



# Cytokinins, the Cinderella of plant growth regulators

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**Abstract** Cytokinins (CKs) are a complex group of compounds. The complexity of CKs is not just present at the level of chemical structures, but as a family of compounds. CKs occur in plants as nucleotides, nucleosides (ribosides) that are produced during de novo biosynthesis, free bases, and conjugates that are in stored/inactivated forms. Almost all organisms make cytokinins. CKs are structural components of the tRNA, and they are located next to the anticodon loop beginning with U of a subset of tRNAs in most eukaryotes and bacteria. The biosynthesis of CKs uses several pathways, and there is evidence that its biosynthesis is also regulated by other growth regulators such as auxins, strigolactones and abscisic acid. Its signaling pathway involves a phosphotransfer signal cascade. Despite advances made so far in the knowledge of CKs, there is still a long way to go. In this review, we summarize the most up-to-date knowledge on the biosynthesis of CKs and the signaling pathway that leads to the response to the presence of CKs in plant tissue, and we identify areas that require more research to complete our understanding of the role of the CKs in plants.

**Keywords** Cytokinins · Biosynthesis · Regulation · Signalling

## Abbreviations

HMBDP	4-Hydroxy-3-methyl-2-( <i>E</i> )-butenyl diphosphate
6-BA	6-Benciladenine
ABA	Abscisic acid
APT	Adenine phosphoribosyltransferase
iPMP	Adenosine-5'-monophosphate
AHK	Arabidopsis histidine kinase
AHP	Arabidopsis histidine-containing phosphotransfer protein
ARR	Arabidopsis response regulators
ABC	ATP-binding cassette
ARF3	AUXIN RESPONSE FACTOR3
CHASE	Cyclases/histidine kinases associated sensory extracellular
CKX	CYTOKININ OXIDASE/DEHYDROGENASE
CRE1	Cytokinin response1
DZ	Dihydrozeatin
DMAPP	Dimethylallyl pyrophosphate
ER	Endoplasmic reticulum
ENTs	Equilibrative nucleoside transporters
GeBP/	Glabrous1 enhancer-binding protein
GPL	
HPT	Histidine phosphotransfer
AHK	Hybrid his kinases

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iP	Hydroxymethylbutenyl diphosphate isopentenyladenine
iPRDP	Isopentenyladenosine-5'-diphosphate
iPRTP	Isopentenyladenosine-5'-triphosphate
IPT	ISOPENTENYL TRANSFERASE
KIN	Kinetin
LOG	LONELY GUY
mT	<i>meta</i> -Topolin
MEP	Methylerythritol phosphate
MVA	Mevalonate
<i>Ot</i>	<i>ortho</i> -Topolin
PUPs	Purine permeases
SPY	Spindly
SL	Strigolactone
UGT	Uridine diphosphate glycosyltransferase
WOL	Wooden leg1
Z	Zeatin
<i>cZ</i>	<i>cis</i> -Zeatin
<i>tZ</i>	<i>trans</i> -Zeatin
tZRPs	<i>tZ</i> -Riboside 5'-phosphates

## Introduction

Since kinetin and its effect on the differentiation of tobacco calli were discovered by researchers at the University of Wisconsin (Miller et al. 1955b; Skoog and Miller 1957), cytokinins (CKs) have been the focus of many plant development and differentiation studies. CKs serve as plant growth signals in a variety of biological processes, such as cell division and differentiation, vascular and flower development, nutrient homeostasis, leaf expansion, circadian clock, abiotic stress responses, stomata aperture, seed germination, shoot apical meristem maintenance, branching and nodulation, leaf senescence, lateral root development, regulation of polar auxin transport, and root growth (Bishopp et al. 2011; Fusconi 2013; Han et al. 2014; Kieber and Schaller 2018; Liu et al. 2019; Mok and Mok 2001; Nitschke et al. 2016; Sakakibara 2006; Sasaki et al. 2014; Werner and Schmülling 2009; Wybouw and De Rybel 2019).

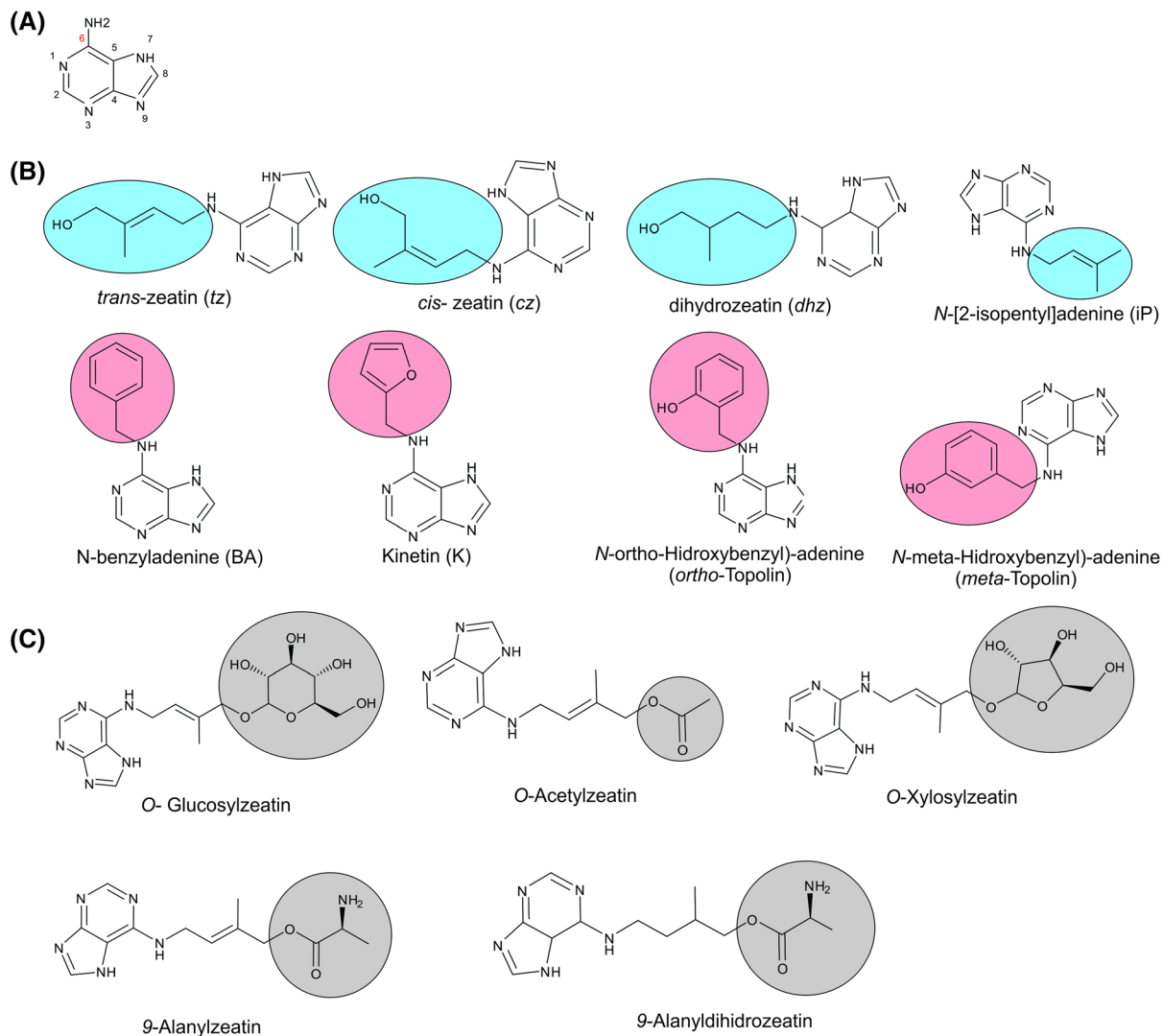
Unlike auxins, CKs are a very complex group of compounds, both in terms of chemical structures and of compound mixtures. Eudicotyledons and monocotyledons use different sets of CKs, at concentrations around 1% that tend to be higher in monocotyledons

(Osugi and Sakakibara 2015). Most of the CKs are conjugated, mainly as glucosides (Bielach et al. 2012; Jiskrová et al. 2016; Osugi and Sakakibara 2015) with, among others, sugars and sugar phosphates, either on side-chain hydroxyl oxygens or on purine ring nitrogens (Mok and Mok 2001; Sakakibara 2006). In another type of complexity, the *trans* and *cis* zeatin isomers are synthesized by different pathways (Kasahara et al. 2004; Sakakibara 2006), and they play different roles in regulating growth (LaCuesta et al. 2018). Finally, due to their chemical diversity, CKs can also establish complex relationships with other growth regulators, such as auxins, strigolactones, gibberellins, abscisic acid (ABA) and nitrate (Noriega and Pérez 2017; Xu et al. 2015).

## Structure and conjugates of cytokinins

CKs are derivatives of purine bases with an isoprenoid or aromatic side chain at the  $N^6$  position (Fig. 1). The most common class of isoprenoid CKs comprises zeatin (Z), dihydrozeatin (DZ) and  $N^6$ -( $\Delta^2$ -isopentenyl) adenine (iP). The first natural CK identified was zeatin, present in the immature endosperm of *Zea mays* (Miller 1961) in *cis*- or *trans*-configuration (depending on which of the two methyl groups is hydroxylated). Previously, it was shown that *trans*-zeatin (*tZ*) is the predominant CK in plants and that *cis*-zeatin (*cZ*) is less active, however the recent development of sensitive, rapid, high-throughput analytical methods has advanced our knowledge of the distribution and possible physiological functions of *cZ* (High et al. 2019; Schäfer et al. 2015; Shefflin et al. 2019; Wang et al. 2019).

Aromatic CKs have an aromatic benzyl or hydroxybenzyl group at the  $N^6$  position. kinetin (KIN; 6-furfurylamino-purine) was the first synthetic aromatic CK discovered through bioassay of coconut milk concentrates in tobacco pith tissue culture (Miller et al. 1955a). The main natural aromatic CKs present in plant tissues are *meta*-topolin (mT; 6-[3-hydroxybenzylamino]purine), *ortho*-topolin (*Ot*; 6-[2-hydroxybenzylamino]purine), 6-benzyladenine (6-BA) and 6-BA derivatives (Aremu et al. 2014; Sáenz et al. 2003; Strnad, 1997), although these CKs are less abundant than the isoprenoid CKs (Sáenz et al. 2003). These structural variations of the side chain change the



**Fig. 1** Chemical structures of cytokinins. **a** Adenine structure and the numbering system for the nomenclature of CKs. **b** Representative Isoprenoid CKs and aromatic CKs. Only common names are given with commonly used abbreviations in

parentheses. Note that the isoprenoid side chain is inside a blue oval and the aromatic chain inside the pink oval. **c** Structure of CK conjugates. The molecule conjugated with the CK is inside the gray oval

physicochemical properties of the CKs, in particular their polarity.

CKs occur in plants as nucleotides, nucleosides (ribosides), free bases and their respective conjugates. The CK bases can be conjugated with sugars (glycosylation, xylosylation) or amino acids (alanylation) (Fig. 1). The CKs are deactivated when conjugated with a sugar moiety, mostly glucose. The glycosylation at the *O*- or *N*-position is performed by the enzyme uridine diphosphate glycosyltransferase (UGT) (Entsch and Letham 1979; Hou et al. 2004).

CK *N*-glucosides are formed irreversibly, in route to CK degradation (Hou et al. 2004), while CK-*O* glucosides can be reversibly de-glycosylated and represent a storage form (Brzobohaty et al. 1993; Falk and Rask 1995). Recent studies have shown that CK glycosylation occurs in the cytosol and serves as a rapid mechanism maintaining intracellular homeostasis, because glycosylation causes the CKs to be more hydrophilic and easily recognized by transmembrane transporters, effecting the release of CK glucosides from the cytosol or their storage in hydrophilic

organelles such as vacuoles (Jiskrová et al. 2016; Lim and Bowles 2004; Šmehilová et al. 2016). The CKs can also be irreversibly inactivated by conjugation to alanine in the  $N^9$  position (Entsch et al. 1983). The hydroxyl group of the CK side chain can also be conjugated with xylose, and the resulting *O*-xylosylzeatin shows higher activity than zeatin in bioassays with *Phaseolus vulgaris* calli and a little conversion into zeatin. These findings suggest a possible biological function of *O*-xylosylzeatin, however its precise role is still unknown (Mok et al. 1987; Turner et al. 1987).

CKs are present in plant tissues at very low concentrations, in most cases at the level of pmol  $g^{-1}$  FW, making their isolation and quantification difficult. However, new procedures of extraction, purification and detection have allowed the detection of CKs and conjugates even at very low concentrations in minimal amounts of plant material from diverse species (Albacete 2017; Cao et al. 2017; Dobrev et al. 2017; High et al. 2019; Sheflin et al. 2019; Svacinova et al. 2012; Wang et al. 2019). The spatial distribution of CKs and their derivatives varies according to the physiological age of the plant and its interaction with abiotic factors such as stress or availability of nitrate.

### Cytokinin biosynthesis pathways

Almost all organisms make CKs, as they are structural components of tRNA, located next to the anticodon loop beginning with the U of a subset of tRNAs in most eukaryotes and bacteria (Persson et al. 1994). This fact led to the initial proposition that the primary source of CKs could come from the degradation of tRNA (Swaminathan and Bock 1977). However, subsequent studies showed that there is a *de novo* biosynthesis pathway that does not involve the degradation of tRNA (Taya et al. 1978).

It was initially thought that the primary site of CK biosynthesis was the root. However, further research suggested CK biosynthesis in various locations of active cell proliferation, such as shoots and young leaves (Blackwell and Horgan 1994; Koda and Okasawa 1980; Sakakibara 2006, 2010). Studies of the chemical profile of the xylem and phloem sap showed that *tZ*-type CKs such as *tZ* and *tZR* predominate in the xylem sap, while *iP*-type CKs such as *iPR*

and *iP*-ribotides predominate in leaf exudates (Corbesier et al. 2003; Hirose et al. 2008).

Eukaryotic cells have the capacity to organize metabolic and signaling processes using subcellular compartmentation (Skalicky et al. 2018). This feature is of particular importance in the regulation of CK biosynthesis and homeostasis. The biosynthesis, conjugation, and storage of CKs take place in different compartments of the cell (Table 1) (Skalicky et al. 2018).

The first step in the biosynthesis of isoprenoid CKs is the *N*-prenylation of AMP, ADP or ATP, isoprenoid moieties donated by dimethylallyl pyrophosphate (DMAPP) or 4-hydroxy-3-methyl-2-(*E*)-butenyl diphosphate (HMBDP). DMAPP is a metabolic intermediate of the cytosolic mevalonate (MVA) and the plastidial methylerythritol phosphate (MEP) pathways, while HMBDP is an intermediate of the MEP pathway (Krall et al. 2002; Sakakibara et al. 2005, 2006) (Fig. 2). Isotopic marker studies in *Arabidopsis thaliana* seedlings showed that the prenyl side chains of *tZ*- and *iP*-type CKs are mainly produced through the MEP pathway, whereas a significant fraction of *cZ* derivatives are synthesized through the MVA pathway (Fig. 3) (Kasahara et al. 2004). The initial prenylation produces isopentenyl adenosine-5'-monophosphate (*iPMP*), isopentenyladenosine-5'-diphosphate (*iPRDP*) or isopentenyladenosine-5'-triphosphate (*iPRTP*). It is catalyzed by adenosine phosphate-isopentenyltransferase (EC 2.5.1.27; *IPT*) (Fig. 2), which was identified for the first time in the slime mold *Dictyostelium discoideum* (Taya et al. 1978). Later, the *Agrobacterium tumefaciens* gene *tmr* (subsequently termed *ipt*) was shown to encode an enzyme with similar activity (Akiyoshi et al. 1984; Barry et al. 1984), and overexpression of *IPT* genes in a variety of plants was found to cause an increase in CK content (Sun et al. 2003).

Plants have two classes of *IPT* enzymes differing in the substrates they use (Miyawaki et al. 2004). Adenosine-phosphate *IPT*s (*AP-IPT*s) use ATP, ADP or AMP as a substrate, and studies of various *IPT* mutants indicate that they participate in the biosynthesis of *iP* or *tZ* (Kakimoto, 2001; Takei et al. 2001a). In contrast, tRNA-*IPT*s catalyze the isopentenylation of UNN-codon tRNAs at adenine (A37), causing the subsequent release of CK nucleotides by the degradation of the tRNA (Murai 1994) and formation of *cZ* (Fig. 2) (Miyawaki et al. 2006).

**Table 1** Subcellular compartmentation of the enzymes of biosynthesis and catabolism of cytokinins

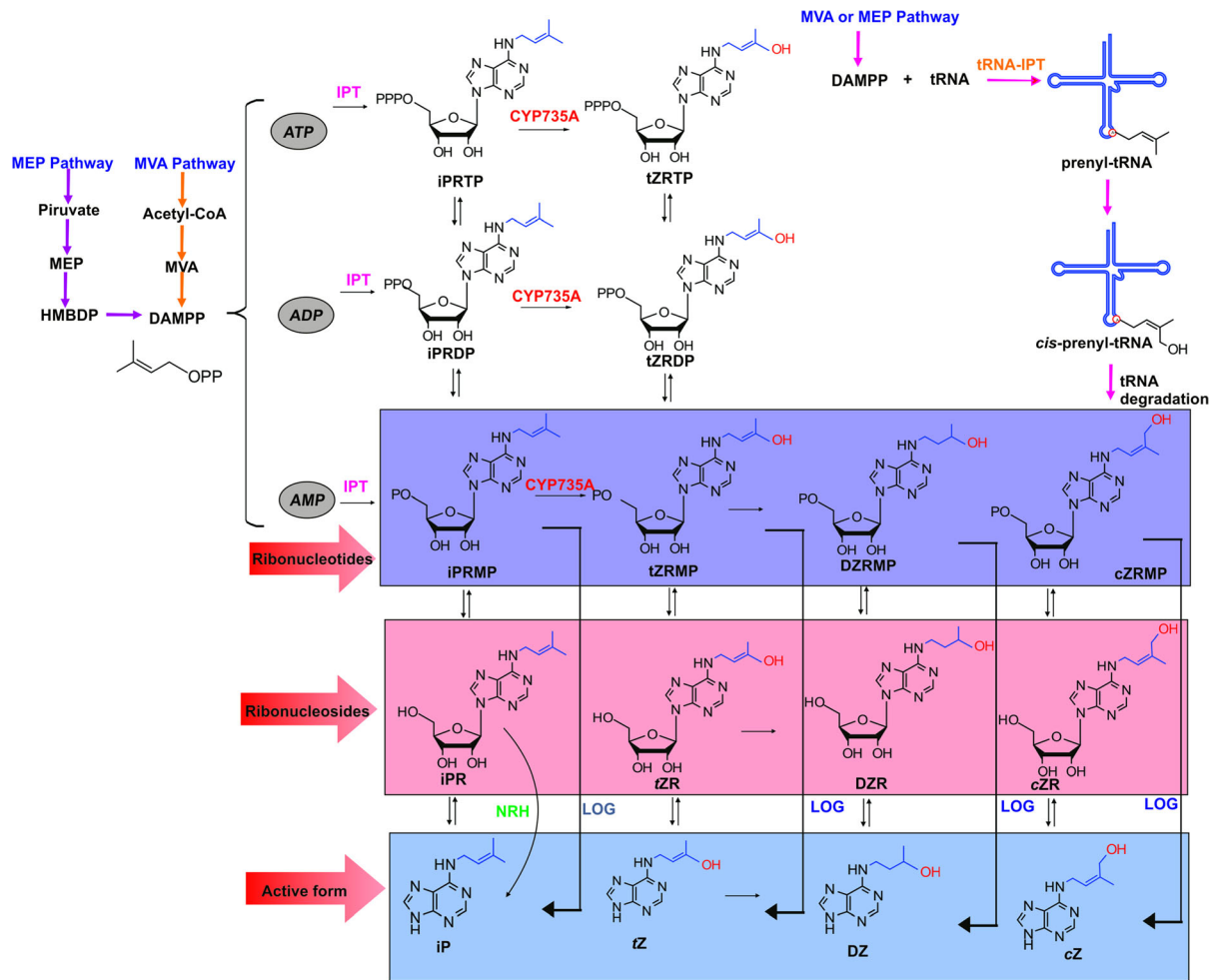
Enzyme	Subcellular compartmentation	References
AtIPT1, 3, and 5	Chloroplasts of mesophyll cells	Kasahara et al. (2004)
AtIPT2	Cytoplasm	Miyawaki et al. (2006)
AtIPT7	Mitochondria	Kasahara et al. (2004)
AtIPT3 farnesylated	Nucleus	Galichet et al. (2008)
Cytochrome P450 monooxygenases CYP735A1 and CYP735A2	Endoplasmic reticulum	Takei et al. (2004b)
AtLOG1 to 9	Nucleus and cytosol	Kuroha et al. (2009)
At uridine diphosphate glycosyltransferase (AtUGT) 85A1	Cytosol	Jin et al. (2013), Šmehilová et al. (2016)
Adenine phosphoribosyltransferases (AtAPT1-3)	Cytosol	Allen et al. (2002), Ko and Helariutta (2017), Moffatt et al. (1991), Zhang et al. (2013)
Direct glycosylation at N7 or N9 might also be catalyzed by UGT76C1 or UGT76C2	Cytosol	Mok and Mok (2001)
CKX2 and CKX4-6	Apoplast	Werner et al. (2003)
CKX1 and 3	Vacuole/ER	Niemann et al. (2018), Schmülling et al. (2003), Werner et al. (2003)
CKX7	Cytosol	Köllmer et al. (2014)

In *A. thaliana*, the *IPT* family comprises seven *AP-IPTs* (*IPT1*, *IPT3*, *IPT4*, *IPT5*, *IPT6*, *IPT7*, and *IPT8*) and two *tRNA-IPTs* (*IPT2* and *IPT9*) (Kakimoto 2001; Takei et al. 2001a). Phylogenetic studies of *IPT* genes in several angiosperms, such as *A. thaliana*, *Oryza sativa*, and *Solanum lycopersicum*, revealed a multigene family with complex evolutionary history marked with duplication events which may be associated with functional redundancies (Ghosh et al. 2018; Matsuo et al. 2012; Nishii et al. 2018). Furthermore, *AP-IPTs* showed differential expression in different organs and, thus, homolog-specific control of the local CK concentration (Miyawaki et al. 2004). For example, the *A. thaliana IPT3* had high level of expression in the phloem, roots, hypocotyl, cotyledons and leaves (Miyawaki et al. 2004; Takei et al. 2004a), while several *O. sativa IPTs* were highly expressed in the shoot apical meristem, roots and leaf primordia (Ghosh et al. 2018). These data suggest that the precursor of *iP*-type CKs can be synthesized in a wide range of plant parts.

The enzymes AtIPT1, AtIPT3, AtIPT5 and AtIPT8 are located in plastids, and an *Allium cepa* epidermal cell assay showed that AtIPT3 contains a C-terminal CaaX motif that is farnesylated, directing the modified protein to be located in the nucleus and the cytoplasm, while the

nonfarnesylated protein is located in the plastids (Galichet et al. 2008). In contrast, AtIPT4 and AtIPT2 are restricted to the cytosol and AtIPT7 to the mitochondria (Kasahara et al. 2004) (Fig. 3). Similarly, in *O. sativa* OsIPT2, OsIPT71, OsIPT72, and OsIPT8 proteins were also predicted to be localized in the cytoplasm, and the proteins OsIPT1, OsIPT3, OsIPT5 in the chloroplast (Ghosh et al. 2018).

The initial products in the biosynthesis of CKs, *iPRMP*, *iPRDP* and *iPRTP*, are subsequently hydroxylated in the isoprenoid side chain by a cytochrome P450 enzyme, forming *tZ*-riboside 5'-phosphates (*tZRPs*) (Fig. 2). *A. thaliana* has two cytochrome P450 monooxygenases, CYP735A1 and CYP735A2, functioning in this step of CK biosynthesis (Takei et al. 2004b). Double mutants affected in both genes showed a lower concentration of *tZ* and its conjugates, causing a delay in the shoot growth and underlining that they play a role in CK biosynthesis. Recent studies in *Jatropha curcas* showed that *JcCYP735A* mutants have decreased in amounts of *tZ* and *tZR* and reduced growth (Cai et al. 2018). Finally, diverse other reports confirmed the existence of *CYP735A* orthologs in such diverse plant species as *Brassica oleracea*, *O. sativa*, *Glycine max* and *S. lycopersicum*, suggesting that the



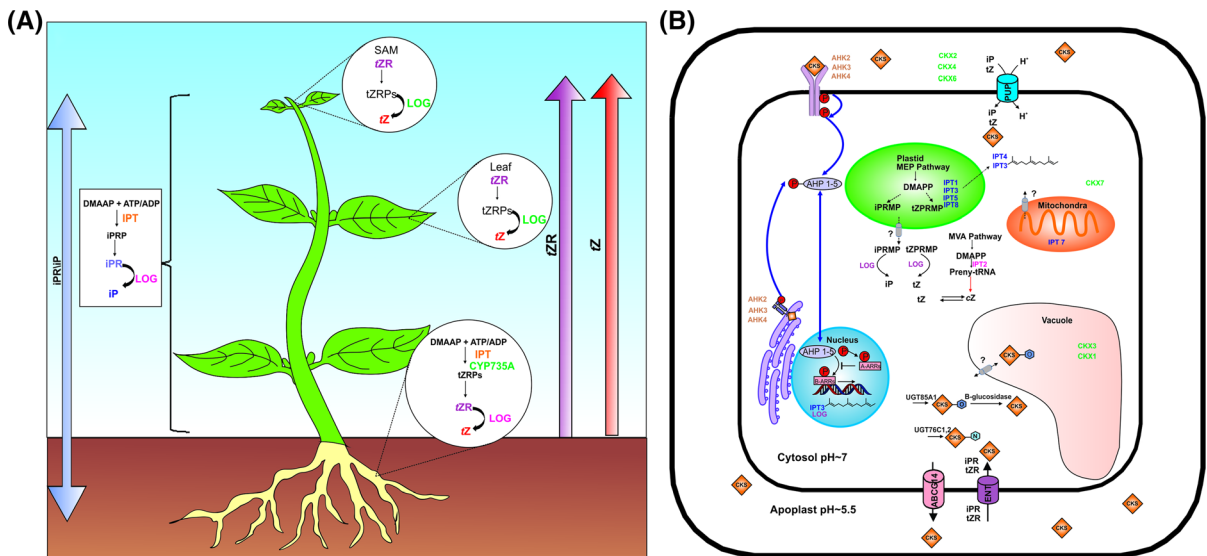
**Fig. 2** Model for cytokinin biosynthesis in plants. CK biosynthesis starts by transferring an isopentenyl moiety from dimethylallyl diphosphate (DMAPP) to AMP/ADP/ATP. In *A. thaliana* the isopentenyl moiety is transferred mainly to ATP/ADP. This reaction is catalyzed by the phosphate-isopentenyl-transferase (IPT) enzymes (pink). The initial products iPRMP, iPRDP, and iPRTP are subsequently hydroxylated in the isoprenoid side chain by cytochrome P450 monooxygenases CYP735A (red) for the formation of the corresponding *tZ*-nucleotides. The ribotides (purple box) can be converted to their free bases by two pathways: the two-step activation is catalyzed

function of CK *trans*-hydroxylation may be highly conserved (Guttikonda et al. 2010; Nelson and Werck-Reichhart 2011; Simm et al. 2016; Yu et al. 2017). Interestingly, *CYP735A* genes is predominantly expressed in root vasculature of *Arabidopsis* (Kiba et al. 2013; Matsumoto-Kitano et al. 2008), alongside other CK biosynthetic enzymes like IPT5 and LOG3. Together, these findings match previous reports that *iZ*-type cytokinins are mainly synthesized in roots

by 5'-ribonucleotide phosphohydrolase for the formation of ribonucleosides (pink box) and by adenosine nucleosidase for the formation of the active form (blue box). So far only one has been identified, a nucleoside N-ribohydrolase (NRH), which catalyzes the hydrolysis of iPR to iP (green). The direct activation pathway is catalyzed by 5'-monophosphate phospho-ribohydrolases (LOGs) (blue). The formation of CKs of the *cis*-Zeatin-type begins with the prenylation of adenine 37 on specific (UNN-) tRNAs by tRNA-isopentenyltransferase (tRNA-IPT) (orange) and subsequent release of CK nucleotides by tRNA degradation. (Sakakibara 2006). (Color figure online)

(while iP-type cytokinins are mostly formed in shoots) (Fig. 3), thus further confirming the tissue- and organ-specific regulation of CK biosynthesis.

The CK ribotides can be converted into the corresponding free bases by two pathways, the two-step activation pathway and the direct activation pathway (Chen, 1997; Kurakawa et al. 2007). In the two-step activation pathway, CK ribotides are hydrolyzed to CK ribosides by 5-ribonucleotide



**Fig. 3** Cytokinin biosynthesis, transport, and homeostasis in plants. **a** Sites of biosynthesis and distribution. *tZR* and its inactive precursor *tZR* are synthesized mainly in the root; both are transported through the xylem towards the shoot. Later *tZR* is activated by *LOG* enzymes; *iZR* acts directly on leaf size, and root-derived *tZR* controls both leaf development and SAM activity. On the other hand, *iPR* and *iP* are synthesized mainly in the shoot and are distributed to the active site through the phloem ( modified from Davière and Achard P 2017). **b** Model of cellular homeostasis of cytokinins. The first reaction of the de novo biosynthesis of CKs is catalyzed by the *IPT* enzymes (blue), which are located mainly in plastids and produced *trans*-zeatin-type and *iP*-type CKs through the MEP pathway; nevertheless, *IPT7* is localized in mitochondria and *IPT3* undergoes a process of farnesylation that causes its translocation in the cytosol and nucleus. The *cis*-zeatin-type CKs are mainly synthesized through the MVA pathway in the cytosol. The *LOGs* enzymes (purple), which transform CK nucleotides into an active form, are located in the cytosol and nucleus. *CKX* enzymes (green), which degrade CKs, are preferably located in the apoplast, although they are also present in the cytosol and

phosphohydrolases. Subsequently, CK ribosides are hydrolyzed to free bases by adenosine nucleosidase (EC 3.2.2.7) (Chen and Kristopeit 1981) (Fig. 2). To date, there are few reports on the functionality of the two-step activation pathway. The first study on the role of the two pathways came from studies in *A. thaliana* (Jung et al. 2009). The enzyme uridine-ribohydrolase 1 (URH1; EC 3.2.2.3) can hydrolyze the CK derivative isopentenyladenine-riboside, as well as uridine and inosine ribosides, and changes in the expression of this gene retard germination (Jung et al. 2009). More recently, two NRH subclasses have been identified in plants (Kopečná et al. 2013), one hydrolyzing the

vacuole. The conjugation of free bases occurs mainly with glucose, a reaction that is catalyzed by uridine diphosphate glycosyltransferases (*UGTs*), the *CK-O* glucosides localized primarily in the vacuole; nevertheless, they can be reversibly deglycosylated by a  $\beta$ -glucosidase enzyme in the cytosol. So far three intracellular CK transporters have been identified: *ABC14* is an exporter of *tZR*-type CKs, while purine permeases (*PUPs*) and equilibrative nucleoside transporters (*ENTs*) have been proposed for import of apoplastic CKs to the cytosol. It is possible that there is an intracellular transport system of the plastids, mitochondria, and vacuole to the cytosol; however, there is still no evidence for this. CK receptors *ARABIDOPSIS HISTIDINE KINASES* (*AHKs*) are localized at the plasma membrane, and the endoplasmic reticulum initiates the signaling cascade. CKs are perceived by the *AHK* receptors, which autophosphorylate and subsequently transfer the phosphate (blue arrows) to proteins (*AHP1* to *AHP5*), which enter the nucleus and phosphorylate type-B *ARABIDOPSIS RESPONSE REGULATORS* (*B-ARRs*) that activate transcription of CK response genes or *B-ARR* negative regulators of CK signaling. (Color figure online)

purine ribosides inosine and xanthosine and the other uridine and xanthosine, while both can hydrolyze CKs ribosides. Their substrate specificities depend on the amino acid at position 249. A tyrosine residue in this position is associated with preferential hydrolysis of purine ribosides, while the presence of aspartate confers high activity on uridine (Kopečná et al. 2013). However, it appears that the hydrolysis of CK ribosides is not the primary function of these enzymes.

There is a direct CK biosynthesis pathway producing free bases in a reaction catalyzed by phosphoribohydrolases encoded by members of the *LONELY GUY* (*LOG*) gene family. The protein cytokinin

riboside 5'-monophosphate phosphoribohydrolase (LOG, EC:3.2.2.n1) was discovered through the analysis of *O. sativa* mutants (Kurakawa et al. 2007) characterized by premature termination of the shoot meristem. LOG encodes an enzyme able to produce CKs from their ribosides in one step, by hydrolyzing the bond between the N<sup>6</sup>-substituted bases and the ribose 5'-monophosphate (Kuroha et al. 2009; Seo and Kim 2017). The direct hydrolysis of the CK ribotides into free CKs has significant biological implications, since it provides a direct mechanism for regulation of CK biosynthesis, where CKs ribotides can be transported to the site of action and then converted into free CKs. Studies with mutants of *A. thaliana* and *Medicago truncatula* showed that *LOG* genes participate in the nodule primordium development and lateral root formation (Kuroha et al. 2009; Mortier et al. 2014; Tokunaga et al. 2012), while *LOG* overexpression reduced apical dominance, retarded leaf senescence and increased cell division rates in root vasculature and embryos (Kuroha et al. 2009).

Although the initial step of CK de novo biosynthesis mainly occurs in plastids, further processes occur in the cytosol. Accordingly, the CK-activating LOG enzymes are localized mainly in the cytosol (Kuroha et al. 2009), like the antagonistic adenine phosphoribosyltransferases (APT1, APT2, APT3; EC: 2.4.2.7) catalyzing the conversion of free CKs into corresponding nucleotides (Allen et al. 2002; Moffatt et al. 1991; Zhang et al. 2013). Together, these findings imply a transport system that moves precursors from the plastids to the cytosol, however evidence for this transport system is lacking to date (Fig. 3).

In *A. thaliana*, *tZR* is the most abundant CK detected in the xylem, comprising approximately 80% of the total CKs, while *tZ* is second-most abundant (Osugi et al. 2017). *tZR* and *tZ* are transported through the vascular system from the root to the shoot using ABCG14, an ATP-binding cassette transporter (ABC) (Ko et al. 2014). Recent studies have shown that *tZR* and *tZ* are the primary long-distance signaling forms in the xylem which, together, control leaf size and meristem activity-related traits (Osugi et al. 2017). On the other hand, studies on the expression of the *LOG* family genes in *A. thaliana* and *O. sativa* suggest that the production of free CKs occurs in virtually all parts of the plant (Kurakawa et al. 2007; Kuroha et al. 2009; Yamburenko et al. 2017). Therefore, the plants use *tZ* and *tZR* as messengers, which are transported from the

root to the shoot, and iP-type CKs as systemic messengers distributed through the phloem to the active site (Fig. 3). Although the general features of terpenoid CK biosynthesis have been established, there are still several aspects of the process that are not fully understood. Also, we have almost no information on the biosynthesis of aromatic CKs.

### Regulation of cytokinin biosynthesis

Ever since the discovery of CKs, their interaction with other growth regulators has been studied, in many cases focusing on the relative hormone concentrations in various plant developmental processes. Early on, it was shown that endogenous CK accumulation was inhibited when the tissue was cultured on auxin-containing medium (Beinsberger et al. 1991; Hansen et al. 1985; 1987; Zhang et al. 1995). Further studies into the underlying mechanisms proved that auxin reduces the expression level of *IPT* genes (Song et al. 1995; Zhang et al. 1996). Later, in vivo deuterium incorporation experiments in *A. thaliana* showed that exogenous auxin exercises a rapid negative control over the CK biosynthesis pathway (Nordström et al. 2004). Auxin also represses local CK biosynthesis in the nodal stem of *Pisum sativum* through the expression of the *PSIPT1* and *PSIPT2* genes (Tanaka et al. 2006). However, in actively growing tissues, such as developing roots and shoots, the CKs induce auxin biosynthesis, suggesting that a regulatory feedback loop maintains auxin and CK concentrations optimal for cell- and tissue-specific developmental processes (Jones et al. 2010). Subsequent studies demonstrated that auxin controlled the spatiotemporal distribution of CK biosynthesis through the negative regulation of *AtIPT* expression by AUXIN RESPONSE FACTOR3 (ARF3) during de novo SAM formation (Cheng et al. 2013). The ARF3 protein is a member of the transcription factor family that activates or represses auxin response gene expression (Leyser 2018). Recent studies have shown that ARF3 also regulates CK biosynthesis directly by repressing the expression of *IPT* genes and indirectly repressing *LOG* genes during floral meristem formation (Zhang et al. 2018). On the other hand, auxin upregulates *CYP735A* genes in *A. thaliana* and *O. sativa*; however, the molecular mechanism involved is still unknown (Takei et al. 2004b; Tsai et al. 2012).



There is evidence that CK biosynthesis is also affected by other growth regulators such as strigolactones (SLs) and abscisic acid (ABA). In roots of SL-deficient or SL-insensitive mutants of pea and Arabidopsis, CK amounts are dramatically reduced, although there are no differences in the CK levels in the shoot apices (Foo et al. 2007; Morris et al. 2001). Subsequent studies showed that in *P. sativum* the expression of the CK biosynthesis gene *PsIPT1* is increased in the nodes and internodes of the shoot of the SL mutant (Dun et al. 2012); however, the expression of *PsIPT1* and *PsIPT2* genes in the stem is not affected by SL defects. Furthermore, the addition of synthetic SL did not affect transcript levels for either *IPT* genes, similar to the effect in *O. sativa* (Dun et al. 2012; Xu et al. 2015; Young et al. 2014). Recent studies in *Zantedeschia* have suggested an antagonistic interaction between SLs and CKs during adventitious bud development, since high SL levels were detected in a little branched cultivar while, conversely, the CK concentration was low compared in a more highly branched cultivar (Manandhar et al. 2018). Further research is needed to determine how SLs and CKs interact, as it is possible that SLs affect the CK metabolism through additional undiscovered members of gene families involved in their homeostasis or by regulation at the post-translational level.

Regarding the effect of ABA in CK biosynthesis, it is known that ABA treatments cause downregulation of essential CK biosynthetic genes such as *IPT* and *CYP735A*, which triggers a reduction in CK concentration (Nishiyama et al. 2011; Takei et al. 2004b; Tsai et al. 2012). In addition, a decrease in ABA content causes an increase in CK levels during bud sprouting in grapevine (Noriega and Pérez 2017), suggesting an antagonistic role between both growth regulators.

The biosynthesis of CKs is also modulated by the availability of mineral nutrients and environmental changes. Nitrate promotes the accumulation of CKs in the roots of *Hordeum vulgare*, *Z. mays* and *A. thaliana* (Takei et al. 2001b), similar to its function in tobacco and sunflower leaves (Salama et al. 1979; Singh et al. 1992). In Arabidopsis, the expression of *AtIPT3* and *AtCYP735A2* is rapidly induced by nitrate, leading to the accumulation of *tZR* and the *tZRMP* in roots. However, the amounts of *AtIPT5* transcript in lateral root primordia increases with the availability of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  in long-term treatments (Kiba et al. 2010; Miyawaki et al. 2004; Takei et al. 2004a; Wang et al.

2004). Furthermore, in monocotyledonous plants like *O. sativa*, nitrogen increased CK amounts by enhancing the expression of *OsIPTs* (Xu et al. 2015).

Several studies have shown that CKs regulate various processes in the plants in response to nitrate, for example dictating leaf size in *S. lycopersicum* and *N. tabacum* (Rahayu et al. 2005; Walch-Liu et al. 2000), and SAM size and branch number in *A. thaliana* (Landrein et al. 2018; Müller et al. 2015). Furthermore, when little nitrate is available overall, then CKs enhance the number and development of lateral roots in those areas rich in  $\text{NO}_3^-$  (Kiba and Krapp 2016; Ruffel et al. 2011). Recent results have shown that nitrate availability controls the ratio of *tZ* to *tZR* in xylem sap (Osugi et al. 2017), further underlining that CKs act as long-distance signals mediating the shoot response to  $\text{NO}_3^-$  perception in roots. New studies have revealed that the CK biosynthetic and transport mutants *IPT3,5,7* and *ABCG14* in the presence of nitrate failed induce genes that respond rapidly to long-distance N-supply signals, indicating that N assimilation pathways in shoots are CK-dependent. Furthermore, levels of *tZ* in shoots of both mutants decreased, suggesting that *tZ* is an important element of the long-distance N-signaling network controlling root responses (Poitout et al. 2018). On the other hand, the expression of *AtIPT3* is also induced by the provision of other nutrients like iron, sulfate and phosphate to the roots (Kiba et al. 2010; Séguéla et al. 2008). In addition, salicylic acid induces CK biosynthesis, increasing the level of expression of the *IPT7* gene in *A. thaliana* and *Sorghum bicolor*. In both species, silicon accumulation delays dark-induced leaf senescence (Markovich et al. 2017). Therefore, to manage resource availability, *IPT* genes can affect overall plant development by controlling the biosynthesis of different active forms of CKs in response to either scarcity or excess of a nutrient.

### Cytokinin catabolism

One of the factors controlling intracellular CK levels is their conjugation, mainly with  $\beta$ -D-glucose at the *O*- and *N*-positions. *O*-glycosylation occurs at the hydroxyl group of the Z and DHZ side chains, while *N*-glycosylation occurs at positions  $N^3$ ,  $N^7$  or  $N^9$  of the purine ring (Entsch et al. 1979; Entsch and Letham 1979). The glycosylation reaction is catalyzed by UGT

enzymes (Lairson et al. 2008; Mok et al. 2000) and, for example, the *O*-glycosylation of Z is carried out by zeatin *O*-glucosyltransferase (EC: 2.4.1.203). In Arabidopsis, five genes encoding CK-specific UGT enzymes (*UGT76C1*, *UGT76C2*, *UGT85A1*, *UGT73C5*, and *UGT73C1*) have been identified (Hou et al. 2004; Jin et al. 2013; Li et al. (2015); Wang et al. 2011). *UGT76C1* and *UGT76C2* are specific cytokinin *N*-glucosyltransferases conjugating CKs on the  $N^7$  and  $N^9$  positions of the adenine moiety (Hou et al. 2004; Wang et al. 2011; 2013), while *UGT85A1*, *UGT73C5*, and *UGT73C1* catalyze the *O*-glycosylation of *tZ* and DHT (Hou et al. 2004; Jin et al. 2013). Both loss-of-function and gain-of-function mutants of CK-specific UGTs (*ugt76c2* and *ugt76c1*) change the expression levels of AHK2, AHK3, ARR1, CYP735A2, LOG2, IPT5, and CKX3, suggesting a tight control of CK homeostasis (Wang et al. 2011, 2013). Several studies have shown that CK-specific UGTs are mainly located in the cytosol (Jin et al. 2013; Šmehilová et al. 2016).

The CK *O*-glucosides can be de-glycosylated and converted into active forms by  $\beta$ -glucosidase enzymes (Brzobohaty et al. 1993; Kiran et al. 2006). New methods of quantification and purification in isolated organelles have shed light on the intracellular distribution of CKs, showing that *O*-glucosides are mainly found in the vacuole. Therefore, this organelle is an essential reservoir for storing CK conjugates, and it is possible that there are specific transporters for *O*-glucosides from the cytosol to the vacuole (Fig. 3) (Jiskrová et al. 2016; Kiran et al. 2012).

Further mechanisms regulating endogenous CK levels involve CK oxidase/dehydrogenase (CKX; EC: 1.5.99.12) enzymes catalyzing the irreversible oxidative breakdown of CKs into adenine and unsaturated aldehyde 3-methylbut-2-enal (Bilyeu et al. 2001; Brownlee et al. 1975; Galuszka et al. 2001, 2004; Niemann et al. 2018; Reid et al. 2016). CKXs are encoded by a multigene family, and *A. thaliana* has seven members located mainly in the apoplast, cytosol and vacuole. However, CKX1 is also localized in the endoplasmic reticulum (ER), from there CKX1 control the subcellular levels of CK, which triggers the signaling from the ER (Niemann et al. 2018; Werner et al. 2003).

## Cytokinin transport

CK homeostasis and signal perception rely on complex patterns of intercellular movement as well as local (short-distance) transport between neighboring cells and long-distance translocation for example between roots and shoots (Table 1) (Liu et al. 2019; Skalicky et al. 2018). The transport of CKs between organs and the intracellular trafficking involved are very actively being researched, and several review papers have recently summarized progress in this area (Durán-Medina et al. 2017; Kang et al. 2017; Ko and Helariutta 2017; Liu et al. 2019; Park et al. 2017). Here, only a brief summary of our present knowledge of CK transport will be provided.

CKs are transported from their biosynthesis site to their site of action by passive diffusion and through active transport mechanisms, and different CKs are translocated using various mechanisms. For example, the *tZ*-type CKs synthesized in roots are transported apoplastically to shoots, where they act by promoting growth (Beveridge et al. 1997; Hirose et al. 2008). Thus, the main CKs transported in the xylem sap are *tZ* and *tZR*, accounting for 15% and 80% of CKs there, respectively (Beveridge et al. 1997; Hirose et al. 2008; Kuroha et al. 2009; Osugi et al. 2017). Conversely, the *iP*- and *cZ*-type CKs synthesized in shoots travel through the phloem to roots (Corbesier et al. 2003; Hirose et al. 2008).

Three types of transporters are involved in CK trans-membrane transport and intercellular translocation. Among these, the ENT (Hirose et al. 2005; Sun et al. 2005) and PUP (Bürkle et al. 2003; Zürcher et al. 2016) transporters import apoplastic CKs nucleoside or nucleobase CKs into the cytosol, respectively (Fig. 3). Yeast studies showed that the ENT transporters of *A. thaliana* and *O. sativa* participate in the uptake of *iP*-ribosides and *tZ*-ribosides (Qi and Xiong 2013; Sun et al. 2005), while PUPs transport *tZ* and *iP* (Bürkle et al. 2003). However, PUPs and ENTs have also been implicated in the transport of other molecules, so they must be considered non-specific for CK transport (Durán-Medina et al. 2017; Gillissen et al. 2000; Girke et al. 2014). Very interestingly, the Arabidopsis PUP14 was recently found to modulate CK signaling (Zürcher et al. 2016) by regulating the availability of CKs in the apoplast, where they are perceived by ARABIDOPSIS HISTIDINE KINASE (AHK) receptors. The Arabidopsis AHK2, AHK3 and

AHK4 proteins are located in the plasma membrane and the ER, and they are involved in transcriptional activation of various target genes (Fig. 3b) (Caesar et al. 2011; Lomin et al. 2018; Pernisová et al. 2018; Wulfetange et al. 2011; Yamada et al. 2001).

The third type of CK transporters, ATP-binding cassette (ABC) transporters, G subfamily, affects the acropetal long-distance transport of CKs from the root to the shoot (Ko et al. 2014; Zhang et al. 2014). In particular, the *AtABCG14* and *OsABCG18* proteins participate in xylem loading and are essential for the long-distance transport of root-derived CKs and, thus, the positive regulation of shoot growth (Kang et al. 2017; Zhao et al. 2019).

Interestingly, the *Arabidopsis* genome encodes numerous members of the three families of transporters, including 21 PUPs, 8 ENTs and 28 half-size ABCG-type transporters (Kang et al. 2011; Li et al. 2003; Zürcher et al. 2016). The numbers of homologs in each family varies drastically between plant species (Liu et al. 2019), with only four ENTs in rice for example (Hirose et al. 2005).

### Cytokinin signaling: a two-component system

The elucidation of the CK signaling pathway started two decades ago with the studies of CK effects on *Arabidopsis* tissues (Inoue et al. 2001; Kakimoto 1996). This pathway involves a phosphotransfer signal cascade, similar to that in bacteria and most fungi (including yeast). The plant signal cascade involves histidine kinases (HKs), histidine phosphotransfer (HPT) proteins, and a specific type of response regulators (RRs). To elucidate the different components of the CK signal cascade, experiments were performed in *Arabidopsis* mutants in combination with protoplast transient expression assays (Argyros et al. 2008; Ferreira and Kieber 2005; Higuchi et al. 2004).

The cytokinin signaling system found in plants is very similar to the two-component system for signal transduction occurring in bacteria (Grefen and Harter 2004; Schaller et al. 2007; Stock et al. 2000). The CK bind to the CHASE domain of HK receptors and the signal is transmitted across the membrane (Bartrina et al. 2017; Kieber and Schaller 2018; Steklov et al. 2013). The HK cytokinin receptors have a conserved cytokinin-binding extracytosolic CHASE (cyclases/

histidine kinases-associated sensing extracellular) domain (Kieber and Schaller 2018).

CKs binding to the hybrid histidine kinase receptors [AHK2, AHK3, AHK4 (CRE1/WOL)], results in the autophosphorylation of a conserved histidine (His) residue in their kinase domain. The phosphate is then first transferred to a conserved aspartic acid residue of the AHKs and then to a His residue of AHP proteins (Argyros et al. 2008). The modified AHPs move to the nucleus, where they transfer the phosphate group to two classes of ARR (Argyros et al. 2008; Gordon et al. 2009).

HK proteins are known to participate in different biological processes in various plant species, including responses to environmental changes and growth regulator signaling. The HK proteins can be divided into two groups, the ethylene and the non-ethylene receptors, and both groups are conserved between plant species with high structural similarity (Nongpiur et al. 2012). Eight transmembrane HKs belonging to the latter group have been identified in *Arabidopsis*, and more in other species like *G. max* with 17 AHKs (Müller and Sheen 2007a). Only some of the *Arabidopsis* AHKs function as CK receptors, with AHK2, AHK3 and AHK4 each binding to different CKs. The AHK4 receptor (also known as CRE1 or WOL1) was the first component of CK signaling identified, phosphorylating AHPs when CKs are present and, conversely, dephosphorylating AHPs in their absence (Higuchi et al. 2004). AHK single and double mutants were not affected in CK signaling, but triple mutants showed severe phenotypes, especially in roots and shoots, suggesting highly redundant functions of these receptors (Ferreira and Kieber 2005). Root elongation in particular was reduced in *ahk4* single mutants, but not in *ahk2* and *ahk3* single and double mutants (Ferreira and Kieber 2005).

The HK receptors possess a conserved region crucial for CK binding of 200 to 230 amino acids, designated as cyclases-/histidine kinases-associated sensory extracellular (CHASE) domain (Anantharaman and Aravind 2001; Hutchison and Kieber 2002). The same domain is present in a variety of receptor-like proteins from prokaryotes and eukaryotes (Anantharaman and Aravind 2001; Mougél and Zhulin 2001). The CHASE domain in the histidine kinase PcrK sensing CKs in the bacterial pathogen *Xanthomonas campestris* pathovar *campestris* was crystallized and its structure determined at 2.55 Å

resolution (Chen et al. 2019). The PcrK protein is a homodimer with overall topology likely similar to AHK4, but also with several significant differences. Among these, the bacterial protein has a ligand-binding pocket with limited size to allow only the binding of iP, whereas the plant CK receptor can interact with a broad spectrum of CKs. All bacterial PcrK homologs have a positively charged residue Arg175 in the ligand-binding cavity important for binding negatively charged and hydrophobic groups, which is replaced by a negatively charged residue Asp262 in AHK4 (Chen et al. 2019).

Although the CHASE domain appeared a long time ago, it is still not clear how land plants acquired it, but there are several theories. One of them is that the *Ectocarpus siliculosus* virus-1 could have infected and integrated its genome into his host, the brown algae; and the other one, is through their chloroplasts, due to a cyanobacterial ancestry (Heyl et al. 2007; Schaller et al. 2011). In *A. thaliana* has been reported three histidine kinases containing the CHASE domain (AHK2, AHK3, and CRE1/AHK4) in which the difference among them is the number of transmembrane segments at their N-terminus. The CHASE domain at these proteins is the recognition site in CK receptors, and also plays an essential role in the regulation of plant growth and development, such as the vascular morphogenesis of the root (Mougel and Zhulin 2001; Müller and Sheen 2007b; Schaller et al. 2011).

There are five canonical AHP genes (AHP1-5) in the Arabidopsis genome comprising the conserved domain necessary for the phosphotransfer signal cascade, along with one pseudo-AHP gene. The latter, AHP6, lacks the His residue and does not participate in phosphate group transfer, therefore interfering and competing with the other AHPs and acting as a negative regulator of CK signaling. Nonetheless, mutants devoid of this protein show abnormalities in vascular tissue patterns (Hutchison et al. 2006; Hwang et al. 2012; Müller and Sheen 2007a). Further experiments with mutants deficient in different combinations of the other AHPs confirmed that AHP1-5 all play similar roles as positive CK signaling regulators, and that loss of their function causes damage to plant growth and development.

Phosphorylation of AHP proteins leads to nuclear translocation, followed by further transfer of the phosphate group first to B-ARRs, which in turn induce

further transfer to a conserved Asp residue of A-ARRs. More than 20 ARR genes have been identified and classified into four groups according to their C-terminal domain and similarities in their core domain sequence, including 10 A-ARRs (ARR3-9, ARR15-17), 11 B-ARRs, 2 C-ARRs and 9 pseudo-ARRs in Arabidopsis. The A- and B-ARRs are best studied (Argyros et al. 2008; To and Kieber 2008), establishing that B-ARRs have long C-termini with a Myb-like domain for DNA binding (also known as GARP), while type A-ARRs have short C-termini. Despite this difference, all ARR genes share a conserved domain for phosphorylation. B-ARRs are positive regulators of CK signaling, while A-ARRs are negative CK signaling regulators (Argyros et al. 2008; To et al. 2004). A-ARRs lack a DNA-binding domain and are affected in the presence of light. It is thought that they inhibit CK signaling by competing with the B-ARRs for phosphate groups transferred through the signaling cascade (Osugi and Sakakibara 2015; To et al. 2004; To and Kieber 2008). However, they can participate in signaling by integrating the ethylene and CK signaling pathways in a plant response to environmental stress and varying nitrogen levels, resulting in changes in the endogenous levels of CKs (Shi et al. 2012; To and Kieber 2008).

The Arabidopsis genome contains eleven B-ARRs (ARR1, ARR2, ARR10–14 and ARR18–21), of which seven belong to subfamily I and two each to subfamilies II and III. Characterization of B-ARR mutants confirmed that especially ARR1, ARR2, ARR10, ARR11 and ARR12 play pivotal, partially redundant roles (Argueso et al. 2010; Ishida et al. 2008; Yokoyama et al. 2007). It has recently been found that the CK signaling pathway is mediated by S-PHASE KINASE-ASSOCIATED PROTEIN1 (SKP1)/Cullin/F-box (SCF) E3-ubiquitin ligase complex and also includes a proteasome-mediated degradation, similar to other plant growth regulators like auxins (Kim et al. 2013a, b; Osugi and Sakakibara 2015). The F-box family protein named KISS ME DEADLY (KMD) regulates and interacts with B-ARRs in Arabidopsis and rice, thus affecting their ubiquitination-dependent degradation via the 26S proteasome and regulating their local levels and activity. KMD is interacting most strongly with ARR1, ARR2 and ARR12, which have been identified as the most important ARR genes in CK response (Kim et al. 2013a, b; Osugi and Sakakibara 2015).

ARRs can also interact with nuclear Cytokinin Response Factors (CRF), a family of APETALA2/ERF transcription factors comprising six members in *Arabidopsis* (Argueso et al. 2010; Müller and Sheen 2007a; Nongpiur et al. 2012). Mutants deficient in CRFs have altered expression of several (but not all) CK-related genes similar to B-ARR mutants. However, the mechanisms of CRF regulation and signal transduction are not yet known (Argueso et al. 2010; Hwang et al. 2012; To and Kieber 2008).

There are reports of other transcription factors involved in CK signaling, such as the GLABROUS1 enhancer-binding protein (GeBP/GPL) and splindy (SPY). The inactivation of GeBP/GPL transcription factors causes a reduction in the sensitivity to exogenous CKs and an increase in the expression of A-ARRs, antagonizing the negative regulation of CK signaling (Argueso et al. 2010). In *Arabidopsis*, loss-of-function mutants in SPY cause CK insensitivity, suggesting that SPY may be acting as a positive regulator of the CK signal cascade and also as a regulator of CK/gibberellin homeostasis (Greenboim-Wainberg et al. 2005).

The mechanistic understanding of the two-component CK signaling system has enabled the development of interesting molecular tools, most notably a synthetic CK reporter coupled with GFP (TCS::GFP) for *in vivo* tracking of the CK signaling output (Brenner et al. 2012; Hwang et al. 2012). This reporter was designed based on the conserved DNA-binding domain of the B-ARRs (A/G)GAT(T/C), with six direct repeats of this motif. Interestingly, the CK signaling output was first detected in the hypophysis and, thus, coinciding with relatively low auxin activity, showing that auxin is an antagonist to CKs (Brenner et al. 2012; Müller and Sheen 2008). However, the signal of the synthetic CK reporter was weak in some developmental tissues where high CK activity had previously been reported, and GFP expression was found to decrease over generation, suggesting that the repetitive DNA-binding motif may have led to silencing. To test this, a novel TCS reporter was developed that exhibited strong expression patterns that are stable from one generation to another (Zürcher et al. 2013).

Despite all the efforts made to elucidate the complete CK signaling pathway, questions remain. In particular, it is not known how the different components of the CK signaling pathway work, which

combinations of proteins participate in a given biological event and, specifically, which CK forms trigger certain response mechanisms in different plant models.

### Cytokinin crosstalk with ABA

Under normal conditions, plant cells receive signals and stimuli from adjoining tissues, and they respond to these signals such that physiological conditions are stabilized in the whole plant. However, this status can be disrupted if isolated cells or tissues are subjected to stress, due to changes in nutrient concentrations or environmental conditions, and affected cells initiate alternative regulatory mechanisms for acclimatization (Nic-Can et al. 2016). Those mechanisms include the reprogramming of gene expression and physiological as well as metabolic changes that let the cells survive and differentiate (Tardieu et al. 2018; VanWallendael et al. 2019). Stress tolerance involves mechanisms operating at different spatial and temporal scales that, together, greatly affect plant growth. Several PGRs play central roles in this response, and crosstalk among them is crucial to maintain the health of the plants. Crosstalk between CKs and ABA has been investigated in some detail, as it occurs in several processes both in response to stress and independent of it.

During the artificial bud sprouting induced in *Vitis vinifera*, ABA biosynthesis decreased while expression of CK-related genes increased, including the CK biosynthesis genes ISOPENTENYL TRANSFERASE (VvIPTs) and LONELY GUY (*VvLOG1*), the CK catabolism gene CYTOKININ OXIDASE (*VvCKX3*), and the key auxin biosynthesis gene *VvYUC3*. Together, these findings suggest that bud sprouting is preceded by a decrease in ABA content and an increase in CKs as well as auxin levels (Noriega and Pérez 2017). Bud outgrowth inhibition by low photosynthetic photon flux density (PPFD) was associated with lower CK and sugar contents and higher ABA content in the stem of *Rosa hybrida* ‘Radrazz’. CKs exogenously supplied to the stem restored bud outgrowth under low PPFD, and ABA supply antagonized the effect of CKs, further underlining that CKs and ABA play opposite roles (Corot et al. 2017).

The complexity of crosstalk among PGRs is manifested in the maintenance of stem cell and vascular differentiation during adventitious root

formation in Marubakaido apple rootstock, where crosstalk among CKs, auxins, plant peptide hormones and ABA occurs (Saito et al. 2019). A similar degree of complexity was also reported for the regulation of root growth under osmotic stress conditions, where ABA functions in interaction with a PGR network of CKs, ethylene and auxin (Rowe et al. 2016).

CKs regulate several aspects of plant growth and development, including plant response to abiotic stress (Albacete 2017), e.g., CKs negatively regulate salt (Aldesuquy et al. 2014; Tounekti et al. 2011) and drought stress signaling (Davies and Zhang 1991; Rivero et al. 2010). Consequently, CK-deficient plants are stress-tolerant, showing increased cell membrane integrity and ABA hypersensitivity (Nishiyama et al. 2011). Conversely, wild-type Arabidopsis plants show a decline in CKs both under stress and after ABA treatment, as a consequence of decreased expression of ISOPENTENYL-TRANSFERASE and CYTOKININ OXIDASES/DEHYDROGENASES genes (Nishiyama et al. 2011). Interestingly, also the uridine diphosphate glycosyltransferase 76C2, a CK glycosyltransferase, is also suppressed under these conditions, revealing novel cues in abiotic stress adaptation (Cai et al. 2018). These results suggest that a reciprocal regulation mechanism exists between the CKs and ABA metabolism in response to stress, plant growth and development (Nishiyama et al. 2011). The consequence of the stress-induced changes in CKs and ABA is leaf abscission, likely to reduce water loss and to enhance the survival of perennial plants, albeit at the expense of reduced growth (Rivero et al. 2009). On the other hand, the addition of kinetin induces tolerance against various abiotic stresses such as drought in *Nicotiana tabacum* (Rivero et al. 2010), and salinity in *Triticum aestivum* (Aldesuquy et al. 2014) as well as *Salvia officinalis* (Tounekti et al. 2011).

The presence of heavy metal in the soil is another source of stress for plants (Kumar et al. 2019), and both Cadmium and Zinc are important contaminants in shoreline areas. Presence of the latter metal reduces the endogenous CKs concentration in *Kosteletzkya pentacarpos* whereas, conversely, the addition of exogenous CKs improves plant growth, stomatal conductance, net photosynthesis, total ascorbate and reduced oxidative stress in Zn-treated plants maintained in the absence of NaCl. The presence of Cd in addition to Zn induces specific physiological responses involving, in particular, increased levels of

ABA (Zhou et al. 2019). The presence of salt has severe impact on senescing PGR, independent of heavy metal pollution (Albacete et al. 2009; Lutts and Lefèvre 2015; Rivero et al. 2009; Singh et al. 2018). Finally, the addition of exogenous CKs also increases plant resistance to heavy metals in *Lupinus termis* (Gadallah and El-Enany 1999), *Pisum sativum* (Al-Hakimi 2007), *Zea mays* (Lukatkin et al. 2007), *Alyssum murale* (Cassina et al. 2011), *Helianthus annuus* (Cassina et al. 2012), *Solanum melongena* (Singh and Prasad 2014) and *S. lycopersicum* (Singh et al. 2018).

## Conclusions and perspectives

Our understanding of the CK biosynthesis and signaling pathways has improved substantially over the last several years. However, some of the roles CKs play, especially during the early stages of cell fate programming and the induction of somatic embryogenesis, are still elusive (Pernisová et al. 2018). This is due, in part, to the complexity of the set of structures with CK activity, where in particular the biosynthesis of aromatic CKs is largely unknown. It will be important to investigate how CK biosynthetic intermediates are intracellularly mobilized and whether specific transporters are required for this. Important questions to be addressed are whether multiple copies of PUPs and ENTs are involved in transport, whether other transporters are involved, and in how far the CK transporters are regulated independently? (Kang et al. 2017).

Another important subject that needs more attention is the occurrence of CK isomers on monocotyledons and eudicotyledons, and their potentially different functions in various plant systems. Furthermore, CK distributions in individual cell compartments should be mapped in more detail, including in the apoplastic space (Zürcher et al. 2016), to understand the physiological role of these molecules. The use of new techniques, such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry imaging, could help to localize and quantify PGRs (Shiono et al. 2017). Since the vacuole appears to be a reservoir for storing CK conjugates, import and export from this organelle likely involve active transport systems; however no such transporters have been found to date.

Even though it is known that different copies of IPT genes are expressed throughout the Arabidopsis plant (Miyawaki et al. 2004, 2006; Takei et al. 2004b), the biosynthesis of the iP-ribosides is restricted to seedling roots (Gelová et al. 2017; Žďárská et al. 2013). Together, these findings suggest active transport toward the shoot, but the physiological functions of this transport system remain to be explored.

Another emerging area of research addresses the potential crosstalk between different growth regulators. Elucidating the interaction between CKs, auxins, ABA, strigolactones and gibberellins, and how they affect the homeostasis of each other, will lead to a better comprehension of plant development. Since auxins control the biosynthesis of CKs and SL in the stem, it must be clarified if these growth regulators serve as second messengers for auxins (Domagalska and Leyser 2011).

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