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REVIEW PAPER

The role of *cis*-zeatin-type cytokinins in plant growth regulation and mediating responses to environmental interactions

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Abstract

Cytokinins (CKs) are well-established as important phytohormonal regulators of plant growth and development. An increasing number of studies have also revealed the function of these hormones in plant responses to biotic and abiotic stresses. While the function of certain CK classes, including *trans*-zeatin and isopentenyladenine-type CKs, have been studied in detail, the role of *cis*-zeatin-type CKs (cZs) in plant development and in mediating environmental interactions is less well defined. Here we provide a comprehensive summary of the current knowledge about abundance, metabolism and activities of cZs in plants. We outline the history of their analysis and the metabolic routes comprising cZ biosynthesis and degradation. Further we provide an overview of changes in the pools of cZs during plant development and environmental interactions. We summarize studies that investigate the role of cZs in regulating plant development and defence responses to pathogen and herbivore attack and highlight their potential role as 'novel' stress-response markers. Since the functional roles of cZs remain largely based on correlative data and genetic manipulations of their biosynthesis, inactivation and degradation are few, we suggest experimental approaches using transgenic plants altered in cZ levels to further uncover their roles in plant growth and environmental interactions and their potential for crop improvement.

Key words: Abiotic stress, *cis*-zeatin, c-io^{6}A37-tRNA, herbivory, pathogen, plant growth, prenylated tRNA.

A brief history of the analysis of *cis*-zeatin derivatives

cis-Zeatin-type cytokinins (cZs) are a group of cytokinins (CKs) that have largely been ignored when compared to *trans*-zeatin (tZ) isomers or other highly active CKs. The lack of interest in cZs is based mainly on their lower activity in the classical CK bioassays. However, the research on cZs has also been restricted by the availability of appropriate methods to analyse their levels in plant tissues. From an analytical perspective, tZ and cZ-derivatives are mainly distinguishable by their chromatographic behaviour. Therefore, the isolation and identification of cZs was tightly linked to the development of new methods in analytical chemistry that have been able to separate the different Z isomers. Although difficult to

reconstruct, it is likely that previous analyses based on low-resolution chromatographic methods likely reported the combination of both isomers in their reported zeatin (Z) levels, whereas some analyses might have also neglected them.

More than half a century ago, the main procedure to determine CKs was based on bioassays (reviewed in Letham, 1978). Tissue extracts were embedded in auxin-containing solid medium on which plant tissues were allowed to grow. The mass increase of the plant material in comparison to treatments with known substances (often kinetin) revealed cell division and therefore CK activity (Gyulai and Heszky, 1994). *Zea mays* caryopses, for example, were shown to contain high cell division activity and therefore one of the first CKs purified from these tissues was called Zeatin (Letham, 1963). Mass spectrometry (MS) and nuclear magnetic resonance techniques allowed much better structural resolution and contributed to the identification of *cis*-zeatin riboside (cZR) in RNA extractions of plant tissues (Hall *et al.*, 1967). In 1971, the chemical synthesis of cZ allowed comparisons between biological activities of cZ and tZ (Leonard *et al.*, 1971). Soon thereafter, cZ was identified as the first bacterial Z isomer purified from cultures of the plant pathogen *Rhodococcus fascians,* (former *Corynebacterium fascians*) (Scarbrough *et al.*, 1973). Trimethylsilyl derivatives of CKs for gas chromatography (GC)-MS improved the separation and quantitation of cZ from various tissues, such as wheat (*Triticum aestivum*) caryopses (Armstrong and Skoog, 1975) and hop (*Humulus lupulus*) cones (Watanabe *et al.*, 1978). With the establishment of selected ion monitoring (SIM) in MS, it was possible to more accurately quantify different CKs from plant extracts, including cZR (e.g. Dauphin *et al.*, 1979; Hashizume *et al.*, 1979). Tay *et al.* (1986) noted that measurements of cZR could have resulted from enzymatic breakdown of tRNA during extraction. Using a modified extraction method, the authors concluded that cZR does not occur as free CK in tobacco shoots. Although it is now well established that cZR occurs as free CK in tobacco and many other plant species, this publication motivated various groups to re-evaluate their extraction procedures. As a result, extraction buffer systems, which minimize enzymatic reactions (Bieleski, 1964) were widely applied and are still used today.

In the late 1980s a significant improvement for the quantification of cZs and other CKs was achieved by using deuterium-labelled internal standards. These internal standards demonstrated high concentrations of cZ, cZR, *cis*-zeatin-*O*-glucoside (cZOG) and *cis*-zeatin riboside monophosphate (cZRMP) in rice tissues (Takagi *et al.*, 1989). The accuracy of CK analysis was further enhanced by the development of different derivatization strategies (e.g. Hocart *et al.*, 1986; Letham *et al.*, 1991). These methods also helped to characterize the first cZ-specific *O*-glucosyltransferase (cZOGT, Martin *et al.*, 2001) and contributed to the identification of cZ derivatives as the most abundant CKs in chickpea (*Cicer arietinum*) tissues (Emery *et al.*, 1998), or in specific organs, such as male flower buds of *Mercurialis* (Durand and Durand, 1994).

A major breakthrough in the analysis of CKs, including cZ derivatives, was the establishment of highly selective and sensitive tandem MS techniques. While the chromatographic conditions in some reports did not distinguish between *cis* and *trans*- Zs (Prinsen *et al.*, 1995; Van Meulebroek *et al.*, 2012), this was achieved by others (e.g. van Rhijn *et al.*, 2001; Dobrev *et al.*, 2002; Mader *et al.*, 2003; Novak *et al.*, 2003). Simplifications of the CK extractions (Dobrev and Kaminek, 2002) and the development of a high-throughput analysis (Kojima *et al.*, 2009; Schäfer *et al.*, 2014a, b) have added new impetus to the field and increased the number of publications that report the levels of cZ derivatives in plant and microbial sources.

Immunoassays were also developed to detect CKs, including cZs (Weiler, 1980). However, the practicability of these assays was often compromised by the relative cross reactivities of the antibodies to different CKs, which required chromatographic separation of extracts prior to the analysis (Wagner and Beck, 1993). Immunoaffinity co-purification of CKs provides a useful step in various CK extraction protocols. Such methods were coupled with other chromatographic methods to analyse cZ derivatives from various sources, including potato tuber sprouts (Nicander *et al.*, 1993, 1995). The first monoclonal antibodies directed against a *cis*-derivative of a plant CK were developed by Banowetz (1993). Immunopurification methods coupled with LC-MS analysis of CKs are still commonly used today (Novak *et al.*, 2003) and contribute to the increasing number of publications regarding the levels of cZ and its derivatives as well as other CKs in tissues from various sources, including pea (*Pisum sativum*) roots (Stirk *et al.*, 2008); algae (*Chlorella minutissima*, Stirk *et al.*, 2011); moss (*Physcomitrella patens*) (von Schwartzenberg *et al.*, 2007; Lindner *et al.*, 2014) and the plant pathogen *R. fascians* (Pertry *et al.*, 2009).

Clearly the recent development of sensitive, rapid, high-throughput analytical methods has largely been responsible for our current understanding on the distribution and function of cZ-type CKs in plants. In this review, we provide an overview of the recent findings on the distribution, metabolism and activities of cZs in plants and interacting organisms.

Distribution of cZ type CKs

cZ-type CKs have not only been detected in plant species throughout the plant kingdom, but also in interacting organisms, such as bacteria (Scarbrough *et al.*, 1973) and fungi (Strzelczyk *et al.*, 1989). Gajdošová *et al.* (2011) analysed the abundance of the *cis* and *trans* forms of Z and its derivatives in shoots and leaves of more than 150 representative species of different plant groups (angiosperms, bryophytes, eudicots, ferns, gymnosperms, lycophytes, magnoliids and monocots). Some plants such as *Cryptomeria japonica* (gymnosperm, Cupressaceae) and *Quercus robur* (angiosperm, Fagaceae) contain similar amounts of *cis*- and *trans*- Zs, but also plants with strong preferences for one of the isomers were found. In *Ginkgo biloba* (gymnosperm, Ginkgoaceae) and *Oenothera biennis* (angiosperm, Onagraceae) for example, >90% of the Z occurs as *trans*-isomer, whereas in *Pinus sylvestris* (gymnosperm, Pinaceae) and *Urtica dioica* (angiosperm, Urticaceae) the *cis*-isomer dominated the CK spectrum. In maize and oat cZ-type CKs even strongly exceed the combined amounts of

isopentenyladenine (IP)-, tZ- and dihydrozeatin (DHZ)-type CKs. Interestingly, these patterns are not associated with the evolutionary history of the plants. At present it is not clear which properties are related to the abundance of the *cis*-isomer in particular plant species. It might be possible that specific environmental conditions (e.g. water and temperature regime, nutrient availability), biotic interactions (e.g. pathogen/herbivore pressure, plant-plant competition, mycorrhiza formation, interaction with nodulating bacteria) or lifestyle (e.g. slow/fast growing, annual/perennial) of the plants are associated with the abundance of cZs. It is also possible that the high levels of *cis*-isomers in many crops (sweet potato, Hashizume *et al.*, 1982; rice, Takagi *et al.*, 1985; potato, Nicander *et al.*, 1995; chickpea, Emery *et al.*, 1998; maize, Veach *et al.*, 2003; pea, Quesnelle and Emery, 2007) may be a result of the plant breeding process itself.

cZ metabolism

The biosynthesis and metabolism of CKs was described in detail elsewhere (e.g. Sakakibara, 2006; Frebort *et al.*, 2011). Here we will focus mainly on the specific requirements for the metabolism of cZ-type CKs (Fig. 1). The rate-limiting step for CK biosynthesis is the prenylation of adenine nucleotides by isopentenyltransferases (IPTs). In plants, there are two possible isoprene sources: the cytosolic mevalonate (MVA) and the plastidic methylerythritol phosphate (MEP) pathway. Additionally, there are two alternative adenine substrates: ATP/ADP and tRNA; and the IPTs are accordingly classified as adenylate-IPTs and tRNA-IPTs. Evidence from *Arabidopsis thaliana* suggests a preference of IPTs to specific nucleotide substrates in combination with one specific isoprene source (Kasahara *et al.*, 2004; Miyawaki *et al.*, 2006). cZ-type CKs are synthesized via tRNA-IPTs (tRNA delta2 isopentenylpyrophosphate transferases; also known as IPPTs; EC 2.5.1.75, Kasahara *et al.*, 2004). These enzymes can be found in almost all organisms except Archaea, while adenylate-IPTs have only been found in higher plants (Frebort *et al.*, 2011). Prenylation by IPTs is not a random modification observed on all tRNAs. It targets a specific base, adenine 37, on the anti-loop of tRNAs of codons starting with uracil (i^6A37; Persson *et al.*, 1994; Taller, 1994). Studies in other organisms, including yeast, bacteria and mammalian cell culture, have shown that prenylation of tRNA is important for translation, avoiding frameshifts and nonsense suppression of UAA (Laten *et al.*, 1978; Waas *et al.*, 2007; Guy *et al.*, 2012). Interestingly, the role of tRNA modifications is often reported to be particularly apparent under specific conditions, such as stress or during interactions with other organisms (El Yacoubi *et al.*, 2012). However, it is not clear whether the functions of prenylated tRNA are associated with those of free cZs in plants. With the exception of AtIPT4 and AtIPT7, which are localized in the cytosol and mitochondria, respectively, all *Arabidopsis* adenylate-IPTs are localized in plastids where MEP biosynthesis occurs. In contrast, the functional tRNA-IPT in *Arabidopsis* (AtIPT2) is localized in the cytosol (Miyawaki *et al.*, 2004, 2006; Kumari *et al.*, 2013). Nevertheless, *in silico* analysis of tRNA-IPTs in monocot species, such as maize and rice, predict plastid localizations (Gramene, Phytozome v9.1, UNIPROT and chlorop v1.1; Emanuelsson *et al.*, 1999). Similarly, all known IPTs in moss are tRNA-IPTs, and PtIPT1 was shown to be localized in the chloroplast (Lindner *et al.*, 2014). Knocking down PpIPT1 decreased cZ-type CK levels in moss, consistent with its role

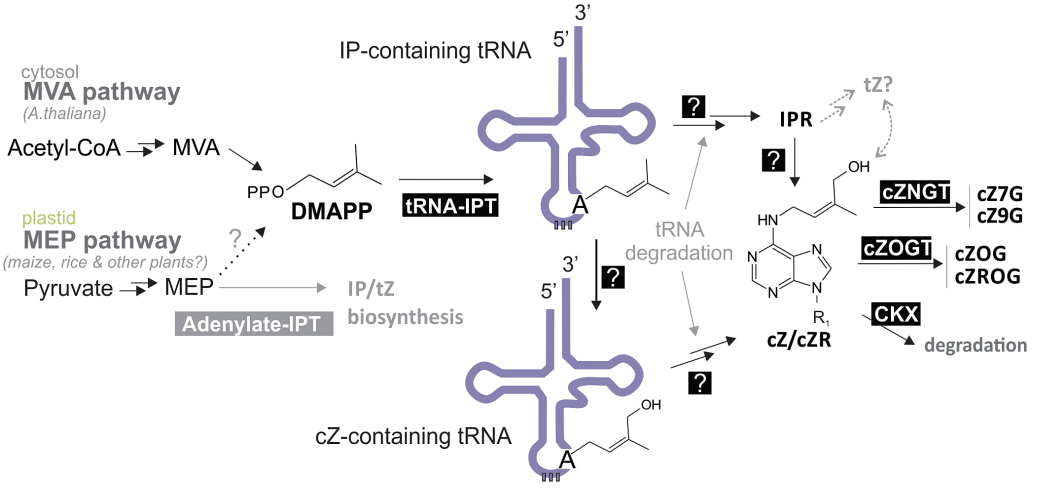

Fig. 1. Overview of *cis*-zeatin metabolism. tRNA-isopentenyltransferases (tRNA-IPTs) catalyse the prenylation of adenine 37 on specific (UNN-)tRNAs leading to the formation of isopentenyl adenine (IP)-containing tRNA. In *Arabidopsis thaliana*, the isopentenyl group is derived from the mevalonate (MVA) pathway in the cytosol; but predicted localization of enzymes in other plants suggests the use of isoprene moieties derived from the methylerythritol phosphate (MEP) pathway (broken arrow). The MEP pathway also contributes to IP and *trans*-zeatin (tZ) biosynthesis. Once isoprenylated tRNA is synthesized, it can be further modified and the CKs can be released by unknown enzymes ('?' in a black box; e.g. tRNA degrading enzymes). Hydroxylation of the prenyl side chain is suggested to occur on the IP-containing tRNA (i^6A37-tRNA), leading to the formation of cZ-containing tRNA (c-io^{6}A37-tRNA). Inactivation and catabolism of cZ is mediated by cZ-*O*-glucosyltransferases (cZOGT), cZ-*N*-glucosyltransferases (cZNGT), and cytokinin oxidase/dehydrogenase (CKX). Glucosylated forms of cZ-type CKs: cZOG (cZ-*O*-glucoside), cZROG (cZ-riboside-*O*-glucoside) and cZ7G/cZ9G (cZ-*N*9/*N*7-glucosides). Multiple arrows indicate multiple biochemical steps; dotted lines show unexplored metabolic flow. DMAPP, dimethylallyl-diphosphate; IPR, IP-riboside; cZR, cZ-riboside, A, adenine. (This figure is available in colour at *JXB* online.)

in cZ biosynthesis (Lindner *et al.*, 2014). Future studies will reveal if the different localizations of tRNA-IPTs in different plant species are correlated with the amount and the importance of cZ-type CKs in these species.

In addition, it is likely that isoprenylation by IPTs is not the rate-limiting step of cZ biosynthesis but rather the degradation of specific tRNAs. Consistent with this observation, tRNA-IPTs are constitutively expressed and not affected by plant hormones or nutrient status in *Arabidopsis* (Miyawaki *et al.*, 2004, 2006), even though cZ levels change under stress conditions (see the following paragraphs about biotic and abiotic stress). Increased tRNA turnover has been found under various stress conditions (Fournier *et al.*, 1976; Lee and Collins, 2005; Hopper *et al.*, 2010; Phizicky and Hopper, 2010). Such stress conditions can also lead to increased cZs levels in various plant species (Fournier *et al.*, 1976; Lee and Collins, 2005; Hopper *et al.*, 2010; Phizicky and Hopper, 2010, see the following paragraphs about biotic and abiotic stress). Two of the three known tRNA turnover pathways, rapid tRNA decay surveillance pathway (RTD) and endonucleolytic cleavage (EC), are induced upon stress and are known to act specifically upon modified tRNAs (Persson *et al.*, 1994; Lee and Collins, 2005; Alexandrov *et al.*, 2006), such as isoprenylated tRNAs. Alternatively, stress-induced ribonucleases, as described for angiogenin and tiRNAs, could lead to the release of tRNA-derived CKs (Yamasaki *et al.*, 2009). As mentioned before, ribonuclease activity in plant extracts can also contribute to the levels of free cZs. Future research should focus on the specific tRNA turnover pathways possibly involved in cZ release, and on the timing and mechanisms of hydroxylation of the isoprene moiety (Fig. 1).

Active CKs can be metabolized via oxidation by cytokinin oxidase/dehydrogenase (CKX; EC 1.4.3.18/1.55.99.12), or by the activity of glycosyltransferases. While the *O*-glucosylation of CKs, including cZ(R) is reversible and *O*-glucosides are generally considered as storage products (Mok *et al.*, 1992), *N*-glucosides are thought to represent deactivation products (Letham *et al.*, 1983; Vankova, 1999). The detection of cZ(R)-*O*-glucosides (cZOG, cZROG) and *N*-glucosides (cZ7G, cZ9G) indicated that cZ-type CKs are not mere tRNA degradation products, because glucosylated forms are not found in tRNA (Takagi *et al.*, 1989; Wagner and Beck, 1993; Nicander *et al.*, 1995). Entsch *et al.* (1979) characterized a pea glucosyltransferase enzyme that conjugated cZ (although with lower activity than tZ). *O*-glucosyltransferases with affinity for cZ are characterized in maize (Martin *et al.*, 2001; Veach *et al.*, 2003) and rice (Kudo *et al.*, 2012). 7 and 9-*N*-glucosyltransferases with affinity for tZ and other CKs were identified in *Arabidopsis* (Hou *et al.*, 2004), however their activity towards cZ was not tested. Limited knowledge also exists about the function of cZ degradation pathways via CKX. Recently, the *Arabidopsis* genes *CKX1* and *7* were shown to have high preference for cZ (Gajdošová *et al.*, 2011). Accordingly, overexpressing *CKX7* highly decreased levels of free cZ(R) in *Arabidopsis* (Köllmer *et al.*, 2014). In summary, this information illustrates that although many cZ-derived metabolites are commonly measured in various plant species, our knowledge of the genes that contribute to the regulation of cZ and derivatives is very limited.

It was proposed by Bassil *et al.* (1993) that cZ(R) and tZ(R) could be converted by a *cis-trans* isomerase, as observed in beans. Although other studies could find no or only negligible conversion between the Z isomers in tobacco BY-2 cultures, oat leaves, rice seedlings and maize cultured cells (Yonekura-Sakakibara *et al.*, 2004; Gajdošová *et al.*, 2011; Kudo *et al.*, 2012), it cannot be excluded that isomerization might occur under specific conditions or in particular tissues or plants. Additionally, it remains an open question if cZ regulation also relies on within-plant transport. cZR was reported as an abundant CK in phloem sap of *Arabidopsis* (Hirose *et al.*, 2008), but was also reported to occur in the xylem sap of *Arabidopsis*, wheat and oat (Parker *et al.*, 1989; Hirose *et al.*, 2008). Additionally, *Arabidopsis* purine permease 1 (AtPUP1) and AtPUP2 were proposed as potential transporters for various CKs including cZ (Burkle *et al.*, 2003) and they may play a role in the loading and unloading required for long-distance transport. However, definitive functional studies on a potential role for long-distance transport of cZs remain to be done.

cZ perception and signalling

To activate the CK-specific phosphorelay, cZs should be able to bind and activate the CHASE-domain containing histidine kinases (CHKs), which serve as CK receptors. Indeed, it could be shown that cZs can bind to CHKs and activate downstream elements of the signalling cascade, although with different sensitivity depending on the plant species and the specific receptor (Spichal *et al.*, 2004; Yonekura-Sakakibara *et al.*, 2004; Romanov *et al.*, 2006; Lomin *et al.*, 2011; Stolz *et al.*, 2011). The *Arabidopsis* CK receptors AHK2 and AHK3, for example showed higher cZ affinity when compared to their paralogue AHK4/CRE1, however in all cases cZ affinity was severalfold lower than its *trans*-isomer (Romanov *et al.*, 2006; Stolz *et al.*, 2011). Crystal structure analysis of the AHK4-CHASE domain in complexes with CKs revealed that the hydroxyl-group of cZ in contrast to tZ cannot form an additional hydrogen bond with Thr294, which is likely the reason for the lower cZ affinity of this receptor (Hothorn *et al.*, 2011). In contrast, the maize receptor ZmHK1, a closely related homologue of AHK4, was shown to have a similar sensitivity to cZ compared to tZ (Yonekura-Sakakibara *et al.*, 2004; Lomin *et al.*, 2011). Also the rice CK receptors OsHK3 and OsHK4 have a cZ affinity, similar to other tested CKs (Choi *et al.*, 2012). Activity measurements with the P_{ARR5}::GUS reporter construct verified that cZ can activate the CK signalling cascade in *Arabidopsis* (Spichal *et al.*, 2004). This was also confirmed by showing strong, tZ-comparable activity of cZ in eliciting the transcript accumulation of the maize response regulator *ZmRR1* (Yonekura-Sakakibara *et al.*, 2004) and of the rice response regulators *OsRR1*, *OsRR2*, *OsRR6*, and *OsRR9/10* (Kudo *et al.*, 2012). Interestingly, also PpHCK4 (from *P. patens*), a member of a recently discovered subgroup of CHKs, which was only found in the early diverging land plant *Marchantia polymorpha* and the moss *P. patens*, strongly responds to cZ (Gruhn *et al.*, 2014). Additional differences were reported for the receptor affinity to CK-ribosides. While AHK4 does not respond to CK-ribosides (Yamada *et al.*,

2001), ZmHK2 showed similar sensitivities to free bases and ribosides (Yonekura-Sakakibara *et al.*, 2004). CHKs were reported to have a high degree of redundancy, but specific functions can also be a single receptor (Riefler *et al.*, 2006). The differential receptor affinities of cZs might therefore allow functional specialization, as indicated already for tZ and IP (Stolz *et al.*, 2011). Alternatively, cZs might also function as modulators to fine-tune 'general' CK-pathway activity under specific conditions, but otherwise be functionally redundant with other CKs. How far species-specific differences in the cZ-affinities of CK receptors are related to a functional differentiation of cZs is currently unknown. The presence of receptors with a high cZs affinity could for example indicate a broader physiological role, whereas low affinity receptors, especially in combination with a low *cis*/*trans* ratio in some plants might indicate only subsidiary functions for cZs. cZ can compete with tZ for receptor binding in the bacterial assay (Romanov *et al.*, 2006) and can partially antagonize tZ-induced chlorophyll accumulation in squash (*Cucurbita maxima*; Kuraishi *et al.*, 1991). Moreover it was suggested that cZs might also play a role as a competitor to the more active CKs, thereby preserving specific CK functions that only require a low CK threshold (Gajdošová *et al.*, 2011).

Roles of cZ in plant growth

CKs are well known for their essential function in plant development and growth. cZs have long been thought to be biologically inactive and were considered as possible remnants of tRNA degradation (Skoog *et al.*, 1966; Vreman *et al.*, 1972, 1978; Tay *et al.*, 1986). Comparing the activity of cZ-type CKs with tZ- or IP-type CKs in classical activity assays (summarized in Gyulai and Heszky, 1994), such as *Phaseolus* (Mok *et al.*, 1978) or tobacco cell-culture assay (Leonard *et al.*, 1971; Schmitz *et al.*, 1972; Gajdošová *et al.*, 2011), it was revealed that cZs have little or no activity compared to IP and tZ, which are generally considered to be the most active natural CKs. Comparing the activities of cZs with their *trans* counterparts in various bioassays, Gajdošová *et al.* (2011) report in general between 3 and >50 times higher activities of the tZs (in accordance with their EC50 values), but the activities strongly depend on the particular bioassay. Several recent studies showed a developmental regulation of cZs in different model plants. In *Arabidopsis*, cZs are high in seeds and after imbibition (24 h), low in growing young plants and increase again when plants stop growing and start to senesce (Gajdošová *et al.*, 2011). Similarly, cZ concentrations are high during seed development in specific chickpea cultivars (Lulsdorf *et al.*, 2013). Micropropagated plantlets of *Musa* have high levels of cZ, which were replaced by IP upon acclimatization (Aremu *et al.*, 2014). In addition, cZs levels change significantly during development in the maize grain, as well as in shoot and root tissues (Saleem *et al.*, 2010; Zalabák *et al.*, 2014). Dwarf hop varieties contain significantly higher amounts of cZs (Patzak *et al.*, 2013) and cZR is a major CK in unfertilized hops (Watanabe *et al.*, 1981). These results reveal that cZ-type CKs tend to accumulate under the particular circumstances associated with limited growth. However, the accumulation of cZ(R) during radicle emergence and early seedling establishment in *Tagetes minuta* (Stirk *et al.*, 2005) also shows that cZs can be associated with fast-growth developmental stages. More data on the levels of specific CKs, including cZs, during the entire developmental phase of plants, instead of levels in very specific growth stages, are needed to draw general conclusions about their levels during plant growth.

Physiological processes influenced by CKs also include the inhibition of senescence (Gan and Amasino, 1995). cZ also suppressed senescence-induction in maize leaves (Behr *et al.*, 2012), and in an oat-leaf assay (Gajdošová *et al.*, 2011), although with lower activity than tZ, but did not inhibit senescence of detached flowers of *Dianthus* (Upfold and Van Staden, 1990). cZ only slightly affected fruit development in *Cucumis sativus* (Ogawa *et al.*, 1990). Decreasing the amount of cZ by overexpressing cZOGTs in rice delayed leaf-senescence and led to short root phenotype, longer roots, and a bigger number of crown roots (Kudo *et al.*, 2012). Suppressing cZ levels by overexpressing *CKX7* also affected root development in *Arabidopsis* (Köllmer *et al.*, 2014). Additional support for cZ functions in plants came from *Arabidopsis* T-DNA insertion lines with impaired cZ biosynthesis (*ipt2, 9*; Miyawaki *et al.*, 2006; Köllmer *et al.*, 2014), which showed chlorotic phenotypes, a shortened primary root, likely a result of the reduced root meristem size and ectopic protoxylem formation. This suggests an active role of cZ-CKs in *Arabidopsis* and rice growth and development. However, the phenotypic changes in *CKX7* overexpression lines might be more related to the reduction of cytosolic CK levels than to that of cZs in particular and the *ipt2, 9* plants might have been compromised by the reduced level of prenylated tRNA (Köllmer *et al.*, 2014). In the moss *P. patens*, in which cZs are the major CK-type, *PpIPT1* (tRNA-IPT) knockout plants with reduced levels of cZs also show developmental disturbances (Lindner *et al.*, 2014), however, these might be caused by concomitant changes in levels of other active CKs.

Some studies suggest a role of cZs in dormancy and seed germination. cZR decreases after decapitation in released buds of *Cicer arietinum* and might be a possible inhibitor of lateral bud growth (Mader *et al.*, 2003). In *Brassica napus*, cZR concentrations increased greatly after the onset of vernalization (Tarkowska *et al.*, 2012). In the seeds of oat, lucerne, *Tagetes* and pea, cZs dominate the CK profile, suggesting a role in seed physiology (Stirk *et al.*, 2005, 2008, 2012). In *Lolium perenne*, highly dormant seeds have been shown to have higher levels of cZR compared to seeds at a less dormant stage (Goggin *et al.*, 2010). cZs were also found at higher levels in dormant potato tubers compared to non-dormant ones and injection of cZ induced premature sprout formation (Mauk and Langille, 1978; Suttle and Banowetz, 2000).

In summary, these reports suggest that cZs might play a role during times of limited growth or dormancy, as is found in buds, tubers and seeds. It was proposed by Gajdošová *et al.* (2011) that cZs could help to maintain a basal level of CK activity under these conditions, but experimental proof remains lacking.

Roles of cZ in abiotic stress

As mentioned previously, the function of cZ(R) in plants was assumed to be the maintenance of minimal CK activity (e.g., ensuring resource supply by minimal sink activity or a suppression of premature leaf-senescence) under growth-limiting conditions, including abiotic stresses. Due to the high energy requirements during stress adaptation, effective stress responses (at least at the early stage) are associated with suppression of growth and reallocation of resources to the formation of stress-protective compounds. So probably in this period tZ(R), which exhibits very high cell division promoting activity, is often replaced by the much less active cZ(R). These CK dynamics may not only be illustrated by various temperature stress experiments, but also under biotic stresses (see below). Adjustment of the CK pool during the cold stress is tissue specific. Transfer of winter wheat to 4°C was associated with a rapid increase of cZ(R) in the main meristematic tissues crucial for over-wintering, namely crowns, in which maximum levels were attained already during the early stress response (after one day of cold exposure) (Kosova *et al.*, 2012). Simultaneously, the majority of the main protective proteins—dehydrins (especially WCS120) accumulated. In leaves, mild, gradual elevation of cZ(R) was detected, attaining maximum concentrations after three days of cold. The levels of tZ(R), however, dropped almost immediately upon cold exposure. During the subsequent acclimation phase, a moderate increase of tZ(R) was detected, associated with the plant's adaptation to low temperature. A peak of cZR (representing approximately 88% of the active CKs) was found in shoot apices of *Brassica napus* plants also after prolonged incubation at a low temperature (Tarkowska *et al.*, 2012). This cZR peak, however, seems to be associated with the onset of the transition between vegetative to generative developmental phases, as mentioned in the previous section. Similarly, the peak of predominantly cZR was found at the very beginning of the vegetative to reproductive developmental transitions in cold treated *Triticum monococcum* (Vankova *et al.*, 2014). Association of the cZ(R) peak with developmental changes was confirmed by comparison of the spring and the winter lines. In the spring wheat line, without vernalization requirement, a peak of cZR was detected in leaves, crowns and roots after 21 days at 4°C, while in the winter line, the maximum of cZR was delayed, occurring after the fulfilment of vernalization requirement (42 days).

Strong increases in cZR, together with moderately active IP and IPR, was found in young pea leaves after 4-day cold stress as well as after prolonged heat stress (Vaseva *et al.*, 2009). Elevation of cZ(R) concentrations with simultaneous down-regulation of tZR was reported at the early phase of heat stress response in leaves as well as in roots of tobacco plants (Dobra *et al.*, 2010). This change in CK pool coincided with high expression of heat shock factors and heat stress-associated proteins. Thus, responses to both temperature extremes seem to affect cZ/tZ profiles.

The effect of salt stress on the dynamics of cZ/tZ CKs was tested in maize plants (Vyroubalova *et al.*, 2009). In roots, tissues directly exposed to the salt stress, rapid elevation of the cZ precursor, cZRMP, was followed by elevation of cZ and especially of cZR, which reached maxima after 3 h (coinciding with minimum tZ and tZR levels). In leaves, the peak of cZR also coincided with the minimum of tZ. After 3 days, when acclimation took place, levels of cZ-type CKs were down-regulated, while peaks of tZ and tZR were detected. Thus in salt stress, rapid down-regulation of growth is also associated with cZ/tZ changes. Response to salt stress may be also affected by other external factors, such as CO_2 levels (Pinero *et al.*, 2014): sweet pepper (*Capsicum annuum*) plants at lower CO_2 content exhibited almost doubling cZR levels in comparison with high CO_2 supplementation.

During drought stress, increase of cZ levels was detected in roots (Havlova *et al.*, 2008; Mackova *et al.*, 2013). The levels of cZ(R) were highly up-regulated in tobacco roots also in response to combined drought and heat stress. After re-watering, cZs were down-regulated, with a simultaneous increase of tZ-type CKs and a stimulation of growth, even to a higher extent than in control (non-stressed) plants. Up-regulation of cZs was also found as a crucial response of *Plectranthus ambiguus* to nitrogen deficiency (Papparozzi, personal communication).

The above-mentioned patterns of cZ and its riboside suggest a role in maintenance of certain physiological functions of CKs under stress or growth-limiting conditions. The described mechanism should, however, be treated with caution, as some plant species do not respond with an elevation of cZs (e.g. soybean, Le *et al.*, 2012) and until now, experimental tests that analyse abiotic stress resistance of plants with manipulated cZ levels are lacking. Additionally, plants with generally high levels of cZs (e.g., maize, Veach *et al.*, 2003) indicate that cZs are not only involved in stress responses and during periods of growth limitations.

Roles of cZ in pathogen resistance

Recently, a function of CKs in the regulation of plant immunity against pathogens has been identified. Several active CKs, including 6-benzylaminopurine (6-BAP), kinetin and tZ have been demonstrated to efficiently increase resistance against pathogens in *Arabidopsis* and tobacco (Choi *et al.*, 2010; Großkinsky *et al.*, 2011, 2013; Argueso *et al.*, 2012). In these CK-mediated resistance phenotypes, interactions with other phytohormones, such as abscisic acid (Großkinsky *et al.*, 2014) or salicylic acid (Choi *et al.*, 2010; Großkinsky *et al.*, 2011; Argueso *et al.*, 2012) have been shown. In contrast, information on the role of cZ in plant immunity is limited. Pre-treatment with cZ can considerably suppress symptom development of *Pseudomonas syringae* infection in cultivated tobacco (Großkinsky *et al.*, 2013). Similar effects were observed in cZ pre-treated or *AtIPT2* expressing (*SAG-IPT2*) plants of the wild tobacco (*Nicotiana attenuata*, Supplementary Fig. S1). The cZ effect on *P. syringae* symptoms in *N. tabacum* was, however, significantly lower compared to the highly active tZ. Concomitantly, cZ had no effect on the *in planta* proliferation of the pathogen as it had been shown for more active CKs including tZ (Großkinsky

et al., 2011, 2013). These data indicate that cZ does not directly activate anti-pathogen defence in these plant species, but mainly suppress symptom development, for example by suppressing the pathogen-induced cell death response similar to that described by Barna *et al.* (2008) for Z and thereby maintaining tissue integrity during an infection.

Interestingly, as previously mentioned, the ability to produce cZs (among others) has been identified in several pathogens. No specific biological role has been attributed to cZs found in *Magnaporthe grisea* hyphae and its culture filtrates or in rice tissue post *M. grisea* infection (Jiang *et al.*, 2013). However, they correlated with the proliferation and symptom maintenance of *R. fascians* infection in *Arabidopsis* (Pertry *et al.*, 2009). This was consistent with the considerably lower amounts of cZs (in addition to others) produced in culture by the non-pathogenic *R. fascians* strain D188-5 compared to the virulent strain D188. Furthermore, production as well as interconversion of CKs, including cZ and derivatives, has been described for *Colletotrichum graminicola* (Behr *et al.*, 2012). The biological relevance of the modulation of cZ levels by fungal production and conversion has further been related to the infection process. Comparable to *C. graminicola* infection, treatment of maize leaves with cZ (but also *t*Z or 6-BAP) resulted in delayed senescence, as evidenced by the formation of photosynthetically active green islands (Behr *et al.*, 2012), indicating the potential modulation of the host physiology by the fungus via cZ production. This physiological modulation could also include the regulation of the host's carbohydrate metabolism as the CK-related delay of senescence is mediated by invertase activity (Balibrea Lara *et al.*, 2004). Since invertases play important roles for the tolerance/resistance against biotic (Roitsch *et al.*, 2003) as well as abiotic (Albacete *et al.*, 2014) stress situations, they could be an important target for the specific modulation of host physiology via cZ in plant-microbe interactions.

Role of cZ in herbivore resistance

In addition to their potential role in abiotic stress responses and pathogen resistance, cZs are also indicated to play a role in plant-herbivore interactions. Very high cZ levels in the larval body of the aphid, *Pachypsylla celtidis* indicate that they might be involved in induced gall formation in hackberry (*Celtis occidentalis*, Straka *et al.*, 2010). Insects and their endosymbiotic bacteria were reported to utilize CKs to manipulate a plant's physiology to their advantage and it seems possible that cZs could also be used in combination with other CKs for this purpose (Giron and Glevarec, 2014). In addition to herbivore-mediated manipulations, plant-mediated responses to herbivory were shown to involve cZs. Conrad and Kohn (1975) showed a wound-induced formation of Z-containing tRNA, potentially the *cis*-isomer; however the *cis*/*trans* conformation was not further specified in this study. Recently, Schäfer *et al.*, (2014a) showed that cZs were upregulated in *N. attenuata* and *Arabidopsis* by wounding and application of oral secretions from the tobacco hornworm (*Manduca sexta*) or the grasshopper, *Schistocerca gregaria*, respectively. cZR levels responded in both species, while cZ was much more responsive in *Arabidopsis*. Even 4 h after treatment the cZR levels were still highly up-regulated in *N. attenuata*, while the levels of another herbivory-induced, bioactive CK, IPR, already started to decline. Jasmonic acid (JA) is a key player in the plant response to chewing insect herbivores. Although methyljasmonate (MeJA) application to *N. attenuata* leaves reduced IPR levels and suppressed the herbivory-induced CK-pathway signalling (indicated by *NaARR5* transcripts), the cZR levels were elevated by this treatment. Accordingly, silencing JA biosynthesis and signalling components reduced the herbivory-induced accumulation of cZR. It seems likely that the contrasting regulation of cZR and IPR by JA could be related to their distinct metabolic origin (Kasahara *et al.*, 2004; Miyawaki *et al.*, 2004). Herbivory, as well as jasmonates were reported as potent suppressors of plant-growth (Hummel *et al.*, 2007; Meldau *et al.*, 2012; Attaran *et al.*, 2014), which is consistent with the observation that cZs are particularly associated with growth-limiting conditions.

In recent years, CKs were shown to amplify plant defence responses against herbivores (Smigocki *et al.*, 1993; Dervinis *et al.*, 2010). Therefore cZs might also be involved in the regulation of anti-herbivore defences. JA-mediated defence responses, such as proteinase inhibitor accumulations, were found to be promoted by CKs (Dervinis *et al.*, 2010). When we applied cZR to *N. attenuata* leaves we found an increase in the MeJA-mediated induction of the phenolamide pathway and trypsin proteinase inhibitor activity (Supplementary Fig. S2). These data suggest that cZs are potentially involved in defence metabolite accumulations after herbivore attack. However, external applications do not allow for the precise regulation of intracellular CK levels and additional work is necessary to ensure that the observed effects can be triggered by physiologically relevant levels of cZs.

Secondary metabolites are known to play an important role in various stress responses (Bennett and Wallsgrove, 1994; Rangan *et al.*, 2014) and it should be tested if cZs can also amplify the accumulation of other secondary metabolites, potentially to improve plant survival under stress conditions. The frequently observed mild CK activity of cZs (Schmitz and Skoog, 1972; Kamínek *et al.*, 1979) might help plants to retain the resources required for the induction of the defence and stress-resistance metabolites.

Outlook

The CK pathway has frequently been predicted to harbour opportunities for future crop improvement (Yang *et al.*, 2000; Werner *et al.*, 2010; Qin *et al.*, 2011; Wilkinson *et al.*, 2012). However, most of these studies gloss over the differences in specific CK profiles that exist between model and crop plants. cZs are highly abundant in many crop plants. In contrast, in *Arabidopsis* the *trans*-isomer is the most abundant in most growth stages (Gajdošová *et al.*, 2011) and its CK receptors have a lower affinity to cZs than has been reported for some of the CHKs of crop plants

(Yonekura-Sakakibara *et al.*, 2004; Romanov *et al.*, 2006; Lomin *et al.*, 2011; Stolz *et al.*, 2011; Choi *et al.*, 2012). Therefore the functional predictions based on previous investigations in low cZ-containing plants might not reflect the role of cZs in many crops.

Environmental factors can have a tremendous effect on agricultural productivity (Boyer, 1982). Various references indicate that cZs are part of the plant response to growth under limiting conditions and hence they might be potential targets to improve the biotic and abiotic stress resistance of crop plants (Fig. 2). Moreover their role during plant development could also help to improve crop properties. For example, cZs were proposed to play a role in regulating potato tuber dormancy (Suttle and Banowetz, 2000), which makes cZs potential breeding targets to produce plants with improved tuber storage characteristics.

Unfortunately, experimental proof of cZ functions remains rare and many assumptions need deeper and more rigorous experimental examination. Experiments that use plants with manipulated cZ levels, such as by external application of cZs (e.g. Großkinsky *et al.*, 2013), impaired cZ-biosynthesis (*ipt2, 9* mutants, Miyawaki *et al.*, 2006) or increased cZ-degradation/-inactivation (*AtCKX7* overexpression, Köllmer *et al.*, 2014; *OscZOGT1* and *OscZOGT2*, Kudo *et al.*, 2012) are sorely needed to unravel the role of cZs in plant-growth and stress responses. Additionally, plants overexpressing cZ-biosynthetic genes (e.g. *AtIPT2*) and forward genetic approaches to identify regulatory elements of the cZ metabolism could also be illuminating. Importantly, the experimental side-effects of the manipulations, such as the changes in prenylated tRNA or the effects on other CKs must be taken into consideration (e.g. Köllmer *et al.*, 2014). For the analysis of stress-specific functions, the use of conditional expression systems (reviewed in Corrado and Karali, 2009) can be very useful to disentangle stress responses from changes in development. The knowledge gained from these experiments about this widely distributed, but often neglected, hormone will help us understand if it plays a role as 'stress hormone' under growth-limiting conditions and as a mediator of responses to a plant's interactions with other organisms, including attackers, as well as mutualists.

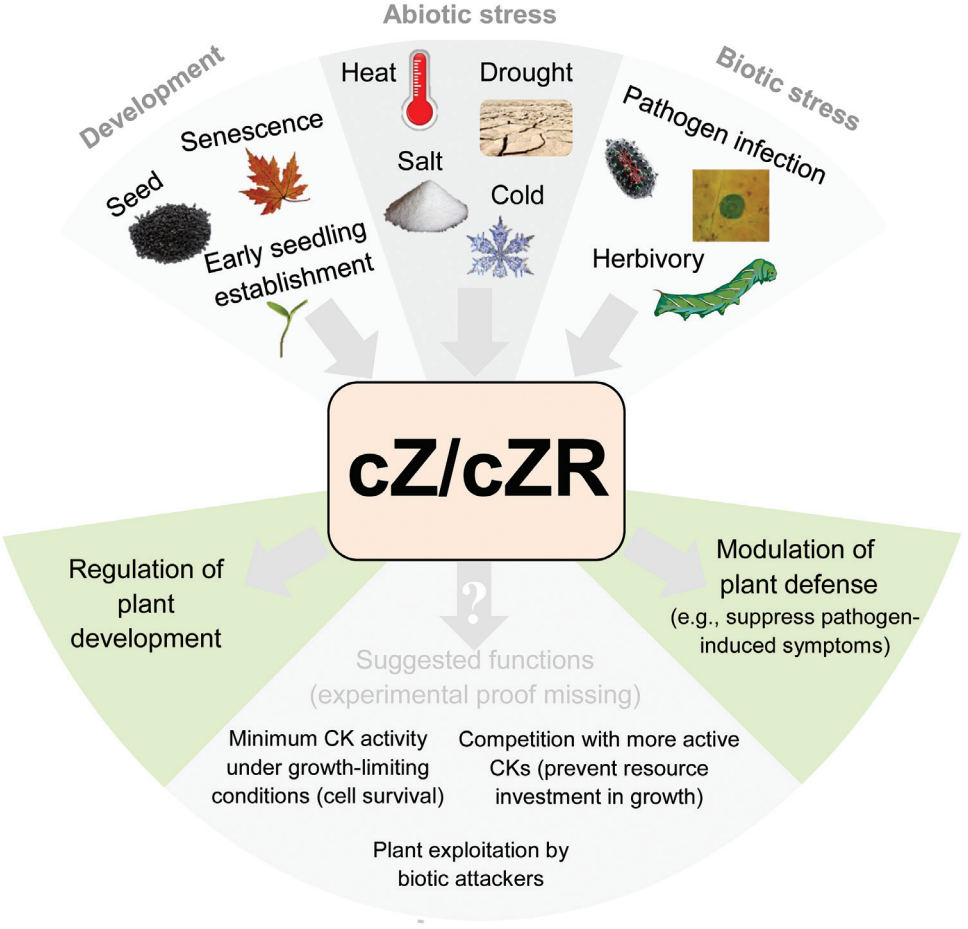

Fig. 2. *cis*-zeatins as potential regulators of plant development and stress responses. *cis*-zeatin (cZ) and its riboside (cZR) are reported to accumulate in particular under various conditions characterized by limited growth, during particular developmental stages, but also in response to abiotic and biotic stresses. cZ/cZR were shown to be involved in the regulation of the plant development and to be able to modulate plant defence responses. Based on their distribution patterns, they were proposed additionally to sustain a minimum cytokinin (CK) activity under growth-limiting conditions, to prevent the redirection of resources e.g., from stress adaptation processes to plant growth and for a means by which phytophagous organisms could manipulate the plant's physiology and morphology for their benefit. However, an experimental confirmation of this hypothesis is still missing. (This figure is available in colour at *JXB* online.)

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Figure S1. Pathogen-resistance of *Nicotiana attenuata* plants treated with *cis*-zeatin and transgenic plants overproducing *cis*-zeatin (SAG-IPT2).

Supplementary Figure S2. *cis*-Zeatin riboside (cZR) amplifies methyljasmonate (MeJA)-induced anti-herbivore defenses in *Nicotiana attenuata*.

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