Cytokinin Regulation of Plant Growth and Stress Responses

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Abstract Plant hormones cytokinins stimulate cell division; regulate shoot and root development; promote leaf growth and flower, fruit, and seed formation; stabilize photosynthetic machinery; suppress senescence; and enhance sink strength and nitrogen acquisition. Cytokinin signaling is mediated by multistep phosphorelay. Binding of the cytokinin molecule to CHASE domain of the histidine kinase receptors triggers an autophosphorylation of the histidine domain and subsequent intramolecular transfer to receiver domain. Phosphoryl group is then transmitted to histidine phosphotransfer proteins and subsequently to type B response regulators (transcription factors) in the nucleus. Phosphotransfer proteins interact also with transcription factors CRFs (cytokinin response factors) that represent signaling side branch. The signal strength is regulated by cytokinin metabolism, which controls levels of active cytokinins, through feedback inhibition of signal transduction via type A response regulators (primary response genes), S-nitrosylation of phosphotransfer proteins, and/or proteasome degradation of type B and type A response regulators. Practical applications of cytokinins include their use in in vitro micropropagation, stimulation of flower branching, crop tillering or berry formation, and prolongation of fruit or tuber shelf life. Targeted elevation of cytokinin levels was found to increase the tolerance of plants to abiotic stresses, at least partially by diminishing the negative stress effects on photosynthesis. Recently, function of cytokinins in biotic stress responses has been also recognized. Full utilization of cytokinin potential to improve plant productivity by regulation of plant development has been until now limited by the necessity of targeting modulation of their levels or signal transduction in a time- and tissue-specific manner.

Keywords Cytokinin • Receptor • Response regulator • Application • Stress response

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In plants, the cytokinins (CKs) were defined as substances stimulating cell division (cytokinesis) in tissue cultures (Miller et al. 1955). Apart of this effect, CKs exhibit a wide range of physiological functions, including regulation of shoot and root apical meristems, stimulation of branching, vascular development, chloroplast differentiation, stabilization of the structure and function of the photosynthetic machinery, delay of senescence, stomata opening, and elevation of the sink strength and nutritional signaling (Mok and Mok 2001; Spichal 2012; Chernyadev 2009; Kiba et al. 2011; Ruffel et al. 2011). Naturally occurring CKs are N^6-substituted adenine derivatives with either isoprenoid or aromatic side chain. Due to their predominance in plant tissues, attention has been focused mainly on isoprenoid CK research. The first identified natural CK, *trans*-zeatin (tZ), was named according to the species from which it was isolated (*Zea mays*, Letham 1963). Later on, other physiologically active CKs were identified: isopentenyladenine (iP), *cis*-zeatin (cZ), and dihydrozeatin (DHZ) as well as their ribosides (Sakakibara 2006). According to the activity in bioassays as well as the affinity to CK receptors, the most active CK is tZ, followed by iP (Spichal et al. 2004). Considerably less active cZ also widely occurs, being highly abundant in some species, especially in the monocots (Gajdosova et al. 2011). Apart from the species specificity, cZ seems to play a role in the stress responses (Dobra et al. 2010). DHZ was found predominantly in dormant seeds and apical buds, where it may serve as a source of active CKs before acceleration of de novo biosynthesis after germination (Frebort et al. 2011). CKs were detected not only in land plants but also in algae (Ordog et al. 2004), mosses (von Schwartzenberg et al. 2007), cyanobacteria, or symbiotic bacteria (Droog et al. 1997). Production of CKs by biotroph pathogens, e.g., *Agrobacterium tumefaciens* (Akiyoshi et al. 1984) or *Rhodococcus fascians* (Crespi et al. 1994), is part of their plant invading strategy. Detailed phylogenetic analysis of the occurrence of CKs and components of their signaling pathway was described by Spichal (2012).

Biosynthesis and Metabolism of CKs

The rate-limiting step of CK biosynthesis is transfer of isopentenyl moiety from dimethylallyl diphosphate (DMAPP) or its hydroxylated derivative (HMBDP) to adenosine 5′-phosphate (ATP and ADP in plants or AMP in bacteria). This reaction is catalyzed by isopentenyltransferase (IPT). In fact, CK biosynthetic genes were first characterized in *A. tumefaciens*. Plant *IPTs* were identified in *Arabidopsis thaliana* based on the homology with bacterial genes (Kakimoto 2001; Takei et al. 2001a). Plant IPTs strongly prefer ATP and ADP to AMP as well as DMAPP. The CK side chain may originate either from the plastid methylerythritol phosphate (MEP) pathway or from the cytoplasmic mevalonate (MEV) pathway. The prevalence of plastid pathway, in case of tZ and iP, was reported by Kasahara et al. (2004). The scheme of CK metabolism is shown in Fig. 1.

Some IPTs (AtIPT2 and AtIPT9) are tRNA-IPTs that prenylate adenine moieties adjacent to the 3′-end of the anticodon of specific tRNAs. This modification

Fig. 1 The scheme of cytokinin metabolism. Donor of isoprenoid side chain can originate either from plastid methylerythritol phosphate (MEP) or from the cytoplasmic mevalonate (MEV) pathway. *IPT* isopentenyltransferase, *CYP450* cytochrome P450 monooxygenase, *LOG* cytokinin phosphoribohydrolase (LONELY GUY), *CKX* cytokinin oxidase/dehydrogenase, *ZOG* zeatin *O*-glucosyltransferase, *N-GT* cytokinin *N*-glucosyltransferase, *GLU* β-glucosidase, *IS* zeatin *cis-trans* isomerase, *ZR* zeatin reductase (adapted from Frébort et al. 2011, J Exp Bot 62: 2431–2452)

supports codon–anticodon interaction, as indicated by the disturbed interactions in bacterial tRNA mutants lacking CK moiety at A_{37} (Einset et al. 1976). Highly prevailing CK moiety in tRNAs is cZ. The tRNA degradation has been considered as an important source of cZ (Kamada-Nobusada and Sakakibara 2009).

In plants, the IPT-catalyzed reaction results in iP nucleotides that may be hydroxylated with cytochrome P450 monooxygenases (CYP735A1 and CYP735A2, Takei et al. 2004). As CK nucleotides are not active, gradual cleavage to nucleosides (i.e., ribosides) and bases by nucleotidase and nucleosidase was anticipated. Later on, CK-activating enzyme phosphoribohydrolase LOG (LONELY GUY), which converts all CK mononucleotides directly to the CK bases, was found (Kurakawa et al. 2007).

Due to the high physiological activity of CKs, the levels of the active forms need to be strictly regulated with respect to the plant developmental stage as well as environmental conditions. The key enzymes downregulating CK levels are cytokinin oxidases/dehydrogenases (CKX), which cleave the N^6 side chain. Oxidative cleavage of CKs was first described by Paces et al. (1971). Later on, the enzyme was identified as cytokinin oxidase (Whitty and Hall 1974). The structure of CKX was first reported by Houba-Hérin et al. (1999). Galuszka et al. (2001) reclassified the enzyme as a dehydrogenase possessing slight oxidase activity. The substrates for CKXs are CKs with unsaturated side chain (iP, tZ, and cZ), while DHZ, containing a saturated side chain, and CKs with aromatic ring at N^6 position are resistant to these enzymes.

Another important mechanism of suppression of CK activity is their glycosylation. Irreversible glucosylation occurs at the adenine ring in N^7 or N^9 position. CK *N*-glucosyltransferase was first isolated from radish (Entsch and Letham 1979). O-glucosylation (or much less frequent O-xylosylation) of the side chains containing a hydroxyl group or quite rarely observed N^3-glucosylation is reversible (Mok and Mok 2001). The enzymes *trans*-zeatin *O*-glucosyltransferase, *trans*-zeatin *O*-xylosyltransferase, and *cis*-zeatin *O*-glucosyltransferase were identified by Martin et al. (1999a, b, 2001, respectively). CK O- and N^3-glucosides can be hydrolyzed back by glucosidases (Brzobohaty et al. 1993). Very rare CK modification is the binding of L-alanine to N^9 position of purine ring reported in lupine (Entsch et al. 1983) or the formation of methylthioderivatives in *R. fascians* (Pertry et al. 2009). Recently, in vitro interaction between CKs and NO (or more precisely with the product of NO reaction with superoxide–peroxynitrite) was reported (Liu et al. 2013). The authors identified several nitro- and nitroso-*trans*-zeatin and iP derivatives and suggested that reaction of CKs with NO can control levels of reactive nitrogen species in vivo.

Signal Perception and Execution of CK-Induced Responses

The CK signaling pathway is mediated by the multistep phosphorelay, similar to a two-component system found in bacteria for perception of extracellular stimuli (Argueso et al. 2009).
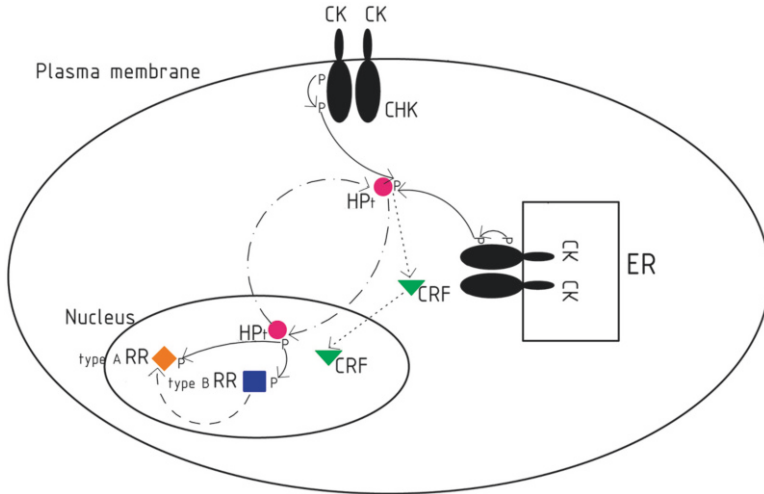
Fig. 2 The scheme of cytokinin signaling pathway mediated by phosphorelay. The binding of cytokinin molecule by a CHASE-domain histidine kinase receptor (CHK) at the endoplasmic reticulum or plasma membrane results in autophosphorylation of the histidine kinase domain and transfer of the phosphoryl group to the receiver domain. Phosphoryl group is then transferred to histidine phosphotransfer proteins (HPts) in the cytoplasm, and the phosphorylated HPts cycle between the cytoplasm and nucleus and pass the phosphoryl group to type B response regulators (type B RRs). The activated type B RRs stimulate transcription of cytokinin primary response genes, including those encoding type A response regulators (type A RRs), which negatively affect signal transduction, at least partially by competing with type B RRs for the phosphate transferred by HPts. The signal from HPts may also be transferred to other transcription factors, the cytokinin response factors (CRFs)

The scheme of cytokinin signaling pathway mediated by phosphorelay is shown in Fig. 2.

Cytokinin Receptors

CK molecule is bound by CHASE-domain-containing histidine kinase receptors (CHKs, Heyl et al. 2013). The receptors contain sensor CHASE domain, which serves for interaction with CKs (Heyl et al. 2007), histidine kinase (HK) domain, and receiver domain (West and Stock 2001; Ueguchi et al. 2001). The binding of CKs to CHASE domain triggers an autophosphorylation of the receptor and subsequent intramolecular transfer of phosphoryl group (Gruhn and Heyl 2013). The phosphoryl residue is transferred to histidine phosphotransfer proteins (HPts) and subsequently to type B response regulators (RRs) (Shi and Rashotte 2012). The first CK receptor was described in *Arabidopsis* as a two-component hybrid molecule that regulates vascular morphogenesis WOL (WOODEN LEG, Mahonen et al. 2000). This CK receptor kinase was named also as CRE1 (cytokinin response 1, Inoue et al. 2001) or AHK4 (*Arabidopsis* histidine kinase 4, Suzuki et al. 2001;

Yamada et al. 2001). Ueguchi et al. (2001) reported AHK4 together with two other CK receptors, the AHK2 and AHK3. The CK receptors exhibit partially overlapping activity, especially in roots, but play specific roles, given by their expression pattern and ligand specificity (Stolz et al. 2011). Recently, receptors were reported to be localized to the endoplasmic reticulum membrane (Caesar et al. 2011; Lomin et al. 2011; Wulfetange et al. 2011). However, their localization to plasma membrane can still be anticipated, too.

The ligand specificity of CK receptors is given by their CHASE domain, which was originally described as "Cyclase/Histidine kinase-Associated Sensing Extracellular" domain (Anantharaman and Aravind 2001; Mougel and Zhulin 2001). This full name was recently suggested to be changed to "Cyclase/Histidine kinase-Associated SEnsing" (Steklov et al. 2013). The crystal structure of the AHK4 CHASE domain was determined by Hothorn et al. (2011), who described also the AHK4 interactions with iP, tZ, benzyladenine, kinetin, and thidiazuron. The N-terminus of the AHK4 molecule folds into a long stalk α-helix, followed by the CHASE domain, which consists of two PAS-like (Per–Arnt–Sim-like) domains connected by a helical linker. The last β-strand of the membrane-proximal PAS domain (proximal to the C-terminus) is covalently linked to the N-terminus of the stalk helix by a disulfide bridge, which brings the flanking membrane helices into close proximity. The membrane-distal PAS domain forms binding cavity for CK molecule. In the lower part of the ligand-binding pocket, the central β-sheet of the PAS subdomain is lined by small hydrophobic residues, such as Ala and Gly. The hydrophobic upper part of the binding site is formed by two β-strands. The purine ring of CK molecule is oriented in the binding cavity by hydrogen bonds with Asp_{262} and Leu_{284}. Approximately 20 amino acid residues are in contact with tZ. Three water molecules in the cavity mediate the additional interactions, including the hydrogen bond between Thr_{294} and side-chain hydroxyl group of tZ, which is the reason for much higher affinity of AHK4 to tZ than cZ (Spichal et al. 2004).

Phylogenetic analysis of ca. 100 receptors (Steklov et al. 2013) indicated that CHASE domain (ca. 220 amino acids) together with adjacent domains (totally about 280 amino acid residues) is enclosed at both sides with transmembrane helices. These hydrophobic regions seem to play a role in the correct subcellular localization as well as in intramolecular signaling. One transmembrane region occurs between CHASE and downstream kinase domains. This means that CHASE domain and catalytic part of the protein are always located at different sides of the membrane. Specific mutations in this downstream transmembrane helix render receptors constitutively active regardless of the CK presence. The number of upstream transmembrane helices may vary among the receptor orthologous groups from 1 to 4. The CRE1/AHK4 orthologs possess only one upstream transmembrane region, whereas AHK2 orthologs have three or four transmembrane helices. In AHK3 orthologs, the number of upstream transmembrane regions may vary. Steklov et al. (2013) suggested that upstream transmembrane helices are predominantly responsible for receptor subcellular localization, while downstream helices are involved in the signal transduction. The conserved N-terminal helix α1, upstream of the CHASE domain, may fix the appropriate conformation of the distal PAS domain and may

regulate its movement upon CK binding. The substrate high-affinity binding results in specific conformational rearrangements of the PAS region in the sensory module. The signaling mechanisms of PAS domains were reviewed by Moglich et al. (2009). Signals originated within the conserved core generate structural and dynamic changes, which are propagated via amphipathic α-helical and coiled-coil linkers at the N- or C-termini of the core to the covalently attached effector domain. Many CK receptors were found to have a short coiled-coil motif that connects transmembrane helix with histidine kinase domain. Steklov et al. (2013) suggested that CK binding can affect the mode of interaction between ligand-binding PAS subdomains in the receptor dimer(s). Such change in the interaction mode of PAS subdomains might induce a mutual rotation of sensory modules relative to each other. The twist of sensory modules can in turn change the mutual position of transmembrane helices and cytoplasmic parts of receptors in dimer. As the histidine phosphorylation obviously occurs in *trans* by the parallel receptor, the change in relative position of receptors in dimer can switch on or off their kinase activity. Thus, formation of CK receptor complex results in HK activation and autophosphorylation of the conserved histidine in the catalytic module (West and Stock 2001). The phosphoryl group is then transferred intramolecularly to the conserved aspartate in the receiver domain. The HK domains of all *Arabidopsis* receptors have conserved histidine residue and five consensus motifs (H, N, G1, F, and G2). The receiver domain has conserved aspartate residue and three regions containing the conserved D, D, and K amino acid residues (Ueguchi et al. 2001). The subsequent transfer of CK signal is based on His–Asp phosphorelay (Grefen and Harter 2004; Muller and Sheen 2007; To and Kieber 2008; Schaller et al. 2011; Gupta and Rashotte 2012).

When ligand specificity of CK receptors was tested, AHK4 showed very high preference for tZ, followed by iP, and very low to cZ (Spichal et al. 2004). AHK3 showed only slight preference for tZ in comparison with tZR, iP, cZ, and DHZ. AHK2 has similar ligand specificity as AHK4 (Stolz et al. 2011). CK receptors differ also in their localization. *AHK4* is expressed predominantly in roots, especially in vascular cylinder and pericycle of primary roots. *AHK3* is expressed in rosette leaves, roots, stems, and flowers (Ueguchi et al. 2001; Higuchi et al. 2004). *AHK2* is expressed in leaves, roots, and flowers (Ueguchi et al. 2001). In accordance with their expression pattern, CK receptors were reported to be involved in the regulation of root vascular morphogenesis (Mahonen et al. 2000) and shoot vascular development (Hejatko et al. 2009), control of root meristem (Dello Ioio et al. 2007) and shoot apical meristem size and activity (Higuchi et al. 2004; Skylar et al. 2010), as well as retardation of leaf senescence (Kim et al. 2006) and abiotic stress responses (Tran et al. 2007; Jeon et al. 2010).

Since its identification, AHK4 role in regulation of root vascular development has been recognized. AHK4 is the main CK receptor involved in the control of root vascular tissues (Mahonen et al. 2000, 2006b). AHK2 and AHK3, together with CKI1 (CYTOKININ-INDEPENDENT1) HK, are important regulators of shoot vascular tissue development. Their mutation results in defects in procambium proliferation and absence of secondary growth (Hejatko et al. 2009). The size of the root meristem was found to be negatively affected by AHK3 signal transduction

(Dello Ioio et al. 2007). AHK3, but not the other CK receptors, plays a major role in CK-mediated chlorophyll retention and leaf longevity (Riefler et al. 2006; Kim et al. 2006). Homologues of AHK4 were reported to be indispensable for root nodulation in *Medicago truncatula* (Gonzalez-Rizzo et al. 2006) and *Lotus japonicus* (Tirichine et al. 2007). Cold-induced expression of a subset of type A *Arabidopsis* RR (*ARR*) genes, including *ARR5*, *ARR6*, *ARR7*, and *ARR15*, was shown to be mediated by the receptors AHK2 and AHK3 (Jeon et al. 2010).

It is interesting that AHK4 may function in the absence of CKs as a phosphatase, which dephosphorylates HPts and further suppresses CK signaling (Mahonen et al. 2006b).

Cytokinin Phosphotransfer Proteins

The components of CK signaling cascade downstream of receptors are the HPts that function as intermediate proteins to transfer the phosphoryl group from hybrid kinase receptors to downstream RRs (West and Stock 2001). In *Arabidopsis*, there are five authentic HPts (AHP1–5), which carry the conserved phospho-accepting His residue (Heyl and Schmulling 2003; Hutchison et al. 2006), and a pseudo-HPt (AHP6), which does not contain the conserved His residue necessary for phosphotransfer activity (Suzuki et al. 2000; Mahonen et al. 2006a). The AHPs have approximately 150 amino acids (Suzuki et al. 2000), except AHP4, which may occur in longer (145 aa) and shorter (127 aa) versions, and AHP5, which exhibits alternative splicing (Hradilova and Brzobohaty 2007). The authentic AHPs are positive regulators of CK signal transduction, which function to transfer phosphoryl group, obtained from AHKs, from the cytoplasm into the nucleus. Their continuous shuttling between the cytoplasm and nucleus was reported to be independent of their phosphorylation status (Punwani et al. 2010). The function of AHP4 is not clear; AHP4 had a slight positive effect in hypocotyl elongation assay, while in lateral root (LR) formation assay it acted as a negative regulator of CK response (Hutchison et al. 2006). However, it needs to be taken into account that *AHP4* transcription levels are very low in most tissues. AHP4 may play a role in specific developmental processes (e.g., anther endothecium formation, Jung et al. 2008). The pseudo-HPt AHP6 is a negative regulator of CK signaling. Its transcription is downregulated by CKs (Mahonen et al. 2006a). Recently, S-nitrosylation of AHP1 by NO at Cys_{115} was reported, which suppressed AHP1 phosphorylation and subsequent transfer of phosphoryl group to ARR1 (Feng et al. 2013). This finding indicates an important mechanism for regulation of CK-induced phosphorelay activity in plants.

The crystal structure of one HPt protein from maize, the ZmHP2, was determined several years ago (Sugawara et al. 2005). ZmHP2 contains four C-terminal helices that form an antiparallel bundle connected to two N-terminal helices by a β-turn. The phospho-accepting residue is His_{80}. The conserved residues surrounding His_{80} possibly act as a docking interface for receiver domains, while the

non-conserved residues seem to be responsible for specific activities of different HPt proteins. More recently, the crystal structure of MtHPt1, an HPt from *Medicago truncatula* (MtHPt1), was reported. The MtHPt1, with His_{79} as its phosphorylation site, consists of six α-helices, four of which form a C-terminal helix bundle. The coiled-coil structure of the bundle is stabilized by a network of S-aromatic interactions involving highly conserved sulfur-containing residues (Ruszkowski et al. 2013).

The *Arabidopsis AHP1* is expressed mainly in the roots; *AHP2*, *AHP3*, and *AHP5* transcripts are widely spread in plants (in roots, stems, leaves, flowers, and siliques). The highest *AHP2* expression is in roots and flowers, while *AHP3* is predominantly expressed in roots and leaves (Suzuki et al. 1998; Hradilova and Brzobohaty 2007). The *Arabidopsis* AHPs, especially AHP2, AHP3, and AHP5, were found to be negative regulators of the drought response (Nishiyama et al. 2013). The loss-of-function mutants of these three *AHP* genes exhibited strong drought tolerance, improved cell membrane integrity under stress conditions, and increased sensitivity to abscisic acid.

AHP6 is expressed in developing protoxylem and pericycle cells, shoot apices, and young leaves (Mahonen et al. 2006a). It promotes protoxylem formation by counteracting CK signaling (Mahonen et al. 2006a). AHP6 also functions as a CK repressor during early stages of lateral root (LR) development. AHP6 is expressed at different developmental stages during LR formation. It is required for the correct orientation of cell divisions at the onset of LR development. Recently, AHP6 was found to influence localization of the auxin efflux carrier PIN1 that is necessary for patterning the LR primordia (Moreira et al. 2013).

Cytokinin Response Regulators

As mentioned above, the HPts transport the phosphate signal, received from receptor AHKs, from the cytoplasm to the nucleus, and transfer the phosphoryl groups to response regulators (RRs) (Gupta and Rashotte 2012). In *Arabidopsis*, there are two main classes of RRs, type A and type B ARRs. Type B ARRs are transcription factors (Sakai et al. 2000) which upon phosphorylation of a conserved Asp residue activate transcription of CK response genes (including type A ARRs). The type B ARRs possess an N-terminal phospho-accepting receiver domain and a C-terminal output domain containing a GARP (GOLGI-ASSOCIATED RETROGRADE PROTEIN) family Myb-like DNA-binding and transactivating region. In addition, there is a conserved nuclear targeting sequence located in the Myb-like/B motif of the type B RRs (Imamura et al. 2001; Hosoda et al. 2002). Three subfamilies of type B ARRs may be distinguished. Subfamily I includes ARR1, ARR2, ARR10, ARR11, ARR12, ARR14, and ARR18. This subfamily is the most important in mediation of CK responses (Hwang and Sheen 2001; Sakai et al. 2001; Argyros et al. 2008). Subfamily II consists of ARR13 and ARR21, and subfamily III of ARR19 and ARR20. *ARR1*, *ARR2*, *ARR10*, and *ARR12* are expressed in young leaves. Their expression is restricted to the vascular tissues and hydathodes during

the leaf maturation. *ARR1*, *ARR2*, *ARR10*, *ARR11*, and *ARR12* are expressed in the roots, especially in root apical meristem and elongation zone (Birnbaum et al. 2003; Imamura et al. 2003; Mason et al. 2004; Tajima et al. 2004). The *ARR1* is expressed at similar level throughout the stele, endodermis, cortex, and epidermis, but *ARR10* is expressed at higher level in epidermis than in the other tissues (Mason et al. 2004; Birnbaum et al. 2003; Argyros et al. 2008).

The role of type B ARRs as positive regulators of CK signaling was demonstrated using *ARR2*-overexpressing plants which proved to be able to stimulate cell proliferation and shoot formation in the absence of exogenous CKs (Hwang and Sheen 2001). The fact that *ARR1*, *ARR2*, *ARR10*, and *ARR12* (but not *ARR11*, *ARR14*, *ARR18*, *ARR13*, *ARR19*, and *ARR20*) were able to complement *arr1arr12* mutant indicates functional diversities among the type B ARRs (Hill et al. 2013). The role of ARR1 and ARR12 in the control of cell division in shoot apical meristem seems to be mediated by transcriptional control of SHY2 (SHORT HYPOCOTYL 2), a suppressor of the auxin response (Dello Ioio et al. 2008). ARR2 was found to be activated downstream of AHK3 in the delay of leaf senescence (Kim et al. 2006). ARR1 and ARR12 were reported to suppress the expression of *AtHKT1;1* (*Arabidopsis thaliana* high-affinity K^+ transporter 1;1) that functions to remove sodium ions from the root xylem (Mason et al. 2010). ARR1 and ARR12 were thus suggested to delay the response to salinity stress.

The effects of the individual type B RRs on meristem size are generally consistent with their absolute transcript abundance, as well as with temporal changes in the expression (Hill et al. 2013). However, the ability of the type B RRs to stimulate transcription of CK response genes may be affected not only by the affinity or specificity to the target but also by potential interactions with HPts or transcriptional coregulators (Dortay et al. 2006; Kim et al. 2006). Promoter deletion analysis of the primary CK response gene *ARR6* showed that a combination of two extended motifs within the promoter is required to mediate the full transcriptional activation by ARR1 and other type B ARRs. The identification of a novel enhancer, which is not bound by the DNA-binding domain of ARR1, indicates that apart from type B RRs additional proteins might be involved in mediating the transcriptional CK response (Ramireddy et al. 2013).

The function of the type B RRs may be also affected by the protein stability (Kim et al. 2012). Recently, specific degradation of type B ARRs upon binding to a family of F-box proteins KMD (KISS ME DEADLY) was reported (Kim et al. 2013). KMD proteins form an S-PHASE KINASE-ASSOCIATED PROTEIN1 (SKP1)/cullin/F-box protein (SCF) E3 ubiquitin ligase complex and directly interact with type B ARR proteins. The *KMD* family members are broadly expressed, predominantly in shoot apical meristem (especially *KMD1* and *KMD2*) and in root tip (especially *KMD2* and *KMD3*) (Kim et al. 2006). They are localized both in the nucleus and in the cytoplasm. KMD proteins interact with ARR1, ARR12, and ARR20, less with ARR2 and ARR10. ARR1 and ARR12 were found unstable, readily to be degraded by proteasome, independently of CK presence. In contrast, degradation of ARR2 by proteasome requires CK-induced phosphorylation (Kim et al. 2012). KMD proteins seem to be key players of an important mechanism that is

responsible for reducing the levels of activated type B RRs, thereby preventing continued transcriptional activation by CKs (Kim et al. 2013). The representative members of type A ARRs, ARR4 and ARR7, were found not to be the substrates of KMD proteins.

Plant hormones regulate most physiological processes in an intensive cross talk. ARR2 seems to represent a link between CK and ethylene signaling pathways (Hass et al. 2004). ARR2 also makes a complex with TGA3 (TGACG-motif-binding transcription factor 3), a salicylic acid response factor. Salicylic acid signaling via NPR1 (NON-EXPRESSOR OF PATHOGENESIS-RELATED GENE1) enhanced binding of ARR2/TGA3 to the PR1 (PATHOGENESIS-RELATED PROTEIN1) promoter. CKs were thus found to promote resistance against *Pseudomonas syringae* in *Arabidopsis* (Choi et al. 2010).

Type B ARRs stimulate the expression of the type A *ARR*s (Hwang and Sheen 2001; Sakai et al. 2001), which are negative regulators of CK signaling and represent a negative feedback loop (CK signal switch-off). In *Arabidopsis*, ten type A ARRs were identified: ARR3, ARR4, ARR5, ARR6, ARR7, ARR8, ARR9, ARR15, ARR16, and ARR17 (To et al. 2004). These ARRs contain a phospho-accepting receiver domain, but no DNA-binding domain as do the type B ARRs. Phosphorylation of type A ARRs, for example, ARR5 and ARR7, at an aspartate of the phosphate receiver domain is a necessary prerequisite of their action as negative regulators (Lee et al. 2007; To et al. 2007). The mode of action of type A RRs seems to include competitive binding of the phosphoryl group from HPts at the expense of type B RRs. ARR5, ARR6, ARR7, and ARR15 were detected only in the nucleus; ARR4 and ARR16 were found both in the cytoplasm and in the nucleus (Hwang and Sheen 2001; Imamura et al. 2001).

Expression of several type A *ARR*s is rapidly induced by CKs, even after the inhibition of de novo protein synthesis (Brandstatter and Kieber 1998; Sakakibara et al. 1999; D'Agostino et al. 2000), suggesting that type A *ARRs* are CK primary response genes. To et al. (2007) specified a subset of type A ARRs stabilized by CKs, in part via phosphorylation (ARR5, ARR6, and ARR7), while ARR4 and ARR9 were not stabilized. The function of CKs as well as of proteasome in regulation of type A RR stability was studied by Ren et al. (2009). They found regulatory effect of CKs in case of ARR5, ARR6, ARR7, ARR8, ARR15, ARR16, and ARR17. Proteasome affected stability of ARR3, ARR5, ARR7, ARR8, ARR15, ARR16, and ARR17 (Ren et al. 2009).

Comparative analysis of *Arabidopsis* plants over-expressing individual members of type A *ARR*s showed their differential roles (Ren et al. 2009). The inhibitory effect of CKs on the primary root elongation was suppressed predominantly by *ARR3* and *ARR5* over-expression, followed by that of *ARR4*, *ARR16*, and *ARR17*. Inhibition of lateral root initiation was affected by most type A RRs, with the exception of ARR4, ARR5, and ARR7. Most type A RRs speeded up flowering, while only ARR16 was active in regulation of dark-induced leaf senescence. The strongest inhibition of CK-induced shoot formation was exhibited by ARR3, ARR5, ARR6, ARR16, and ARR17 (Ren et al. 2009). The expression of *ARR5*, *ARR6*, *ARR7*, and *ARR15* is repressed by transcription factor WUS (WUSCHEL,

"wuscheligen habitus"), in order to maintain optimal CK levels in shoot apical meristem (Leibfried et al. 2005). In root apical meristem, *ARR7* and *ARR15* transcription is positively regulated by auxin to maintain a balance between auxin and CK levels (Muller and Sheen 2008). A subset of type A *ARR*s, especially *ARR5*, *ARR6*, *ARR7*, and *ARR15*, are induced by cold (Jeon et al. 2010). Upregulation of type A *RR*s at the early phase of cold stress response is in accordance with transient downregulation of the active CK levels observed in winter wheat after exposure to cold (Kosova et al. 2012). ARR4 was found to represent a link between CKs and light signaling, interacting with phytochrome B (Fankhauser 2002; Sweere et al. 2001). ARR3 and ARR4 are involved in regulation of circadian rhythms (Salome et al. 2006).

Similar to type A RRs are the type C RRs, which are sometimes included into the type A RR group. Type C RRs have also only receiver domain. This domain is more related to the receiver domain of the hybrid histidine kinase receptors (Kiba et al. 2004; To and Kieber 2008). Additionally, type C RRs are not induced by CKs. This ARR group includes ARR22 and ARR24 (Gattolin et al. 2006). *ARR22* is expressed in flowers and developing pods, where it undergoes alternative splicing. Expression of *ARR24* was found restricted to pollen grains (Gattolin et al. 2006). Transcription of *ARR22* is induced by wounding, which may indicate a possible role of type C ARRs in response to biotic stresses (Gattolin et al. 2006). ARR22 interacts with AHP2, AHP3, and AHP5, acting as phosphohistidine phosphatase (Horak et al. 2008).

CRFs represent a side branch of CK signaling pathway. These proteins can interact directly with HPt proteins. The *Arabidopsis CRF*s are induced by CKs and belong to AP2-/ERF-like (APETALA2/ethylene-responsive factor) transcription factor family, distinct from type B RRs (GARP-/Myb-related family). CRFs share some targets with type B RRs but also activate some other genes (Rashotte et al. 2003, 2006). CRFs occur broadly in land plants (Rashotte and Goertzen 2010) and are involved in the normal development of embryos, cotyledons, and leaves (Rashotte et al. 2006).

Improvement of Plant Productivity with Biotechnological Manipulation of Cytokinin Biosynthesis and Signaling

The multiple physiological functions of CKs, which include regulation of germination, shoot and root development, leaf growth, flower and fruit formation, suppression of leaf senescence, enhancement of sink strength as well as uptake of nitrogen (Mok and Mok 2001), make this hormone class very perspective for practical applications. Unfortunately, effective regulation of particular physiological processes requires very precise time- and site-specific targeting of modulation of CK levels or signaling.

Until now, exogenous applications of CKs have been predominantly used in practice, to enhance shoot formation, branching, and tillering, to improve nitrogen acquisition, or to delay senescence (e.g., see Mala et al. 2013; Malabug et al. 2010; Gapper et al. 2005). Aromatic CKs are preferentially used, as these types of CKs are

not subjected to fast degradation by CKXs. N^6-(3-hydroxybenzyl)adenine (*meta*-topolin) became a very promising alternative for widely used N^6-benzyladenine (Strnad 1997). *meta*-Topoline has a high biological activity, which is in accordance with its relatively high affinity to AHK4 (Mok et al. 2005). As a good substrate of *trans*-zeatin *O*-glucosyltransferase (Mok et al. 2005), *meta*-topoline is metabolized to a storage *O*-glucoside, which can be gradually converted back to the active compound, in contrast to N^6-benzyladenine that is quickly *N*-glucosylated, resulting in a stable metabolite accumulated in basal parts of plants (Werbrouck et al. 1996). Recently, an alternative approach—suppression of degradation of endogenous CKs by inhibition of CKXs—has been tested (Zatloukal et al. 2008; Motte et al. 2013).

Since their discovery in 1955, CKs are routinely used in in vitro cultures for stimulation of shoot differentiation and propagation. Micropropagation techniques are used, for example, for cultivation of ornamental plants (orchids, chrysanthemums or carnations, e.g., see Ferreira et al. 2006), for multiplication of elite clones of forest trees (pine, elm, poplar, eucalyptus, and teak, e.g., see Mala et al. 2013), or for propagation of potato (Baroja-Fernandez et al. 2002). Exogenous CKs are used in classical horticulture to increase branching and thus the amount stem cuttings and flowers (Kaminek et al. 1987). Exogenous CKs have been also used for prolongation of the flower vase life, e.g., of gerberas (Danaee et al. 2011). Benzyladenine together with gibberellin was shown to promote plant growth and yield in three strawberry cultivars (Momenpour et al. 2011). When synthetic CK CPPU [*N*-(2-chloro-4-pyridyl)-*N*-phenylurea] and gibberellic acid were applied to various grapevine varieties at the fruit setting stage, these hormones increased berry size in Perlette, Superior, and Thompson Seedless cultivars. Gibberellin was found to enhance cell expansion, while CKs to increase cell number and density (Ben-Arie et al. 1997). CPPU was also found to increase berry mass and firmness, as well as cluster mass and compactness in *Vitis labrusca* and *V. labrusca* × *V. vinifera* in field trials (Zabadal and Bukovac 2006). Souza et al. (2010) reported positive effect of benzyladenine application on the quality of clusters of cv. Superior Seedless grapes. CKs were tested to increase a wheat grain yield by promotion of tillering. Their effect was significantly positive when the original plant density was low. In case of high plant density, the amount of seeds was also increased, but their size was reduced; thus, the yield was not enhanced.

CK application may also allow reduction of nitrogen fertilization, as CKs promote nitrogen acquisition (Takei et al. 2001b; Sykorova et al. 2008; Kiba et al. 2011; Pavlikova et al. 2012). This may solve the problems associated with high nitrogen levels in the field soils and underground waters which often result from heavy fertilization used to maintain high grain yields.

Surprisingly, CKs have been commercially used also in "non-plant" areas. Their antiaging effects were proved also for human skin, as evidenced by the fact that several types of cosmetics contain CKs (e.g., Pyratine-6 antiaging cream). Some CK analogues were found to block cell cycle progression not only in plant cells (Vesely et al. 1994) but also in humans (Vermeulen et al. 2002). These CK analogues were successfully tested as anticancer agents (Casati et al. 2011; Molinsky et al. 2013).

When genetic approach to elevation of CK levels is applied, it is necessary to prevent too strong *IPT* over-expression. High CK levels cause morphological abnormalities and very high levels may induce cell apoptosis (Mlejnek and Prochazka 2002). This problem was solved by Gan and Amasino (1995), who expressed *IPT* gene under the control of senescence-inducible promoter (*SAG12*) in tobacco. Stimulation of senescence program in plants resulted in an enhanced activity of *SAG12* promoter, leading to an increase of CK biosynthesis. Increased CK levels in turn suppressed the promoter activity, which prevented their overproduction. Repetition of these cycles allowed prolongation of the plant life-span. The *SAG:IPT* construct was successfully used in *Lactuca sativa* to delay developmental and post-harvest leaf senescence in mature lettuce heads (McCabe et al. 2001). No significant effect of transformation on the head diameter or fresh weight of leaves or roots was observed. Postponed plant senescence, accompanied by the delay in the loss of photosynthetic activity, was observed in maize expressing *ipt* under the control of a senescence-enhanced maize promoter (Robson et al. 2004). The elevation of CK content by expression of *SAG:ipt* in cassava plants delayed substantially the post-harvest senescence of cassava tuberous roots (Zhang et al. 2010). Prolongation of the shelf life may be very important in some developing regions, where cassava represents substantial part of the diet. When promotion of photosynthetic activity and delay of senescence are desirable, stimulation of CK biosynthesis is advantageous (Gregersen et al. 2013). In some cases, however, delay of leaf senescence may interfere with the developmental program. The over-expression of *SAG:ipt* in wheat prolonged substantially the vegetative period, increasing the sink strength of leaves, which interfered with grain filling (Sykorova et al. 2008). Due to the smaller seed size, no yield improvement was observed, in spite of their increased number. Apart from the changed sink/source relations, increase in seed number may result in plant exhaustion that limits the seed growth. A similar situation was observed after stimulation of flower branching in chrysanthemum plants by over-expression of *ipt* under *LEACO1* promoter, which resulted in substantial increase in flower number, but their diameter was smaller than in wild type (Khodakovskaya et al. 2009).

Recent climate changes have strengthened the demands for crops with improved stress tolerance, as unfavorable environmental conditions, including various abiotic and biotic stresses, may cause more than 50 % loss of the crop yield, especially in developing countries. As the response to the stress conditions requires vast re-programming of the metabolism to reallocate the energy supplies from the developmental programs to fast and effective stimulation of defense pathways, down-regulation of CK levels, associated with low growth rate, was tested. CK deficiency achieved by over-expression of *CKX* or downregulation of CK biosynthesis was found to increase substantially drought tolerance (Werner et al. 2001; Mytinova et al. 2010; Nishiyama et al. 2011). The same phenomenon has been described with knock-out mutants of CK receptor (Tran et al. 2010) and of AHP-encoding genes (Nishiyama et al. 2013). Apart from drought, also salinity or heat stress tolerance was improved (Nishiyama et al. 2011; Mackova et al. 2013). Constitutive *CKX* expression promotes growth of the root system, a trait that positively correlates with tolerance to water deficit (Tuberosa et al. 2002). However, it has strong negative

effect on the shoot growth. This drawback can be avoided by utilization of root-specific promoters. Targeting of *CKX* expression only to roots (Werner et al. 2010) resulted in plants which maintained enhanced root system, but their shoot phenotype was similar to wild type. Their stress tolerance was lower in comparison with *35S:CKX* plants but still significantly higher than that of wild type (Mackova et al. 2013).

Interestingly, opposite strategy—elevation of CK levels by expression of CK biosynthetic gene (*IPT*)—also resulted in strong elevation of abiotic stress tolerance. In contrast to constitutive elevation of CK levels, which was associated with high sensitivity to drought (*pssu:ipt*, Synkova et al. 1999), *IPT* over-expression under the senescence- or stress-inducible promoters (*SAG12*, *SARK*, or *rd29A*) enhanced tolerance to drought (Rivero et al. 2007, 2009, 2010; Merewitz et al. 2010, 2012; Peleg et al. 2011; Qin et al. 2011; Qiu et al. 2012; Kuppu et al. 2013), heat (Xing et al. 2009), salinity (Ghanem et al. 2011), cold (Hu et al. 2005; Belintani et al. 2012), or flooding (Huynh et al. 2005). The underlying mechanism seems to be diminishing of the stress-induced suppression of photosynthetic activity and stabilization of photosynthetic machinery (Rivero et al. 2009), which improves the energy supply. Moreover, transcription of many stress-inducible genes could be stimulated by CKs (Hare et al. 1997). Recent reports indicate intensive cross talk of CKs with salicylic acid and jasmonic acid and potential positive effect of CKs in biotic stress responses (Choi et al. 2010; Synkova et al. 2006). CK functions in stress responses were recently reviewed by Argueso et al. (2009) or Ha et al. (2012).

The CK functions in regulation of plant development offer unique opportunities to target different processes using suitable promoters. The over-expression of *ipt* under cysteine protease promoter resulted in transgenic rice plants with early flowering and higher number of emerged panicles (Liu et al. 2010). Expression of *ipt* under the control of seed-specific lectin promoter in tobacco promoted cell division in the embryo, resulting in an increase in the number of plerome cell layers and cell number in cotyledons (Ma et al. 2008). Dry weight of seeds was higher and transgenic seedlings grew faster.

The alternative approach to over-expression of biosynthetic gene is silencing of the expression of deactivating gene (*CKX*). Bartrina et al. (2011) reported that *Arabidopsis ckx3ckx5* double mutant formed larger inflorescence and floral meristems. Cellular differentiation was also retarded in this mutant, leading to higher cell number and larger flowers. Silencing of *HvCKX1* expression in barley and wheat resulted in higher grain yield (Zalewski et al. 2010). As *HvCKX1* exhibits high activity in the regulatory aleurone layer of the seeds, the positive effect of downregulation of CKX activity seems to be based on the increase of CK concentration in this layer with positive effect on sink strength and starch accumulation during grain filling (Zalabak et al. 2013).

The perspectives of genetic engineering of CK metabolism for the improvement of agricultural traits of crop plants were discussed by Zalabak et al. (2013), who also provided a comprehensive list of transgenic plants with altered expression of CK-related genes and their traits. The abovementioned data indicate that modulation of CK metabolism and/or signaling may represent a promising strategy for

improvement of plant productivity, especially in combination with suitable tissue- and time-specific promoters that allow direct control of grain filling or stimulation of inflorescence meristems.

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