficiency. This correlated with changes in the abundance of proteins related to carbohydrate metabolism, such as fructose-bisphosphate aldolase 1 (FBA1; catalyzing synthesis of glyceraldehyde phosphate from D-fructose), which was down-regulated in CK-deficient plants, and glucose-6-phosphate isomerase 1 (PGI1; catalyzing D-fructose synthesis) and beta-glucosidase 43 (BGLU43; release of beta-D-glucose from beta-D-glucosyl), which were both up-regulated by CK excess. The decreasing level of hexoses (i.e. Glc and Fru) in CK-deficient plants may have been caused by a higher abundance of NADP-dependent malic enzyme 2 (NADP-ME2), which is involved in pentose phosphate shunt and can lead to the attenuated biosynthesis of starch in sink cells (Wheeler *et al.*, 2005). Thus, regulation of the sugar content by CK can either affect the metabolic energy source for sink tissues or act as a signal in the retrograde regulation of nuclear-encoded photosystem reaction center compartments, such as the photosystem I reaction center subunit III (PSAF), which was stimulated by CK and may serve as a feedback regulation of photoautotrophic growth (Haldrup *et al.*, 2000).

Plant cell expansion is a result of turgor pressure acting as a driving force, accompanied by a delicate balance between cell wall biosynthesis and cell wall remodeling, which allows turgor-driven cell wall extension without any disruption of cell integrity (Dupuy *et al.*, 2010; Bashline *et al.*, 2014). Our data

show that CK excess induced at the cell expansion phase stimulates cell growth by, among other, primary metabolism stimulation rather than through enhanced cell division and endoreduplication. Thus, an increased content of soluble sugars is expected to increase the turgor pressure, which is required for simultaneous biochemical loosening of the cell wall, which is followed by absorption of water and cell wall expansion and which initiates cell enlargement (Cosgrove, 2000). However, wild-type CK levels are necessary to stimulate cell division and endoreduplication, as evidenced by the lowered cell number and endoreduplication index found in plants with CK deficiency induced at both the cell proliferation and cell expansion phases.

In conclusion, our experimental set-up allowed us to obtain deep insights into distinct CK actions throughout leaf development that regulate final leaf size and structure (Figure 9). Transcriptome and proteome profiling targeted to specific developmental windows of a particular leaf resulted in the identification of a set of as yet unrecognized CK-responsive genes and proteins apart of those that have been previously reported (Nishiyama *et al.*, 2012; Rashotte *et al.*, 2003; Lochmanová *et al.*, 2008; Kiba *et al.*, 2005; Nemhauser *et al.*, 2006; Černý *et al.*, 2011; Chen *et al.*, 2010; Černý *et al.*, 2013; Brenner *et al.*, 2005; Brenner *et al.*, 2012; Nishiyama *et al.*, 2011) and that provide a valuable resource for future research. These regulations remained unrevealed in previous profiling studies, because they might have been absent in young seedlings and hidden in bulk rosette samples. The future detailed analysis of selected candidate genes is expected to significantly deepen our knowledge of CK action in plant development.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions

Seeds of *Arabidopsis thaliana* transgenic lines (*proCaMV35S*>GR>*ipt, proCaMV35S*>GR>*HvCKX2*, *proCaMV35S::GFP-TUA5*; Shaw et al., 2003; Craft et al., 2005; Samalova et al., 2005; Müller et al., 2007), TCSn:GFP (Zürcher *et al*., 2013) and corresponding wild type (Col-0) were sown on nylon meshes placed on half-strength Murashige and Skoog (MS) medium containing 0.5% (w/v) MES and 0.8% (w/v) agar. At 2 DAS, plants were moved to growing chambers with a 16-h day/8-h night light regime, 21°C and 19°C, respectively, under standard light intensity (110 µmol m^{-2} s^{-1}) for the day period.

DEX-inducible and wild-type lines were activated at 8 or 14 DAS by transfer of nylon meshes to half-strength MS medium supplemented with either 2.5 µM DEX dissolved in $5x10^{-4}$% (v/v) DMSO or $5x10^{-4}$% (v/v) DMSO (mock).

For in-soil experiments, plants were cultivated in a growth chamber under controlled environmental conditions (50-60% relative humidity, 16-h day/8-h night, 21 °C and 19 °C, respectively, 110 µmol m^{-2} s^{-1}). Plants were activated at 14 DAS by watering once with 50 ml of distilled water supplemented with either 10 µM DEX dissolved in $5x10^{-4}$% (v/v) DMSO or $5x10^{-4}$% (v/v) DMSO (mock).

Phenotype Analysis

After imaging of complete rosettes, cotyledons and leaves were dissected and placed in a row, from youngest to oldest, on plates containing 1% (w/v) agar medium. Blade area of cotyledons and leaves were calculated from leaf series made at 21 DAS from transgenic and wild-type plants. Final leaf series ($n \geq 10$ in 3 biological repeats) were photographed and leaf areas were measured using ImageJ (http://rsb.info.niv.gov/ij/).

Cellular Analysis of Leaf 3

Leaf 3 was dissected at 21 DAS and cleared in 70% ethanol and transferred to lactic acid. After 1-day incubation, the leaves were placed on microscope slides and mounted in lactic acid. The total blade area was measured under a dark-field binocular microscope. The abaxial epidermal pavement cell size and total number were calculated from 10 representative leaves in 3 biological repeats using a microscope equipped with differential interference contrast optics (DM LB with 20x objective; Leica) and drawing tube. Downstream analysis was performed as described in Andriankaja et al., 2012.

Confocal Microscopy

All samples were examined with an Axioimager Z.1 platform equipped with LSM700 module (Carl Zeiss, Germany) using 40x oil objective. The light source included an argon-neon laser with wavelength 488 nm for GFP fluorescence and 639 nm for chlorophyll auto-fluorescence to avoid an interference of the two fluorescence channels. To avoid photo-bleaching effect, leaves were scanned at the same time.

Quantitative RT-PCR

RNA extraction was performed from 8-day-old seedlings (shoot part only; n ≥ 10) harvested 3 h, 6 h, 9 h and 12 h after activation with 2.5 µM DEX and immediately frozen in liquid nitrogen. The total RNA was extracted according to a combined protocol of TRI Reagent RT (Molecular Research Center) and the RNeasy Kit (Qiagen) with on-column DNase (Qiagen) digestion (for the primer pairs, see Supplemental Table S6). All individual reactions were done in triplicate on a LightCycler 480 (Roche Diagnostics) in 384-well plates with LightCycler 480SYBR Green I Master (Roche Applied Science) or the UPL system (Roche Applied Science) and LightCycler 480 Probes Master kit (Roche Applied Science), as described in (Novák *et al.*, 2015).

RNA-Seq Analysis

For RNA-seq analysis, 9 days old seedlings were activated 2.5 µM DEX and placed in RNAlater (Ambion) 3h after activation (stored at 4 °C). The leaf 3 was dissected from i > 250 individual seedlings per variant on a cooling plate under a stereomicroscope with precision needle and removed into the liquid nitrogen. For RNA-seq 3 biological replicates of WT, *ipt* and *HvCKX2* activated by DEX were used. The RNA was extracted according the same method described for qRT-PCR. RNA sequencing was done by GATC Biotech AG (Konstanz, Germany).

The TruSeq RNA Sample Preparation Kit version 2 (Illumina) was used for library preparation. Briefly, poly(A)-containing mRNA molecules were reverse transcribed, double-stranded cDNA was generated and adapters were ligated. After quality control using 2100 Bioanalyzer (Agilent), clusters were generated through amplification using the TruSeq SE Cluster Kit v3-cBot-HS kit (Illumina) followed by sequencing on a Illumina HiSeq2000 with the TruSeq SBS Kit v3-HS (Illumina). Sequencing was performed in Single-End mode with a read length of 50 nt.

The quality of the raw data was verified with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/, version 0.9.1). Next, adapter removal and quality filtering was performed using Trimmomatic (http://www.usadellab.org/cms/?page=trimmomatic) (Bolger *et al.*, 2014): reads were filtered by sliding window trimming (window size 1) which removes leading bases with a quality below Q10, ensuring a minimum length of 38 nt remaining. Reads were subsequently mapped to the Arabidopsis reference genome (TAIR10) using GSNAP (version 2012-07-20) allowing maximally 2 mismatches (Wu and Nacu, 2010). These steps were performed through Galaxy (Goecks *et al.*, 2010). The reads that uniquely map to the genome where used for quantification on the gene level with htseq-count from the HTSeq.py python package (Anders *et al.*, 2014).

After data normalization using TMM, differential expression analysis was conducted with the edgeR package (version 3.1.0) in RStudio (Robinson *et al.*, 2010). A design matrix was created in which the

genotypes (Col-0, *HvCKX2*, and *ipt*) were specified, and in addition at least two biological repeats were performed to include a batch effect. Dispersions were estimated with the Cox-Reid profile-adjusted likelihood method, after which testing for differential expression was done with the generalized linear model likelihood ratio test.

The false discovery rate (FDR)-corrected *P*-value < 0.05 was used as a cutoff (Benjamini and Hochberg, 1995). Go enrichment analyses were conducted with BiNGO (Maere *et al.*, 2005). The details of sequencing procedure and RNA-Seq data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6109.

Photosynthetic Parameters

21-day-old seedlings activated with 2.5 µM DEX were scanned by the WALTZ PAM system after 20 min of dark adaptation. The photosynthetic yield (Fv/Fm) values were determined for each leaf 3 ($n \geq 30$ in 3 biological repeats).

Chlorophyll Extraction

For the chlorophyll extraction, leaf 3 dissected from 21-day-old plants ($n \geq 15$ in 3 biological repeats) was placed in DMSO and stored at 50°C for 2 h. Final content of chlorophyll was measured on a Beckman DU 640B spectrophotometer (chlorophyll *a* A665.1; chlorophyll *b* A649.1) and calculated by *Arnon's* equations (Arnon, 1949; Wellburn, 1994).

Ploidy Analysis

Leaf 3 from 21-day-old plants grown in soil were dissected with a razor blade and frozen in liquid nitrogen for ploidy analysis ($n = 3$ in 3 biological repeats). Leaves were placed in 200 µL of Cystain UV Precise P Nuclei Extraction buffer (Partec), followed by the addition of 800 µL of staining buffer and filtered through a 50-µm filter. Nuclei were analyzed with Cyflow MB flow cytometer (Partec) and the corresponding FloMax software. The endoreduplication index was calculated as $\%4C + 2 \times \%8C + 3 \times \%16C$.

Hormonal Analysis

For hormonal analysis of developing leaf 3, the leaf 3 was dissected from SAM each day 2h after beginning of the day under binocular microscope and immediately frozen in liquid nitrogen. Approximately 1-4 mg of fresh leaves in 3 biological repeats were freeze-dried in a microtube with a screw cap. For hormonal analysis of mature leaf, leaf 3 from 21-day-old wild-type and DEX-inducible lines grown in soil was removed with a razor blade. Approximately 20 mg of fresh leaves in 3 biological repeats were freeze-dried in a microtube with a screw cap. The cytokinin extraction and profiling essentially followed the method previously described by Svacinova et al. (2012). Briefly, the plant material in 2.0-mL micro-centrifuge tubes was mixed with following stable isotope-labelled cytokinin internal standards at a concentration of 0.5 pmol of each compound per 50 µL of Bieleski buffer: [$^{13}C_5$]*c*Z, [$^{13}C_5$]*t*Z, [$^{2}H_5$]*t*ZR, [$^{2}H_5$]*t*Z7G, [$^{2}H_5$]*t*Z9G, [$^{2}H_5$]*t*ZOG, [$^{2}H_5$]*t*ZROG, [$^{2}H_5$]*t*ZMP, [$^{2}H_3$]DHZ, [$^{2}H_3$]DHZR, [$^{2}H_3$]DHZ9G, [$^{2}H_7$]DHZOG, [$^{2}H_3$]DHZMP, [$^{2}H_6$]iP, [$^{2}H_6$]iPR, [$^{2}H_6$]iP7G, [$^{2}H_6$]iP9G and [$^{2}H_6$]iPMP. Samples were then extracted in Bieleski solvent using a MM 301 vibration mill (Retsch GmbH & Co. KG, Haan, Germany). The samples were transferred onto StageTips and purified according to the protocol described in (Svačinová *et al.*, 2012). After elution, eluates were collected and evaporated to dryness in a Speed-Vac concentrator RC1010 (Jouan, Winchester, UK). Thereafter, dry samples were dissolved in 20 ml of 10% methanol, and 10 ml of each sample was injected on to a reversed-phase column (Acquity UPLCW BEH C18, 1.7 mm, 2.1 mm 150 mm, Waters). The samples were eluted in a 24-min gradient composed of methanol (A) and 15 mM ammonium formate pH 4.0 (B) at a flow rate of 0.25 ml min 1, with a binary gradient: 0 min, 5:95 (A:B), 7.0-min isocratic elution, 5:95 (A:B), 9.0-min linear gradient, 20:80 (A:B), 7.0-min linear gradient, 50:50 (A:B) and 1 min isocratic elution, 50:50 (A:B). The effluent was passed through an ultraviolet-diode array detector and the tandem mass spectrometer Xevo TQ MS without post-column splitting. The parameter settings were set as described previously (Svačinová *et al.*, 2012).

Proteomic Analysis

A quantitative proteomic analysis was performed using a gel-free shotgun protocol based on nano-HPLC and MS/MS as described in (Baldrianová *et al.*, 2015). Briefly, two independent replicates each consisting of approximately 100 mg of homogenized leaf 3 of 21-day-old Arabidopsis plants were extracted by acetone/TCA and phenol extraction and subsequently digested in solution with immobilized trypsin beads (Promega). The resulted peptides were desalted, dried and dissolved in 0.5% (v/v) formic acid in 5% (v/v) acetonitrile, then analyzed by nanoflow C18 reverse-phase liquid chromatography using a 15-cm Ascentis Express Column (0.1-mm inner diameter; Sigma-Aldrich) and a Dionex Ultimate 3000 RSLC nano UPLC system (Thermo) directly coupled to a CaptiveSpray nanoESI source (Bruker) and an UHR maXis impact q-TOF mass spectrometer (Bruker). Only high-

confidence peptides with better than a 10-ppm precursor mass accuracy were considered to be significant if there was an absolute DEX/mock ratio ≥1.4 with a Student's *t*-test *P*-value < 0.05. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno *et al.*, 2016) partner repository with the dataset identifier PXD007678 and 10.6019/PXD007678.

Sugar Analysis

For the quantification of soluble sugars, 100 mg of homogenized leaf 3 of 21-day-old Arabidopsis plants were extracted for 30 min at 4 ºC in 1 mL of 80% ethanol (v/v). The content of D-glucose, D-fructose and sucrose was determined in the concentrated extracts on a Tecan Infinite M1000 Pro spectrophotometer (Tecan Group AG, Männedorf, Germany) using the Megazyme Sucrose/Fructose/D-Glucose Assay Kit (Megazyme International Ireland, Bray, Ireland).
In order to quantify the concentration of starch, the samples were then resuspended in 0.5 mL of 0.1 M NaOH and incubated for 30 min at 50 °C. After neutralizing the solution with 1 mL of 0.1 N acetic acid, the starch content was determined on a Tecan Infinite M1000 Pro spectrophotometer using a Sigma-Aldrich Starch (HK) Assay Kit (Sigma-Aldrich, St. Louis, Missouri, USA).

Accession Numbers

Gene sequence data from this article are provided in Supplemental Data Sets and can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the accession numbers: *AHK4*, At2g01830; *ANT*, At4g37750; *ARR15*, At1g74890; *ARR4*, At1g10470; *ARR7*, At1g19050; *ARR9*, At3g57040; *AtCKX5*, At1g75450; *BGLU43*, At3g18070; *BHLH2*, At1g63650; *COL6*, At1g68520; *COR47*, At1g20440; *CUT1*, At1g68530; *CYP78A5 (KLUH)*, At1g13710; *ERD6*, At1g08930; *ESM1*, At3g14210; *EXPA5*, At3g29030; *FAF3*, At5g19260; *FBA1*, At2g21330; *GATA17*, At3g16870; *LSH10*, At2g42610; *NADP-ME2*, At5g11670; *PGI1*, At4g24620; *PRP2*, At2g21140; *PRP4*, At4g38770; *PSAF*, At1g31330; *TGG1*, At5g26000; *TGG2*, At5g25980.

AUTHOR CONTRIBUTIONS

J.S., L.V., H.C., D.I. and B.B. designed the experiments and wrote the manuscript with input from the other authors. J.S., L.V., H.C., J.H., M.Č., O.N., I. S-F. and P.S. performed the research. J.S., L.V., H.C., S.D. and P.S. analyzed the phenotyping data and performed cellular analysis. J.S., L.V., H.C.,

J.H. and F.C. conducted the transcriptome data. J.S. and M.Č. performed the proteome analysis. O.N., L.P. and I. S-F. provided metabolic data.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest related to this research.

SHORT SUPPORTING INFORMATION LEGENDS

Figure S1 Representative rosettes of control and *ipt* or *HvCKX2* transgenic plants after *in-vitro* DEX treatment.

Figure S2 Leaf blade area calculated from leaf series.

Figure S3 Effects of DEX treatment on the expression of selected CK-responsive genes.

Figure S4 Representative rosettes of *ipt* and *HvCKX2* transgenic plants after DEX treatment in soil and fresh weight analysis.

Figure S5 Impact of altered cytokinin levels on total number of leaves, number of flowers/buds, stem length and the root system.

Figure S6 Impact of altered cytokinin levels on distribution of cell area in leaf 3 from 21-day-old plants with altered CK levels activated at 14 DAS in soil.

Table S1 List of differentially expressed genes in activated *HvCKX2* plants.

Table S2 List of differentially expressed genes in activated *ipt* plants.

Table S3 qPCR confirmation of selected transcripts identified by RNA-Seq, main enriched GO categories of the cytokinin-regulated genes and overlap between the genes significantly up- or down-regulated after an alteration of CK levels.

Table S4 List of enriched GO categories of genes differentially regulated in *HvCKX* plants after 3 h of DEX treatment using BiNGO analysis.

Table S5 List of enriched GO categories of genes differentially regulated in *ipt* plants after 3 h of DEX treatment using BiNGO analysis.

Table S6 List of primer sequences used for qPCR validation of selected transcripts identified from RNA-Seq.

Table S7 List of genes involved in CK metabolism and signaling and regulated during the development of leaf 3 (from Andriankaja et al., 2012).

Table S8 List of differentially regulated proteins in *HvCKX2* and *ipt* plants and the overlap of their relative abundances.

Table S9 List of enriched GO categories of *HvCKX2* and *ipt* plants after LC-MS/MS analysis.

Table S10 Summary of previously unpublished regulations of protein abundances and transcript levels by modulation of endogenous cytokinin levels.

Table S11 Dynamics of cytokinin pool during leaf 3 development

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Figure legends

Figure 1. Dynamics of *trans*-zeatin cytokinins and cytokinin signaling during the transition from cell proliferation to cell expansion.

A. Endogenous *trans*-zeatin forms during leaf 3 development. Error bars represent SD; means with different letters are significantly different in three biological replicates (n > 30) determined by one-way ANOVA with Tukey's post-test ($P < 0.05$; for details see Table S11). The levels of *t*ZROG were below detection limits. Abbrev.: *t*Z, *trans*-zeatin; *t*ZR, *trans*-zeatin riboside; *t*ZRMP, *trans*-zeatin riboside monophosphate; *t*ZOG, *trans*-zeatin-*O*-glucoside; *t*ZROG, *trans*-zeatin riboside-*O*-glucoside; *t*Z7G, *trans*-zeatin-7-glucoside; *t*Z9G, *trans*-zeatin-9-glucoside. **B.** The relative change in Two Component Signaling Sensor (TCSn:GFP) output (GFP; green) and chlorophyll autofluorescence (ChlA; red) during leaf 3 development. Fluorescence was quantified as a mean gray value. The plots show means (with standard deviations) obtained from at least 15 leaves per variant ($P < 0.05$; Student´s t-test). **C.** Leaf 3 of TCSn:GFP plants was scanned during the transition from cell proliferation to cell expansion in transmitted light (TL), GFP and chlorophyll *a* (ChlA) channels with final merge. Blue bar = 100 µm.

Figure 2. Experimental setup and cellular changes upon activation of *ipt* and *HvCKX2* transgenic plants.

A. Scheme of the experiment showing the temporal activation by DEX in relation to the specific stages of leaf 3 development, where the blue color represents the proliferation zone (GUS signal pattern of CYCB1;1:D-box:GUS-GFP), white color represents the expansion zone and light blue color represents the cell-cycle arrest front (Andriankaja *et al.*, 2012). The seedlings were transferred on MS plates supplemented with DEX at times indicated and cultivated in the presence of DEX till 21 DAS. **B.** Representative pictures of rosettes with altered CK levels and visualization of the shape of the epidermal cells. Bar = 10 mm. **C.** Average leaf area , **D.** cell area and **E.** number of epidermal cells of leaf 3 of 21-day-old *HvCKX2* and *ipt* plants transferred at 8 DAS to medium with DMSO or 2.5 µM

DEX. Error bars represent SE (n > 10) and asterisks indicate significant differences with the wild type ($P < 0.05$; two-sided Student's *t*-test).

Figure 3. The impact of altered CK levels on epidermal cell morphology.

A. Visualization of GFP-labeled cortical microtubules in pavement cells of 21-day-old inducible lines crossed with TUA5:GFP (green) superimposed with the relative signal of chlorophyll *a* (red) from mesophyll layer underneath. The relative change in fluorescence intensity compares DEX-activated plants at 8 DAS with the wild type ($P < 0.05$; Student's *t*-test). Bar = 20 µm. **B.** Chlorophyll fluorescence analysis of 21-day-old plants activated by 2.5 µM DEX from 8 DAS until 14 DAS. Mean maximum quantum yield of photosystem II (F_v/F_m) was measured after dark adaptation; error bars represent SE (n > 90) and asterisks indicate significant differences ($P < 0.05$; Student's *t*-test) with the wild type. Bar = 10 mm. **C.** Chlorophyll *a* and **D.** chlorophyll *b* content in response to 2.5 µM DEX treatment from microdissected leaves 3. Error bars represent SD (n > 45) and asterisks indicate significant differences with the wild type ($P < 0.05$; two-sided Student's *t*-test).

Figure 4. Gene ontology analysis of the early cytokinin response transcripts in proliferating leaf 3.

A. Representative image of 9-day-old WT plant (Bar = 1 mm) with focus on leaf 3 growing out of the apical meristem (Bar = 0.1 mm). Venn diagram showing the overlap between the genes significantly up- or down-regulated after alteration of CK levels. **B.** Enrichment analysis of biological processes of genes differentially regulated in *ipt* or **C.** *HvCKX2* plants using BiNGO. The color bars indicate level of significance from low (yellow) to high (orange). **D.** RT-qPCR verification of differential gene expression in independent RNA samples. Error bars represent SE (n > 20) and asterisks indicate significant differences with the wild type ($P < 0.05$; Student's *t*-test). See Supplemental Table S3 for gene identifiers and expression values.

Figure 5. Rosette and individual leaf area diverge after inducible changes in the endogenous CK levels.

A. Representative pictures of 21-day-old plants cultivated in-soil and activated by 10 µM DEX compared to mock treatment. Bar = 10 mm. **B.** Representative leaf series of individual treatments (two cotyledons at the left followed by true leaves; bar = 10 mm); **C.** average individual leaf area; **D.** analysis of total rosette area; and **E.** analysis of the fresh weight and **F.** dry weight of the shoot part.

Values are the mean of three biological repeats with their SE; asterisks indicate significant differences with the wild type ($P < 0.05$; two-sided Student's *t*-test).

Figure 6. Hormonal analysis of leaf 3 from 21-day-old plants activated at 14 DAS in soil.

Levels of basic isoprenoid **A.** *trans*-, **B.** *dihydro*-, **C.** *isopentenyl*- and **D.** *cis*-forms of CK measured in 1 g of extracted tissue (pmol/g FW; Mean ± SD). Abbrev: tZRMP, *trans*-zeatin riboside monophosphate; tZR, *trans*-zeatin riboside; tZ, *trans*-zeatin; tZOG, *trans*-zeatin-*O*-glucoside; tZROG, *trans*-zeatin riboside-*O*-glucoside; tZ7G, *trans*-zeatin-7-glucoside; tZ9G, *trans*-zeatin-9-glucoside; DHZRMP, dihydrozeatin riboside monophosphate; DHZR, dihydrozeatin riboside; DHZ, dihydrozeatin; DHZOG, dihydrozeatin-o-glucoside; DHZROG, dihydrozeatin riboside-*O*-glucoside; DHZ7G, dihydrozeatin-7-glucoside; DHZ9G, dihydrozeatin-9-glucoside; iPRMP, $N^6(\Delta^2$-isopentenyl) adenosine monophosphate; iPR, $N^6(\Delta^2$-isopentenyl) adenosine; iP, $N^6(\Delta^2$-isopentenyl) adenosine; iP7G, $N^6(\Delta^2$-isopentenyl) adenine-7-glucoside; iP9G, $N^6(\Delta^2$-isopentenyl) adenine-9-glucoside; *c*ZRMP, *cis*-zeatin riboside monophosphate; *c*ZR, *cis*-zeatin riboside; *c*Z, *cis*-zeatin; cZOG, *cis*-zeatin-*O*-glucoside; *c*ZROG, *cis*-zeatin riboside-*O*-glucoside; *c*Z9G, *cis*-zeatin-9-glucoside. Asterisks indicate statistically significant differences in the 10 µM DEX-treated plants (Col-0, *ipt* and *HvCKX2*) versus the controls (mock) in an ANOVA analysis (Student's *t*-test; *, **, and *** correspond to *P*-values of $0.05 > P > 0.01$, $0.01 > P > 0.001$, and $P < 0.001$, respectively).

Figure 7. Cellular analysis of leaf 3 from 21-day-old plants with altered CK levels activated at 14 DAS in soil.

A. The average pavement cell area, **B.** the total number of pavement cells per leaf and **C.** the stomatal index were compared between wild-type and transgenic plants with significant differences highlighted by asterisks ($P < 0.05$; Student's *t*-test). **D.** Ploidy analysis of leaf 3 from wild-type and inducible lines at 21 DAS presenting the percentage of nuclei. **E.** The average number of endocycles (endoreduplication index) undergone by a typical nucleus, calculated as 1*4C+2*8C+3*16C+4*32C. Error bars represent SE (n = 9) and asterisks indicate significant differences with the wild type ($P < 0.05$; two-sided Student's *t*-test).

Figure 8. Long-term activation of *ipt* and *HvCKX2* induces specific proteome changes in leaf 3 of 21-day-old plants.

A. Venn diagram showing the overlap between the proteins significantly up- or down-regulated after alteration of CK levels. **B.** Functional distribution of CK-regulated proteins for each specific genotype with a color code representing the functional classification according to Bevan et al. (1998), and with

increased (up) or decreased (down) abundances in response to altered CK levels. **C.** The content of soluble sugars D-Glucose (Glc) and D-Fructose (Fru), and **D.** sucrose (Suc), starch and total sugars in leaf 3 from 21-day-old plants activated at 14 DAS by 10 µM of DEX. The significant differences between the DMSO-treated wild type and DEX-treated plants are indicated by asterisks ($P < 0.05$; two-sided Student's *t*-test) and error bars represent SD (n > 15).

Figure 9. Model of regulation of early leaf development by CK in Arabidopsis.

Integration of transcriptome and proteome responses to *ipt* and *HvCKX2* activation at distinct phases of early leaf development reveals a molecular network through which CK shapes leaf size and structure in *Arabidopsis thaliana*.