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Cytokinins and Their Possible Role in Seed Size and Seed Mass Determination in Maize

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1. Introduction

Cytokinins (CKs) are plant hormones promoting cell division and differentiation, morphogenesis in tissue culture, leaf expansion, bud formation, delay of leaf senescence and chloroplast development (reviewed in Rijavec & Dermastia, 2010). Natural CKs are adenine derivatives and based on the side chain moiety, they can be divided into two subgroups: isoprenoid and aromatic cytokinins.

One of the richest sources of CKs in various plants species are developing seeds. In fact, zeatin, a major natural CK, was first discovered in developing seeds of maize (Letham, 1963). Not surprisingly, developing seeds of maize, rice and *Lupinus albus* have played a major role in CK research (reviewed in Emery & Atkins, 2005). A major focus of these studies has been on the possible role of CKs in sink strength of developing seeds. The role of CKs in controlling sink strength is inferred largely from (a) the well known role of CK in stimulating cell division that may lead to increased organ size of the sink tissue, and (b) the coincidence of CK accumulation with seed cell division profiles in several plant species (Dietrich et al., 1995; Arnau et al., 1999; Yang et al., 2002).

Nearly all CK metabolites identified thus far have been reported to be present in developing seeds of different plants. In maize, the zeatin type of isoprenoid cytokinins is most common (Brugière et al. 2003; Veach et al., 2003; Rijavec et al., 2009, 2011). Developmental profiles of various CKs in maize caryopsis, a single seeded fruit of plants from the grass family, have shown high levels of zeatin riboside (Brugière et al., 2008; Rijavec et al., 2009, 2011) during the early stages of endosperm development (Dietrich et al., 1995; Brugière et al., 2003; Rijavec et al., 2011). In maize caryopsis there are also many reports on biochemical and

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molecular aspects of genes and proteins involved in CK biosynthesis, conjugation, and degradation. Enzyme activity for isopentenyl transferase (IPT) that marks the first committed step in CK biosynthesis was reported in immature maize caryopsis by Blackwell and Horgan (1994). Recently, Brugière et al., (2008) described a small family of *IPT* genes from maize based on the similarity with *Arabidopsis* IPT proteins. The *Ipt* genes *ZmIpt1*, *ZmIpt2* and *ZmIpt10* are expressed in the pedicel, endosperm and embryo (Brugière et al., 2008; Šmehilová et al., 2009; Vyroubalová et al., 2009; Rijavec et al., 2011), indicating local biosynthesis of CKs in these parts of the caryopsis. Degradation and reversible/irreversible inactivation are key regulators of CK levels. Several maize CK dehydrogenase genes (*Ckx*) are expressed in the caryopsis pedicel region, endosperm and embryo (Massonneau et al., 2004; Šmehilová et al., 2009; Vyroubalová et al., 2009), and their corresponding protein products irreversibly cleave the N^6-side chain from the main purine ring (Massonneau et al., 2004). Recently, the importance of conjugation has also been described. In maize caryopsis *cis*-zeatin riboside-*O*-glucoside was shown to be a major CK metabolite in caryopsis (Veach et al., 2003), and similarly, increased levels of zeatin-*O*-glucoside have been reported in roots and leaves of maize transformants harboring the *Zog1* gene encoding a zeatin-*O*-glucosyltransferase from *Phaseolus lunatus* (Rodó et al., 2008). Although the exact role of the conjugates CK-*O*-glucosides is not clear, they are generally assumed to be the storage products. Finally, information on the molecular biology of CK-*N*-glucosides, a group of metabolically inactive CKs (Brzobohatý et al., 1994) is scarce (Hou et al., 2004; Rijavec et al., 2011). However, in maize caryopsis there is a notably high concentration of *trans*-zeatin-9-glucoside (Z-9-G) recorded in the developmental phase following intense mitotic activity (Rijavec et al., 2009, 2011).

As in other eukaryotes the plant cell cycle is governed by cyclin-dependent kinases (CDKs). While the catalytic CDK subunits are responsible for recognizing the target motif, which is present in substrate protein, the regulatory proteins — cyclins play a role in discriminating between distinct protein substrates and thus regulate different cell cycle transitions. Plants contain many cyclins. In *Arabidopsis*, for example, at least 32 cyclins in seven classes have been described (reviewed in Inzé and De Veylder 2006). In particular, cyclins from the class D were proposed to have a role as external growth factor sensor that integrates the external signals with the cell cycle machinery (Sherr and Roberts 1999; Planchais et al., 2004). In *Arabidopsis*, the expression of *CycD2* and *CycD3* during the G1 phase is controlled by the availability of sugars (Riou-Khamlichi et al., 2000). In addition, *CycD3*, but not *CycD2*, expression responds to CKs. *CycD3* is elevated in an *Arabidopsis* mutant, exhibiting high CK levels, and is rapidly induced by CK application in both cell cultures and whole plants (Riou-Khamlichi et al., 1999). On the other hand, in maize CKs also stimulated the expression of *CycD2* at the late stages of germination (Gutiérrez et al., 2005). In *Arabidopsis* developing leaves *CycD3* function contributes to the control of cell number by regulating the duration of the mitotic phase and timing of the transition to the endoreduplication stage (Dewitte et al., 2007), in which nuclear DNA content is increased by rounds of full genome duplication without intervening mitoses, giving rise to cells with higher ploidy levels (Joubès and Chevalier 2000). It has also been suggested that cellular expansion and its accompanying endoreduplication are inhibited in *Arabidopsis* plants overexpressing the *CycD3;1* gene (Dewitte et al., 2003).

In this study, the role of various CK metabolites in the control of seed mass in maize was investigated using an allelic series of *miniature 1* (*mn1*), a loss-of-function mutation in the

Mn1 gene that codes for an endosperm-specific cell wall invertase 2 (INCW2) (Miller & Chourey, 1992, Cheng et al., 1996). The *mn1* seed mutation is associated with a conspicuous loss of seed mass at maturity and small seed size, related to the decreased cell number and size in developing endosperm (Vilhar et al., 2002). Several lines of correlative evidence from various plant species are available to show that cell wall invertase plays a major role in controlling sink strength through source-to-sink unloading of sucrose (Chourey et al., 2006). As expected from the invertase-deficiency, the *mn1* mutation also exhibits much altered sugar metabolism relative to *Mn1* (LeClere et al., 2010). The *mn1* mutant accumulates higher levels of sucrose and lower levels of hexoses in the basal region of the caryopsis, while its upper regions accumulate either similar or even increased concentrations of sugars compared to the upper regions of the wild type caryopsis. Given the importance of hexose signaling, these changes may also be associated with the levels of CKs and auxin (Rolland et al., 2002). Indeed, CK metabolism in the *mn1* mutant differs from that of the wild type (Rijavec et al., 2009, 2011) and also has greatly reduced levels of the auxin indole-3-acetic acid (IAA) throughout caryopsis development (LeClere et al., 2008). Given these relationships, it was reasoned that the four lineage-related genotypes of the *mn1* allelic series are an ideal genetic system in which to analyze possible relationship among various CK metabolites, RNA level expression of a few selected genes of CK metabolism and sink strength in developing seeds. In this regard, a previously described *mn1-89* mutation in maize is of special interest. It is an EMS-induced point mutation representing a single amino acid change from the conserved proline to leucine at the position that is critical for either efficient translation or for stabilization of the protein *in planta* (Carlson et al., 2000). The *mn1-89* allele encodes normal or higher levels of *Incw2* RNA compared to the wild type, but exceedingly low levels, ~ 6% of the *Mn1*, of the INCW2 protein and enzyme activity (Cheng et al., 1996). More importantly for the studies here, genetic analyses showed that the *mn1-89* allele is semi-dominant in seed phenotype (Cheng et al., 1996) when crossed to the standard null allele, *mn1-1*, which has approximately ~1% of the wild type levels of invertase activity encoded by the non-allelic gene, *Inc1,* a paralog of the *Mn1* locus (Chourey et al., 2006). The reciprocal hybrids from such crosses yield gene-dose dependent invertase activity levels of ~4 and 2% encoded by two and one *mn1-89* alleles, respectively, present in the triploid endosperms. Thus, the invertase activity is strictly gene-dose dependent in that the number of allele copies determines the amount of enzyme activity.

It has recently been shown that both *Mn1* and *mn1* genotypes have extremely high, but similar CK levels during the very early stages of development from 6 to 8 days after pollination (DAP), which are followed by a marked and genotype-specific reduction (Rijavec et al., 2009). While the decrease of CKs in *Mn1* was associated with their deactivation by 9-glucosylation, the absolute CK concentrations as well as concentrations of the biologically active CKs remained higher in the mutant until 16 DAP. Based on the correlative results from different studies, a developmental model showing possible crosstalk among CKs, cyclins CycD2 and CycD3, and INCW as causal to increase of cell number and sink strength of the *Mn1* developing endosperm has been proposed (Fig. 1) (Rijavec et al., 2009). In the present work the proposed hypotheses that *CycD2* and *CycD3* genes are temporally differentially expressed in the mutant *mn1* caryopsis and that their transcript abundance is associated with the elevated CK level or CK activation were further explored.

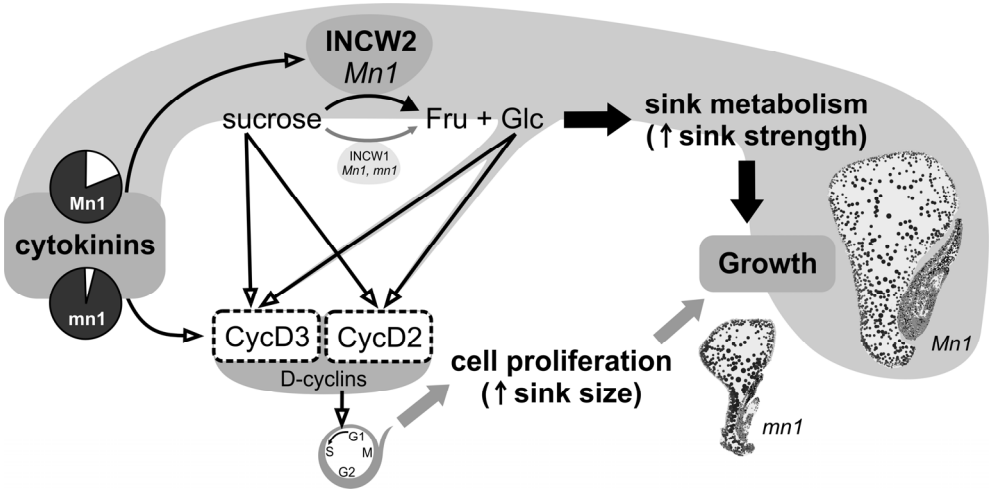

Fig. 1. Model of two alternative developmental pathways induced by cytokinins that lead to different sizes of filial tissues (endosperm and embryo) in wild-type (*Mn1*) and mutant *miniature1* (homozygous for the *mn1* allele) maize caryopsis. Cytokinin pie charts indicate the ratio of metabolically active cytokinins (dark) versus metabolically inactive *trans*-zeatin-9-glucoside (Z-9-G) (white) in both genotypes. The pathway that utilizes INCW2 for increasing cell number and sink strength is shaded gray. The images of *Mn1* and *mn1* caryopsis are bubble graph presentations of their actual longitudinal sections at 16 DAP. Steps in the model presented by dotted boxes and arrows with open heads have been proven in experimental systems other than maize. (From Rijavec et al., 2009; with the permission of the Journal of Integrative Plant Biology).

2. Caryopsis dry mass and *ZmIncw2* transcript level correlate with the gene dose of mutant alleles

A series of mutant maize caryopses (i.e. endosperm genotypes *mn1-89/mn1-89/mn1-89*, *mn1-89/mn1-89/mn1-1*, *mn1-1/mn1-1/mn1-89*, *mn1-1/mn1-1/mn1-1*) were obtained by reciprocal crossing of maize plants homozygous for *mn1-89* and *mn1-1* mutant alleles. The mass of developing caryopses rose from 6 to 28 DAP and was gene-dose dependent (Fig. 2). In mature caryopses dry mass of the wild type *Mn1* was significantly higher than that of the mutants. Seed mass of the homozygous *mn1-89* seed, which was ~80 % that of the *Mn1*, was in accordance with reported phenotypical similarity between this mutant and the wild type (Cheng et al., 1996). Additionally, the caryopsis with slightly more reduced seed mass (i.e. 64 % of the size of *Mn1*) had two copies of the *mn1-89* allele and one of the *mn-1* allele in the triploid endosperm. Seed masses of a heterozygous genotype with two copies of the *mn-1* allele and the *mn1* homozygous genotype were almost indistinguishable in the developmental period from 6 to 28 DAP (Fig. 2), confirming the similarity of caryopsis phenotypes (Cheng et al., 1996). However, the differences among the genotypes were more pronounced in mature caryopses (Fig. 2), in which the dry mass of *mn1-1/mn1-1/mn1-89* represented 41 % of the wild-type (*Mn1*) and that of *mn1-1/mn1-1/mn1-1* only 25.6 %.

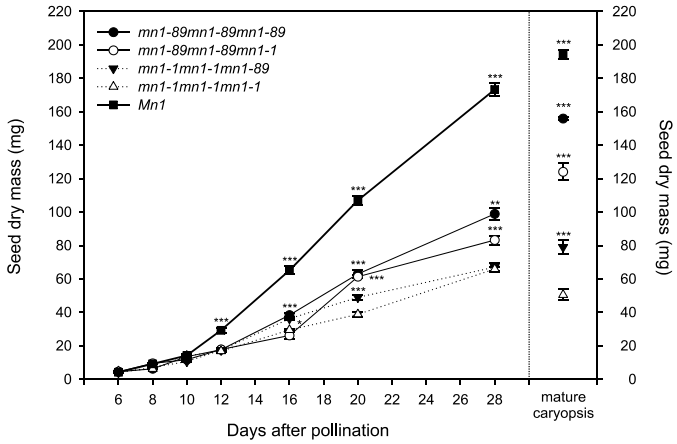

Fig. 2. Dry mass of developing caryopsis. The results are an average mass of 10 dry caryopses ±SE. Maize plants, homozygous for the *mn1-89* and *mn1-1* allele (both in the W22 genetic background) were cross-pollinated to produce four different endosperm genotypes (*mn1-89/mn1-89/mn1-89*, *mn1-89/mn1-89/mn1-1*, *mn1-1/mn1-1/mn1-89* and *mn1-1/mn1-1/mn1-1*). Student's t-test between the wild type Mn1 and each mutant yielded P-values < 0.05 (*), 0.01 (**), or <0.001 (***).

The general temporal profiles of the *ZmIncw2* transcript levels measured with quantitative real-time PCR were similar for all endosperm genotypes (Fig. 3).

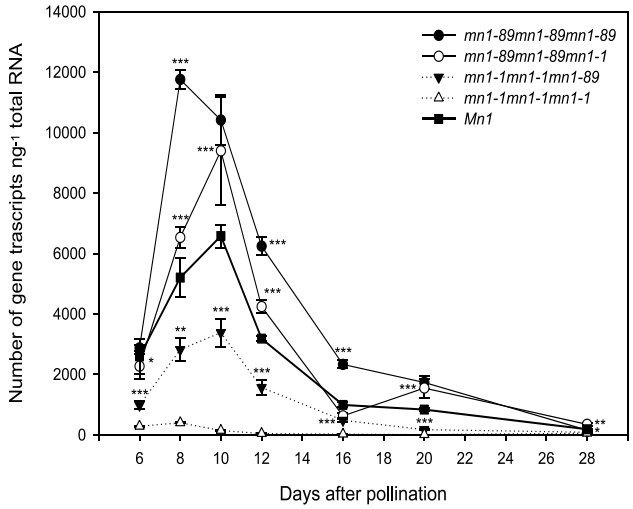

Fig. 3. Temporal expression of *ZmIncw2*. Data represents average transcript abundance ±SE for 6-9 real-time PCR reaction prepared from two individual RNA isolations and 3 subsequent reverse transcription reactions. Gene pecific primers based on the sequences with the GenBank Accesion number AF165179. Student's t-test between the wild type Mn1 and each mutant yielded p-values < 0.05 (*), 0.01 (**), or <0.001 (***).

Transcript levels were low at 6 DAP and between 16 and 28 DAP, while showing a distinct peak in transcript abundance at 8 or 10 DAP (Fig. 3). The highest abundance of the transcript preceded the peak of INCW2 activity at 12 DAP (Cheng et al., 1996). In spite of the similar profiles, *ZmIncw2* transcript levels showed a gene dose dependent trend in the *mn1-89/mn1-1* mutant series. Accordingly, genotypes *mn1-89/mn1-89/mn1-89* and *mn1-89/mn1-89/mn1-1* showed the highest transcript levels and even exceeded those of the wild type, confirming previous observations of high *Incw2* transcript levels in the *mn1-89* homozygous mutant using Northern blotting (Carlson et al., 2000). On the other hand, *Incw2* transcript levels were low in the heterozygous mutant with one *mn1-89* allele and almost undetectable in the homozygous *mn1-1* mutant genotype (Fig. 3). It is noteworthy that there is a discrepancy between protein and enzyme activity levels and *Incw2* transcript levels in *Mn1* and *mn1* endosperms (Chourey et al., 2006), since high RNA level in the homozygous *mn1-89* mutant results in only ~6 % of the *Mn1* invertase activity (Cheng et al., 1996). It has been suggested that a large proportion of INCW2 is dispensable without a significant change in seed phenotype (Cheng et al., 1996).

3. Cytokinin profiles and transcript levels of CK metabolism related genes

The total concentration of nine isoprenoid CKs (*trans*-zeatin, *tZ*; dihydrozeatin, DHZ; N^6-isopentenyl adenine, iP; *trans*-zeatin riboside, ZR; dihydrozeatin riboside, DHZR; N^6-isopentenyl adenosine, iPA; *trans*-zeatin-9-glucoside, Z-9-G; dihydrozeatin-9-glucoside, DHZ-9-G; N^6-isopentenyladenine-9-glucoside, iP-9-G) detected in maize caryopses with applied immunoaffinity chromatography followed by HPLC analyses (Rijavec et al., 2009, 2011) was similar in all examined genotypes (Fig. 4a). Depending on the mutant, a CK peak was recorded in the period between 8 and 12 DAP, followed by a drop of cytokinin content at 16 DAP and a slight increase at 20 DAP (Fig. 4a). Similar CK patterns have been reported in maize caryopses before (Dietrrich et al., 1995; Brugière et al., 2008; Rijavec et al., 2009, 2011). Specific CK concentrations at peak stages were 40.87 ± 5.35, 81.7 ±33.3 and 90,68 ±23.21 pmol CKs per caryopsis from *mn1-89/mn1-89/mn1-1*, *Mn1* and *mn1-1/mn1-1/mn1-89*, respectively at 8 DAP; 93,54 ± 13.91 pmol per caryopsis from *mn1-1/mn1-1/mn1-1* caryopsis at 10 DAP; and 88.62 ±17.99 of total CKs per caryopsis from *mn1-89/mn1-89/mn1-1* at 12 DAP (Fig. 4a). The peak of CK concentration before 12 DAP has been previously suggested to be associated with the induction of programmed cell death in the placento-chalazal region of the caryopsis pedicel (Rijavec et al., 2011), as its essential developmental phase (Kladnik et al., 2004). The proper activity of the placento-chalazal region is crucial for the transport of photosynthates from the maternal to the filial tissues of the caryopsis. An interesting observation in the present research was the lowest detected amount of total CKs at 10 DAP in *mn1-89/mn1-89/mn1-1,* in which the total content never exceeded 41 pmol per caryopsis and differed substantially from the CK concentrations in *mn1-89/mn1-89/mn1-89* (Fig. 4a). However, the overall appearance of caryopses remained similar. It has been shown before that the absolute concentration of CKs in plants is not the crucial factor controlling their development, but that local changes in cytokinin level can have global consequences for the plant (Dermastia & Ravnikar, 1996; Dermastia et al., 1996). Indeed, specific distribution of CK metabolites in various caryopsis tissues has been demonstrated (Rijavec et al., 2009, 2011).

The observed CK distribution was associated with a temporal expression of several CK metabolic genes (Fig. 5). The transcript abundance of isopentenyl transferase 2 (*ZmIpt2*), cytokinin dehydrogenase 1 (*ZmCkx1*), *cis*-zeatin-*O*-glucosyl transferase (*ZmCzog*), histidine kinase 1 (*ZmHK1*) and a putative *N*-glucosyl transferase (*ZmCngt*; Rijavec et al., 2011) were

determined in the period from 6 DAP to 28 DAP using quantitative real-time PCR. The temporal expression profiles of genes *ZmIpt2*, *ZmCkx*, *ZmCzog* and *ZmcHK1* roughly clustered into two groups related to the genotype; the first group consisted of *Mn1*, *mn1-89/mn1-89/mn1-89* and *mn1-89/mn1-89/mn1-1*, and the second group of *mn1-1/mn1-1/mn1-89* and *mn1-1/mn1-1/mn1-1* (Fig. 5).

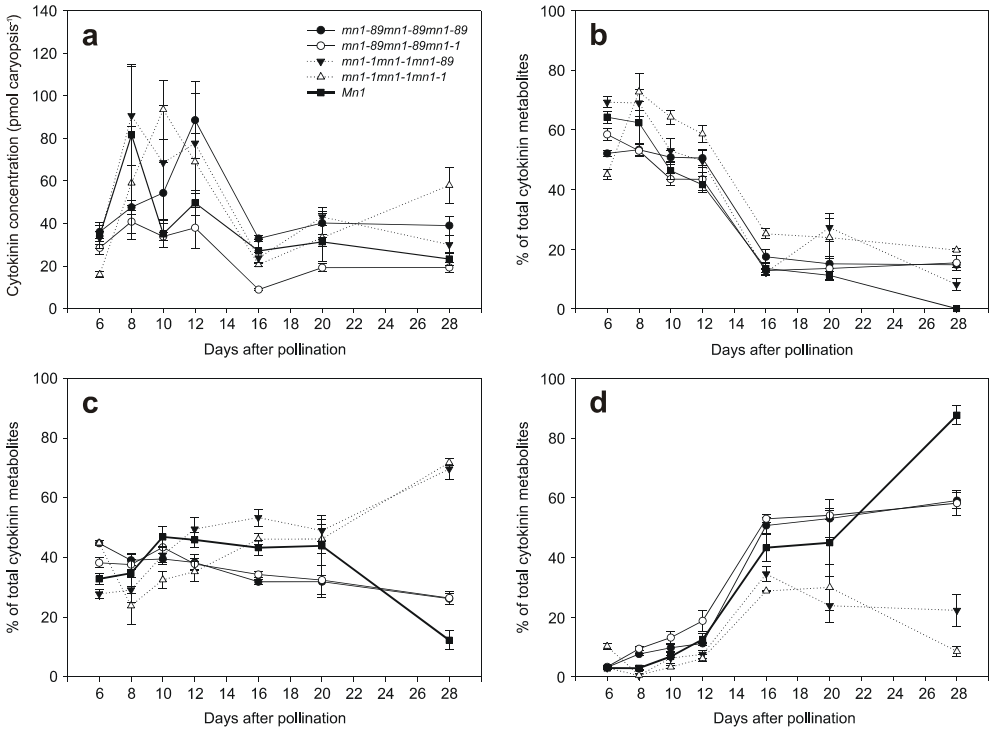

Fig. 4. Temporal profiles of cytokinins in caryopses with genotypes *Mn1*, *mn1-89/mn1-89/mn1-89, mn1-89/mn1-89/mn1-1, mn1-1/mn1-1/mn1-89* and *mn1-1/mn1-1/mn1-1* between 6 and 28 days after pollination. Data represent the average relative amount of cytokinin metabolites ± SE for 3 separate quantifications. a) Total CK concentration per caryopsis of CKs *tZ*, ZR, Z-9-G, DHZ, DHZR, DHZ-9-G, iP, iPA, and iP-9-G; b) percent of CK free bases *tZ*, DHZ and iP from total detected CKs; c) percent of CK ribosides ZR, DHZR and iPA from total detected CKs; d) percent of CK 9-glucosides Z-9-G, DHZ-9-G, iP-9-G from total detected CKs.

The *ZmIpt2* transcript profile of the first group of endosperm genotypes was low at 6 DAP, peaked between 8 DAP and 12 DAP and dropped after 12 DAP. The transcript abundance was similar in the two mutant genotypes and was significantly higher than in *Mn1*. In the second group the transcript profile showed level amount of the *ZmIpt2* transcript between 6 DAP and 12 DAP and, again, steep decrease after 12 DAP (Fig. 5a). The broader expression peak of the CK biosynthetic gene *ZmIpt2* in the wild type, in the homozygous *mn-1* mutant and in *mn1-1/mn1-1/mn1-89* (Fig. 5a) preceded the elevated CK concentration through several DAP (Fig. 4a). On the contrary, a narrow increase and subsequent drop in *ZmIpt2* transcript in *mn1-89/mn1-89/mn1-89* was associated with a

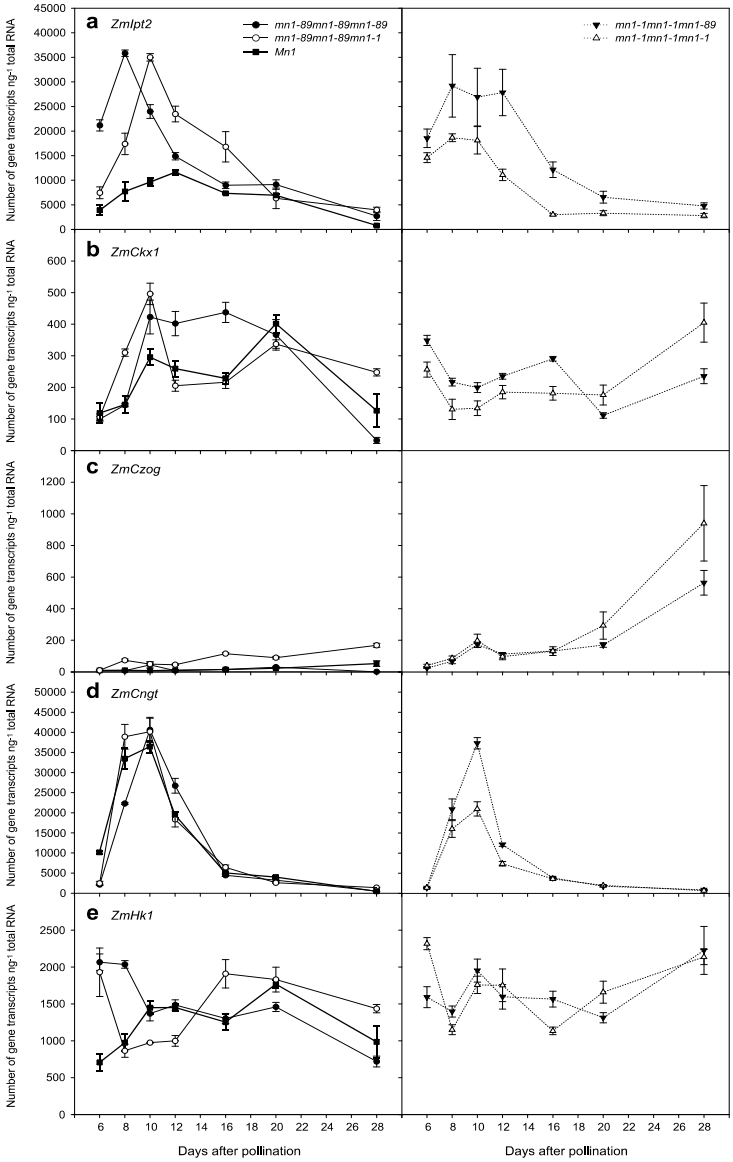

Fig. 5. Temporal expression profiles of cytokinin metabolism related genes. Gene expression of (a) *ZmIpt2*, (b) *ZmCkx1*, (c) *ZmCzog* d) *ZmCngt*, and (e) *ZmHk1*, genes was examined in developing caryopsis 6 to 28 DAP in four *miniature1* endosperm mutants with different relative numbers of *mn1-89* and *mn1-1* mutant alleles. Data represents average transcript abundance ±SE for 6-9 real-time quantification reaction prepared from two individual RNA isolations and three subsequent reverse transcription reactions. Gene-specific primers were based on the sequences with the following GenBank Accesion numbers: *ZmCkx1*, Y18377; *ZmIpt2*, DV527975; *ZmCngt*, BT016809; *ZmHk1*, AB042270; *ZmCzog*, AF318075.

narrow peak of CK concentration at 12 DAP. However, *ZmIpt2* transcript abundance was not related to the quantity of detected CKs. Interestingly, in a heterozygote with two alleles of *mn1-89* no clear increase in CKs was observed (Fig. 4a), but a high and sharp increase of *ZmIpt2* transcript was detected at 10 DAP (Fig. 5a).

The transcript abundance of *ZmCkx1* in the first group of genotypes peaked at 10 DAP. In *mn1-89/mn1-89/mn1-89* stayed approximately at the same level until 20 DAP and then decreased (Fig. 5b). However, in *Mn1* and *mn1-89/mn1-89/mn1-1* the transcript level decreased after 10 DAP, increased again between16 DAP and 20 DAP and declined after that. The absence of a clear CK concentration peak in *mn1-89/mn1-89/mn1-1* (Fig. 4a) might be explained because the highest detected level of the *ZmCkx* transcript peak corresponds with the expression peak of *ZmIpt2* (Fig. 5a), suggesting enhanced regulation of CKs during this time period.. In the group of *mn1-1/mn1-1/mn1-89* and *mn1-1/mn1-1/mn1-1* the transcript abundance was similarly high at 6 DAP, decreased in the period between 6 DAP and 8 DAP, slightly increased after 10 DAP, decrease again between 16 DAP and 20 DAP and at 28 DAP reached the level of 6 DAP (Fig. 5b).

The expression profile of the *ZmCzog* gene was low between 6 DAP and 16 DAP. It remained low until 28 DAP in the first group of genotypes, but it steeply increased after 20 DAP in *mn1-1/mn1-1/mn1-89* and *mn1-1/mn1-1/mn1-1* (Fig. 5c).

Regardless of the genotype, the profile of the transcript *ZmCngt*, encoding the corresponding enzyme *N*-glucosyl transferase, was very low at 6 DAP, followed by a clear expression peak at 8 DAP and rapid drop afterward (Fig. 5d). However, its abundance was similar in all mutants except the homozygous *mn1* mutant, where it was two-fold lower (Fig. 5d). Previously reported high concentration of the putative product of the *N*-glucosyl transferase reaction, Z-9-G, in the developmental phase following intense mitotic activity in maize endosperm (Rijavec et al., 2009, 2011) was confirmed in mutant hybrids. The concentration of this metabolically inactive CK-*N*-glucoside (Brzobohatý et al., 1994) in mutants with the *mn1-89/mn1-89/mn1-89* genotype was close to the concentration previously reported for the wild type (Fig. 4d) (Rijavec et al., 2011). On the other hand, its concentrations were lower in the other examined mutant hybrids, especially at 28 DAP (Fig. 4d), but the peaks corresponded to the gene expression profile of *ZmCngt*.

In addition, the expression of the *ZmHk1* gene was evaluated in caryopses with different genotypes (Fig. 5e). Histidine kinases (HK) are transmembrane receptors that bind cytokinin on the outer side of the cell's membrane and transducer the signal across the membrane to the inner side (reviewed in Rijavec & Dermastia, 2010). When cytokinin is not bound to the receptor, the latter is not active, suppressing downstream CK signaling. The oscillating expression patterns of *ZmHk1* were similar for all genotypes until 20 DAP, but by 28 DAP a distribution into two groups was evident again (Fig. 5e). Whether this higher *ZmHk1* expression was related to more active CK forms in *mn1-1/mn1-1/mn1-89* and *mn1-1/mn1-1/mn1*-1 at this time point is currently not known. However, it has been suggested that the cellular cytokinin level modulates signaling and that mutual control mechanisms exists between metabolism and signaling, which may contribute to fine-tuning of the cytokinin response (Riefler et al., 2006). Study of cytokinin receptor mutants has also led to the conclusion that the seed size is a direct consequence of loss of receptor functions and their role in growth control (Riefler et al., 2006).

Although absolute CK concentrations (Fig. 4a) did not cluster into two groups as did expression levels of CK metabolic genes (Fig. 5), specific groups of CKs, specifically ribosides and 9-glucosides, clearly followed this pattern (Fig. 4c, d). Specifically, in *Mn1* and the hybrids with two or three *mn1-89* alleles the share of CK ribosides continuously decreased after 10 DAP, while in the hybrids with one *mn1-89* allele or in the homozygous hybrid with three alleles of *mn1* their share increased and was 2.7-fold higher at 28 DAP. The share of 9-glucosides, which in maize caryopses were represented solely by Z9G, did not exceed 17% from 6 DAP to 12 DAP. However, in *Mn1*, *mn1-89/mn1-89/mn1-89 and mn1-89/mn1-89/mn1-1* it continuously rose and represented at 28 DAP about 57% in the hybrids and even 88% in the wild type (Fig. 4d). On the other hand, the share of Z-9-G in *mn1-1/mn1-1/mn1-89* and *mn1-1/mn1-1/mn1-1* increased to 34 % and 39 %, respectively, at 16 DAP but subsequently decreased, and in the homozygous genotype represented only 8 % of total cytokinin metabolites at 28 DAP (Fig. 4d).

4. Differential expression of type D2 and type D3 cyclin genes in the *Mn1* and *mn1* caryopsis supports the alternative development of *mn1*

The degree of change in transcript abundance of putative maize cycling genes *ZmCycD2*, *ZmCycD3-1* and *ZmCycD3-2* recovered by homology searches against all translated maize sequences (TBLASTN) with known protein sequences from *Arabidopsis* and rice was quantified by a real-time PCR in *Mn1* and *mn1* caryopsis samples at developing stages from 0 to 32 DAP. The *ZmCycD2* expression profile was similar in both, wild type and mutant caryopsis. The gene was highly expressed at pollination, down-regulated in the period between 0 and 12 DAP, up-regulated from 12 to 20 DAP, and in the wild type the transcript abundance afterward decreased. However, the the *ZmCycD2* transcript in *mn1* mutant caryopses was slightly more abundant from 0 to 8 DAP than in *Mn1*; the gene was more heavily down-regulated at 12 DAP and was up-regulated in the late stages of caryopsis development (Fig. 6a).

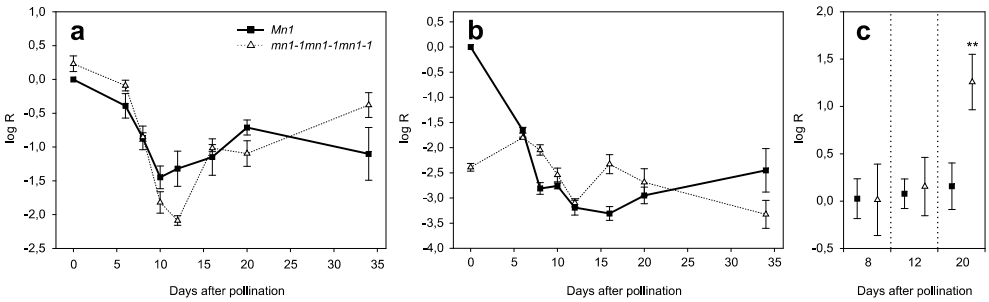

Fig. 6. The expression profiles of a) *ZmCycD2*, b) *ZmCycD3-1* and c) *ZmCycD3-2* genes in caryopsis from 0 DAP to 28 DAP. Three independent RT preparations from three separate RNA isolations were used for transcript quantification each in three replicate real-time PCR reactions. Final data represented the mean ±SE of nine quantification reactions; ** $P < 0.01$. The relative quantification of gene transcripts was done after normalization of all samples to a geometric average of the expression levels of cytochrome oxidase (COX), elongation factor 1α (EF-1α) and cyclophilin (CyP) genes. Gene specific primers based on the sequences with the following GenBank Accesion numbers: *ZmCycD2* (NM_001111578), *ZmCycD3-1* (NM_001158642) and *ZmCycD3-2* (NM_001196691).

The expression level of *ZmCycD3-1*was 2- to 4- fold lower in the mutant caryopsis compared to the wild type at 0 DAP. It increased during 0 - 5 DAP and was higher in comparison to the wild type until 20 DAP, with an additional peak of expression at 16 DAP. However, at 34 DAP the expression of *ZmCycD3-1*in the mature caryopsis was lower than that of the wild type (Fig. 6b).

The expression profile of the gene *CycD3-2* was similar to that of *CycD3-1*, but more differentially expressed (data not shown). Fig. 6c shows its relative expression analysed in detail at three time points. It is evident that its expression at 20 DAP was significantly (2.6-fold) higher in the *mn1* caryopsis in comparison with *Mn1*.

The expression of *CycD2* and *CycD3* has been previously characterized in *Arabidopsis*. It has been shown to be induced by sucrose and glucose, but the increase in *CycD2* levels is more responsive to glucose (Riou-Khamlichi et al., 2000). It has been recently demonstrated in maize caryopsis that in the period between 8 and 12 DAP sucrose levels remain constant in the basal part and only slightly decrease in the upper part of the wild type caryopsis, but increase in both parts of the mutant maize caryopsis (LeClere et al., 2010). On the other hand, in the same time period glucose decrease in both caryopses (LeClere et al., 2010). In rough accordance with those results was a down-regulation of *ZmCycD2* between 8 and 12 DAP (Fig. 6a). The subsequent up-regulation of *ZmCycD2* between 12 and 16 DAP is consistent with the constant concentration of sucrose in the whole *Mn1* caryopsis, the slight decrease of glucose in its upper part and strong increase of glucose in its basal part. Similar patterns of sugars and *ZmCycD2* expression were observed in the *mn1* caryopsis. The differences in the expression profiles of the *ZmCycD3* genes in the *Mn1* and *mn1* caryopsis in comparison with *ZmCycD2* might be at least partially explained by the levels of cytokinins present, immunolocalized as described (Rijavec et al., 2011) (Fig. 7). The immunosignal was similarly high in the pedicel regions, slightly stronger in the mutant embryo but very abundant throughout *mn1* endosperm development. While the signal was relatively strong in the aleurone layer of both caryopses, it was very faint in the upper part of the *Mn1* endosperm. On the other hand it was very strong in the upper endosperm of *mn1*. It has been shown, that *CycD3* expression is induced by cytokinins when sucrose remains present (Riou-Khamlichi et al., 1999).

Another interesting observation from this study is the increased concentration of the *ZmCycD3-2* transcript, which was shown to be significantly higher at 20 DAP (Fig. 6c) in the *mn1* caryopsis in comparison with the wild type. The peak of *ZmCycD3-1* in *mn1* at 16 DAP (Fig.6b) corresponds to the peak of endosperm endoreduplication. In the *mn1* mutant at the endoreduplication developmental stage endosperm comprise 55 % fewer cells with reduced cell size (Vilhar et al., 2002). Despite this, the progress of endoreduplication was not affected in the *mn1* endosperm in comparison with the wild type. In the model of caryopsis growth (Fig. 1) (Rijavec et al., 2009) it has been proposed that growth of *mn1* caryopsis that is highly reduced in size due to the absence of the invertase *Incw2* gene may be partially compensated by *CycD2* and *CycD3* induced cell division regulated by sugars and cytokinins. The results of this and other studies support the model. Specifically, in transgenic tobacco lines expressing *Arath-CycD3* gene the ploidy levels of mature stem tissues were not affected, suggesting no effect on the extent of endoreduplication. Moreover, the *Arath-CycD3* overexpressing lines have an increased cell number together with a reduced cell size (Boucheron et al., 2005). Similar effects were also observed in *Arabidopsis* that expressed very high levels of *CycD3;1* (Dewitte et al., 2003). However, in *Arabidopsis* endoreduplication was also inhibited in plants overexpressing *CycD3;1*.

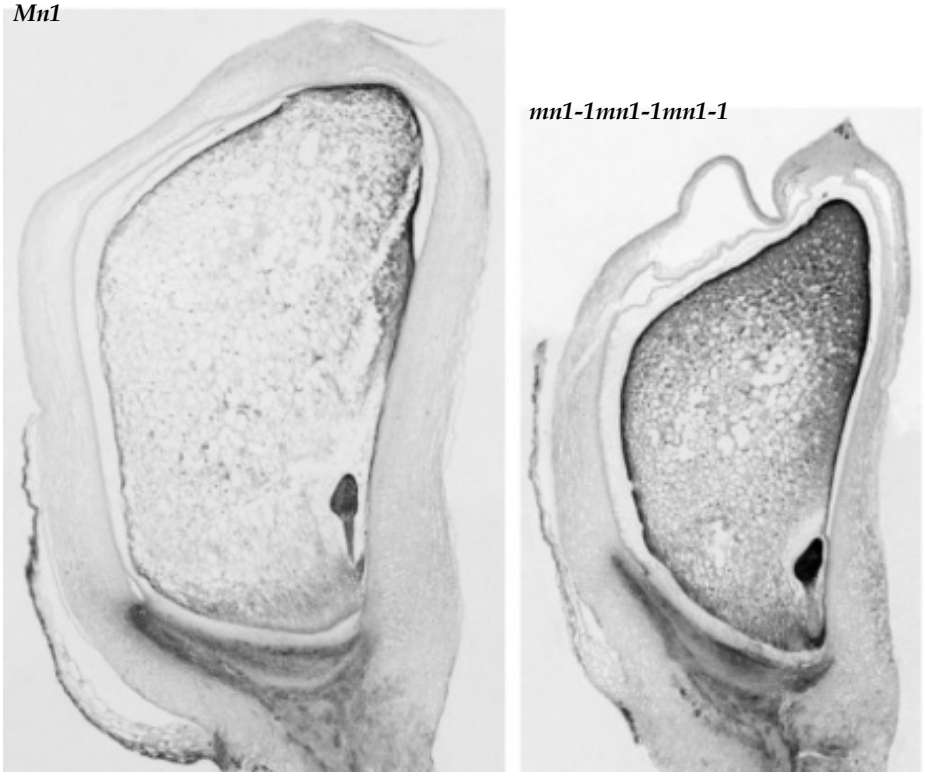

Fig. 7. Immunolocalization of cytokinins on the central longitudinal section of wild type *Mn1* and *mn1-1/ mn1-1/ mn1-1* caryopsis at 12 DAP.

5. Conclusions

The results here confirm the previously demonstrated linear gene-dose relationship of the *Incw2* transcripts with the number of *mn1-89* copies. Additionally we show that the seed mass was markedly different between the homozygous *Mn1* and the *mn1-89* series beginning at 12 to 16 DAP. Quantitative measurements of nine CK metabolites showed that the genotypes with reduced levels of invertase activity have reduced concentrations of the metabolically inactive CK form zeatin-9-glucoside and increased concentrations of active CK ribosides. Quantitative real-time PCR analyses for the CK genes showed that the *cytokinin-N-glucosyl transferase* gene (*ZmCngt1*) was expressed at the highest level at 10 DAP and was greatly reduced in expression in the *mn1* mutant. Additionally, this study supports our model (Rijavec et al., 2009) which proposes that high amounts of CKs and a higher ratio of their active forms may partially compensate the INCW2-deficiency in regulating CycD3 and CycD2 induced cell divisions by CKs and sucrose in the *mn1* endosperm. Consistent with the model, the sink strength is greater in caryopses with the *Mn1* genotype. In contrast, the levels of glucose, fructose and sucrose were higher in the upper part of the mutant caryopsis, due to as yet unknown mechanisms, and may contribute to the increased expression of the CycD3 gene and to normal, although substantially slower, development of *mn1* caryopses.

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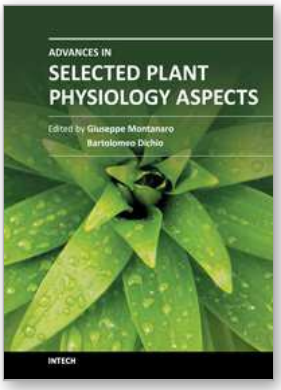

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