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Short Communication

Two Cytokinin Receptors of *Arabidopsis thaliana*, CRE1/AHK4 and AHK3, Differ in their Ligand Specificity in a Bacterial Assay

Lukáš Spíchal [1, 2], Natalia Yu. Rakova [3], Michael Riefler [1], Takeshi Mizuno [4], Georgy A. Romanov [3], Miroslav Strnad [2] and Thomas Schmülling [1, 5]

[1] *Free University of Berlin, Institute of Biology/Applied Genetics, Albrecht-Thaer-Weg 6, D-14195 Berlin, Germany*
[2] *Institute of Experimental Botany, CAS, and Laboratory of Growth Regulators, Palacký University, Šlechtitelu 11, CZ-78371 Olomouc, Czech Republic*
[3] *Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya 35, 127276 Moscow, Russia*
[4] *Laboratory of Molecular Microbiology, School of Agriculture, Nagoya University, Chikusa-ku, Nagoya, 464-8601 Japan*

Strains of *Escherichia coli* that express two different cytokinin receptors of *Arabidopsis thaliana*, CRE1/AHK4 and AHK3, were used to study the relative sensitivity of these receptors to various cytokinins. Both receptors were most sensitive to the bases of the isoprenoid-type cytokinins *trans*-zeatin and isopentenyladenine but differed significantly in the recognition of other cytokinin compounds. In particular, CRE1/AHK4 recognized at 1 μm concentration only *trans*-zeatin while AHK3 recognized *cis*-zeatin and dihydrozeatin as well, although with a lower sensitivity. Similarly, CRE1/AHK4 was not activated by cytokinin ribosides and ribotides, but AHK3 was. Comparisons using the *ARR5::GUS* fusion gene as a cytokinin reporter in *Arabidopsis* showed similar relative degrees of responses in planta, except that cytokinins with aromatic side chains showed much higher activities than in the bacterial assay. These results indicate that the diverse cytokinin compounds might have specific functions in the numerous cytokinin-regulated processes, which may depend in turn on different receptors and their associated signalling pathways. The importance of precise control of local concentrations of defined cytokinin metabolites to regulate the respective downstream event is corroborated.

Keywords: *Arabidopsis thaliana* — Cytokinin — Cytokinin receptor — Plant hormone — Signal transduction.

Abbreviations: BA, N^6-benzyladenine; cZ, *cis*-zeatin; DPU, diphenylurea; DZ, dihydrozeatin; DZR, dihydrozeatin riboside; iP, N^6-(Δ^2-isopentenyl)adenine; iPR, N^6-(Δ^2-isopentenyl)adenosine; K, kinetin; mT, *meta*-topolin; TDZ, thidiazuron; tZ, *trans*-zeatin; oT, *ortho*-topolin; tZR, *trans*-zeatin riboside; tZMP, *trans*-zeatin riboside 5′-monophosphate.

Cytokinins play central roles in plant cell division and in numerous plant developmental processes, such as chloroplast maturation, regulation of shoot and root growth, branching and plant senescence (Mok 1994, Werner et al. 2003). Naturally occurring cytokinins are adenine derivatives with a side chain at the N^6-position (Mok and Mok 2001). Depending on the structure of the N^6-substituent, cytokinins are classified as isoprenoid or aromatic. Isoprenoid cytokinins are the most abundant class. They are either isopentenyl (iP)-type cytokinins, having an isopentenyl N^6-side chain, or zeatin-type cytokinins, having a hydroxylated isopentenyl N^6-side chain. The side chain of a zeatin-type cytokinin occurs in either *cis* or *trans* configuration. Reduction of the double bond in the side chain leads to dihydrozeatin (DZ) (Mok and Mok 2001). Aromatic cytokinins have an aromatic benzyl or hydroxybenzyl group at N^6 [e.g. N^6-benzyladenine (BA), *meta*-topolin (mT)]. They occur more rarely and much less is known about them (Strnad 1997). Isoprenoid and aromatic cytokinins exist as free bases and the corresponding ribosides and ribotides. Non-active storage forms of cytokinins are the *O*- and *N*-glucosides. In addition, there are the structurally unrelated phenylurea-type cytokinins, a class of synthetic cytokinins. These cytokinins [e.g. diphenylurea (DPU), thidiazuron (TDZ)] are highly active in most biotests but do not occur naturally (Mok and Mok 2001).

Typically, a mixture of many different cytokinins in varying proportions is present in plant tissues. There is uncertainty about which cytokinins have genuine biological activity, which makes it difficult to interpret the results of quantitative measurements. The free bases show the highest activities in bioassays and are believed to be the active forms of the hormone. Ribosides and ribotides are generally less active than the corresponding free bases. It could be that these compounds only become active after metabolic conversion to the free base or that they activate the receptor to a lesser extent (Mok and Mok 2001).

Cytokinins vary in their relative activities in different bioassays. For example, *cis*-zeatin (cZ) usually has only low activity compared to *trans*-zeatin (tZ); isomerization of cZ into tZ

[5] Corresponding author: E-mail, tschmue@zedat.fu-berlin.de; Fax, +49-30-8385-4345.

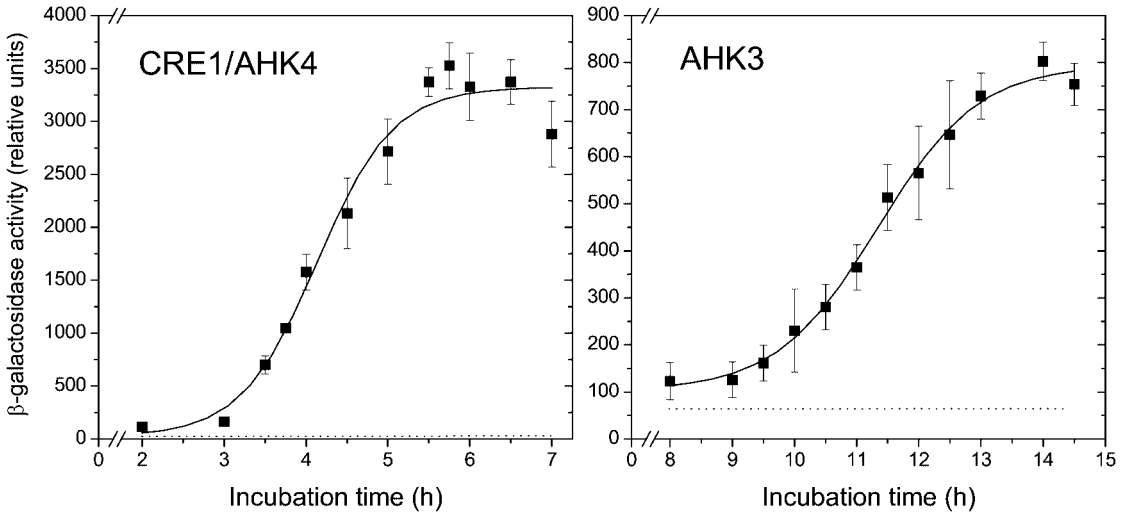

Fig. 1 Kinetics of cytokinin-dependent induction of β-galactosidase activity in *E. coli.* CRE1/AHK4 and AHK3 expressing *E. coli* were incubated with 1 μM *trans*-zeatin for the indicated time. The β-galactosidase activity in non-induced strains (control) is indicated by the dotted line. Error bars show SE ($n = 2$). *E. coli* strain KMI001 harbouring the plasmid pIN-III-AHK4 or pSTV28-AHK3 were described (Suzuki et al. 2001, Yamada et al. 2001). *E. coli* strains were grown overnight at 25°C in M9 media enriched with 0.1% casamino acids (Sambrook et al. 1989) to OD_{600} ~1. The preculture was diluted 1 : 50 in 400 μl M9 medium containing 0.1% casamino acids and 4 μl stock solution of the tested compound or solvent control were added. Cultures were further grown at 25°C. At the end of the incubation period 200 μl of the culture were centrifuged, 100 μl of the supernatant were transferred to a test tube containing 2 μl 50 mM 4-methyl umbelliferyl galactoside and incubated 1 h at 37°C. The reaction was stopped by adding 100 μl 0.2 M Na_2CO_3. Fluorescence was measured using a Hoefer™ DyNA Quant™ 200 (Amersham Biosciences, San Francisco, U.S.A.) at excitation and emission wavelengths of 365 and 460 nm, respectively. The OD_{600} of the remaining culture was determined and the β-galactosidase activity was calculated as nmol 4-methylumbelliferone × OD_{600}^{-1} × h^{-1}.

might be required for activation. Different relative activities were reported for tZ and kinetin (K) in cell division and senescence retardation assays (Letham 1967). Similarly, comparisons between BA and its hydroxylated form mT showed both a decrease or an increase of biological activity as a consequence of hydroxylation, depending on the bioassay employed (Holub et al. 1998). Two *Phaseolus* genotypes showed large differences in the growth response to tZ, iP and DZ (Mok et al. 1978). In castor bean, BA was significantly more effective than K or tZ in inducing shoot formation (Sujatha and Reddy 1998), while all three cytokinins showed a similar ability to induce a cytokinin response gene in *Arabidopsis* (Brandstatter and Kieber 1998, Romanov et al. 2002, this paper). These differences suggest that different cytokinins have specific functions and make it necessary to determine the qualitative and quantitative importance of each cytokinin compound.

Differences in relative activities could be due to the perception and transmission of the signal by different receptors that are involved in triggering these biological processes. Three cytokinin receptors, CRE1/AHK4, AHK2 and AHK3 have been described in *Arabidopsis* (Inoue et al. 2001, Suzuki et al. 2001, Yamada et al. 2001, for recent reviews see Kakimoto 2003, Heyl and Schmülling 2003). All three are sensor histidine kinases and have a predicted extracellular ligand binding domain, the so-called CHASE domain (Anantharaman and Aravind 2001, Mougel and Zhulin 2001). Sequence comparison revealed that the CHASE domains of CRE1/AHK4 and AHK3 have 65% identical amino acids, while CRE1/AHK4 and AHK2 as well as AHK3 and AHK2 share 63% identity (our unpublished result). All three receptor genes are expressed in roots, leaves, stem and flowers, although with different mRNA abundance (Ueguchi et al. 2001, Higuchi et al. 2004).

A number of plant-based bioassays have been used in the past to compare the biological activities of different cytokinin compounds. Most assays measure rather slow reactions, e.g. callus growth or leaf senescence. Cytokinins are rapidly metabolized in plant tissues, therefore it may be difficult to interpret the outcome of these assays. A more rapid bioassay, which depends on the activation of the promoter of the primary response gene *ARR5* and lasts only several hours, has been described as well (D'Agostino et al. 2000). However, this test integrates the responses of several putative cytokinin signalling pathways and may not distinguish between different receptors. Recently, a bacterial test system for cytokinins was described (Suzuki et al. 2001, Yamada et al. 2001). In this system, the cytokinin receptors CRE1/AHK4 or AHK3 are functionally expressed in *E. coli.* Their output signal is coupled to an *E. coli* signalling pathway finally leading to the activation of the reporter gene *cps::lacZ.* This allows for a rapid test of cytokinin activity, without problems associated with hormone penetration and interfering metabolic activities of long-lasting plant bioassays. tZ or iP at 0.1 μM trigger the response in case of CRE1/AHK4, indicating that the system is responsive to physiological concentrations of the hormone (Suzuki et al. 2001).

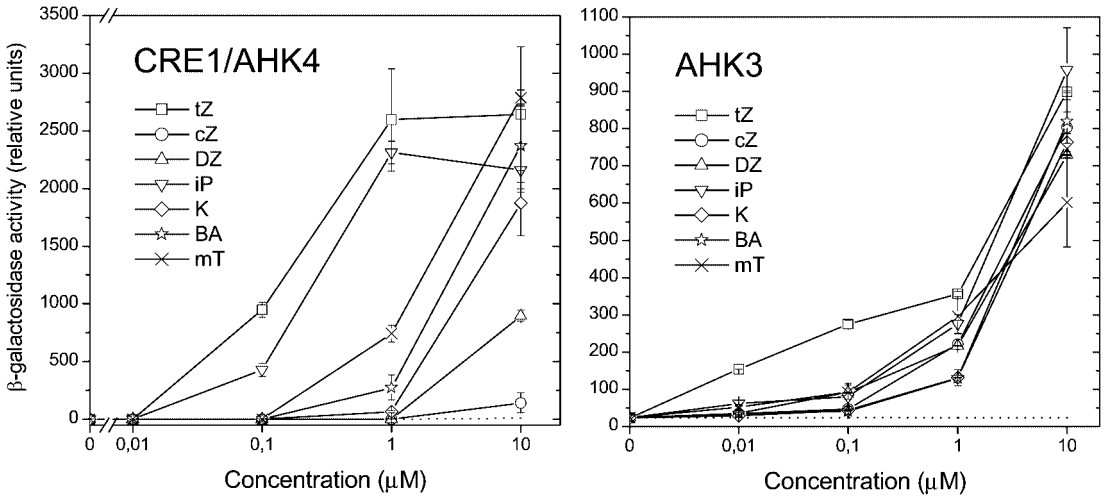

Fig. 2 Dose dependence of cytokinin-induced β-galactosidase activity. CRE1/AHK4 and AHK3 expressing *E. coli* were incubated for 6 h and 12 h, respectively, with different cytokinin bases in concentrations from 0.01 μM to 10 μM. The β-galactosidase activity in non-induced strains (control) is indicated by the dotted line. Error bars show SE ($n = 2$). For description of the method see Fig. 1.

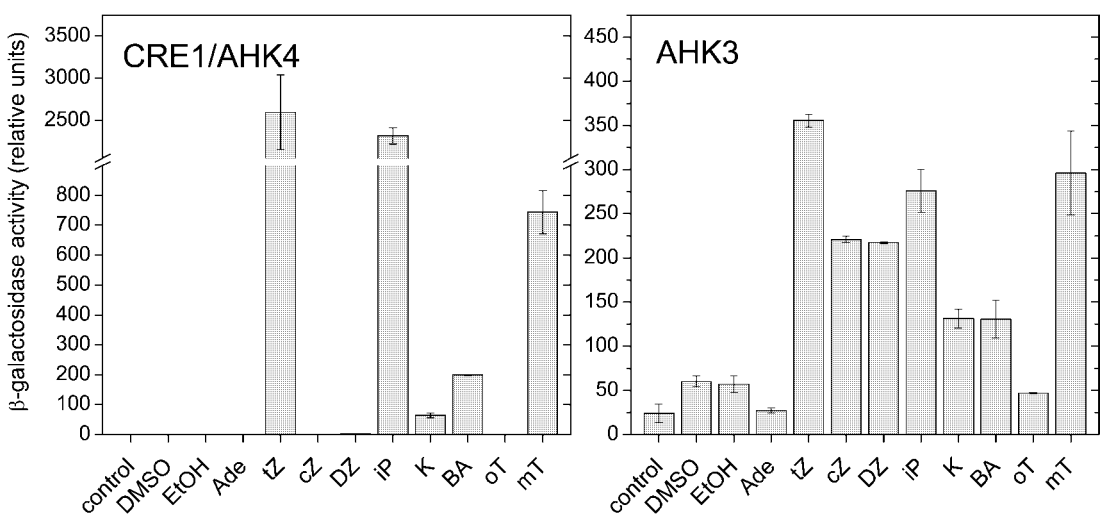

Fig. 3 Comparison of the sensitivities of CRE1/AHK4 and AHK3 to different cytokinin bases in the *E. coli* assay. Incubation times of 6 h and 12 h were used for AHK4 and AHK3, respectively. The tested concentration of cytokinin compounds was 1 μM. For further description of the method see Fig. 1.

The activity of several free bases was shown with this system (Suzuki et al. 2001) but a systematic study comparing the ligand specifity of the receptors with a greater selection of known cytokinins is missing. We have therefore used this test system to systematically investigate the relative activities of different cytokinins. For comparison, the same compounds were also tested in planta in a reporter gene-based assay of *Arabidopsis*.

In order to characterize the experimental system we first analyzed the kinetics of the reaction and established dose-response curves for different cytokinin metabolites. Fig. 1 shows that in both *E. coli* strains, expressing either AHK3 or CRE1/AHK4, the reaction towards 1 μM tZ reached a plateau phase after about 6 h and 14 h. Dose-response curves were established for all tested metabolites between 0.01 μM and 10 μM. Fig. 2 shows examples demonstrating that the response of both bacterial strains increased constantly for most compounds up to a concentration of 10 μM. Active compounds like tZ and iP reached saturation at 1 μM, while higher concentrations of all other active cytokinins (e.g. K, BA, mT; Fig. 2) were needed. We decided to perform all comparisons at a ligand concentration of 1 μM, which is a commonly used non-toxic hormone concentration.

First we compared the relative activities of different cytokinin bases, exploring the relevance of the N^6-linked side chain. Fig. 3 shows that tZ and iP were similarly highly active in the CRE1/AHK4 and AHK3 assay. Other free bases with side-

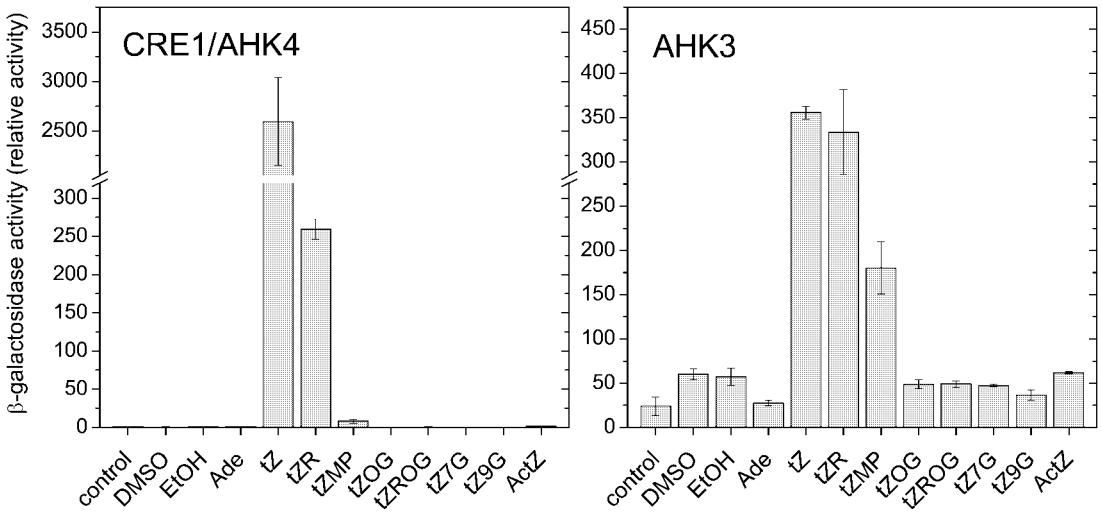

Fig. 4 Comparison of the sensitivities of CRE1/AHK4 and AHK3 to different zeatin derivatives in the *E. coli* assay. Incubation times of 6 h and 12 h were used for AHK4 and AHK3, respectively. The tested concentration of cytokinin compounds was 1 μM. For further description of the method see Fig. 1.

chain modifications, cZ and DZ, were not recognized by CRE1/AHK4 at 1 μM concentrations but showed about half of the activity of the most active free base with AHK3. Even at ten times higher concentration cZ only provoked a very weak response with CRE1/AHK4, while at 10 μM DZ had about 30% of the activity of the most active compounds (Fig. 2). Please note that the purity of cZ was carefully checked by HPLC and was found to be >99.9% pure (data not shown). Aromatic cytokinins (BA, K) had significantly lower activity than isoprenoid cytokinins with both receptors (Fig. 3). A notable exception was topolin. mT, but not oT, showed considerable activation of both receptors. Adenine was unable to activate either receptor (Fig. 3).

Next we compared the activities of different metabolites of tZ. Fig. 4 shows that tZR had only 10% of the activity of tZ with AHK4/CRE1, but about the same activity as tZ with AHK3. Activity of the ribosides with AHK3 was also found for iPR, DZR and aromatic cytokinins, but their relative activities were significantly lower than that of tZR (Table 1). The nucleotide tZMP reached almost 50% of the activity of the free base with AHK3, but was almost completely inactive with CRE1/AHK4 (Fig. 3). In contrast, none of the *O*- and *N*-glucosides was able to trigger a response in the bacterial assay.

Finally, two phenylurea-type cytokinins were tested in the bacterial assay. Of the two compounds, DPU and TDZ, only the latter was highly active, while no activity was detected with DPU (Table 1).

We compared the results obtained in *E. coli* with the biological activity of the metabolites in a rapid and sensitive *Arabidopsis* reporter gene assay. This was done to investigate whether similar relative differences of activity between the cytokinin compounds could be found in planta. The results are compiled in Table 1. For most compounds the relative ability to activate P_{ARR5}*::GUS* reporter gene expression was in between the range observed for both receptors in *E. coli.* For example, in *Arabidopsis* tZR reached 47.9% of the activity of tZ, while in the *E. coli* assay it reached 10% with CRE1/AHK4 and 92.5% with AHK3. However, there were some notable exceptions. All cytokinin glucosides, and in particular the *O*-glucosides, were biologically active in the *Arabidopsis* assay, while they showed no activity in the *E. coli* assays (Table 1). This could be due to rapid breakdown of these metabolites in *Arabidopsis*, yielding biologically active free base. A second exception was the aromatic cytokinins BA, K and oT, which showed only low activity or no activity at all in *E. coli.* They were all highly active in the *Arabidopsis* reporter gene test, reaching almost the maximal activity of tZ (Table 1).

The finding that the free bases of isoprenoid-type cytokinins show high activity with both receptors in *E. coli* and in the *Arabidopsis* assay, confirmed earlier studies including competition assays (Inoue et al. 2001, Suzuki et al. 2001, Yamada et al. 2001) and is in accordance with results of many bioassays which indicated a prevalent role for these compounds (Mok and Mok 2001). Likewise, the inactivity of both *O*- and *N*-glucosides is in agreement with their proposed function as biologically inactive storage forms. Other compounds showed a more complex pattern of activity. In particular, all other naturally occuring cytokinins had significantly lower activity with CRE1/AHK4 than the free bases. The only exception is mT, which was as active as the bases of isoprenoid cytokinins (Table 1).

Ribosides, a transport form of cytokinins (Mok and Mok 2001), have a relatively higher activity with AHK3 than with CRE1/AHK4. We cannot exclude that there is some nucleosidase activity releasing free base from the riboside. However, if there was nucleosidase activity it would be expected to be the

Table 1 Comparison of the relative activities of different cytokinin compounds in CRE1/AHK4 and AHK3 expressing *E. coli* and in the P_{ARR5}*::GUS* reporter gene assay in *A. thaliana*

Abbreviation	Compound	Relative activity (%) [a]		
		CRE1/AHK4	AHK3	*Arabidopsis* plants
tZ	*trans*-zeatin	100.0	100.0	100.0
tZR	*trans*-zeatin riboside	10.0	92.5	47.9
tZMP	*trans*-zeatin riboside 5′-monophosphate	0.3	40.4	100.1
tZOG	*trans*-zeatin *O*-glucoside	0.0	0.0	92.0
tZROG	*trans*-zeatin *O*-glucoside riboside	0.0	0.0	84.1
tZ7G	*trans*-zeatin 7-glucoside	0.0	0.0	10.6
tZ9G	*trans*-zeatin 9-glucoside	0.0	0.0	14.1
tAcZ	*trans*-zeatin *O*-acetyl	0.0	0.1	100.5
cZ	*cis*-zeatin	0.0	54.2	40.5
cZR	*cis*-zeatin riboside	0.0	n.t.	n.t.
DZ	dihydrozeatin	0.1	52.9	11.8
DZR	dihydrozeatin riboside	0.0	20.8	12.5
iP	N^6-(Δ^2-isopentenyl)adenine	89.2	72.9	108.2
iPR	N^6-(Δ^2-isopentenyl)adenosine	10.0	10.1	77.3
K	kinetin	2.5	23.8	94.0
KR	kinetin riboside	0.0	5.0	61.7
BA	N^6-benzyladenine	7.7	23.6	93.6
BAR	N^6-benzyladenosine	0.0	4.0	47.7
oT	*ortho*-topolin	0.0	0.0	52.8
oTR	*ortho*-topolin riboside	0.0	0.0	31.1
mT	*meta*-topolin	28.6	79.8	123.4
mTR	*meta*-topolin riboside	0.0	6.3	83.5
DPU	diphenylurea	0.0	0.0	0.0
TDZ	thidiazuron	81.7	120.7	131.7
Ade	adenine	0.0	0.0	1.6
DMSO [b]	dimethylsulfoxide	0.0	1.2	0.0
EtOH [c]	ethanol	0.0	0.9	0.0

[a] The relative activity (%) was calculated from the mean values of three repetitions of two parallel assays with 1 μM (*E. coli*) or 0.5 μM (*Arabidopsis*) of metabolite. The standard deviation did not exceed 20%, except of DZR, BAR, mTR, KR in the AHK3 assay and tZMP and oT in the CRE1/AHK4 assay, in which the standard deviations were between 22 and 35%. 100% was 2,595 nmol MU × OD_{600}^{-1} × h^{-1} for CRE1/AHK4, 356 nmol MU × OD_{600}^{-1} × h^{-1} for AHK3 and 390±60 nmol MU × mg $protein^{-1}$ × h^{-1} for the P_{ARR5}*::GUS* transgenic *Arabidopsis.*

[b] The concentration of DMSO in the assay was 0.1%.

[c] The concentration of EtOH in the assay was 0.07%. n.t., not tested.

Transgenic *Arabidopsis* plants (*Arabidopsis thaliana* (L.) Heynh. accession Wassilewskija) harbouring the *GUS* reporter gene fused to 1.6 kb of the *ARR5* (P_{ARR5}*-GUS*) gene promoter were described (D'Agostino et al. 2000). Plants were treated as described previously (Romanov et al. 2002). In brief, seeds of homozygous plants were surface sterilized and soaked for 2–3 d at 4°C in 0.2 ml sterilized distilled water in a cell culture cluster (Costar, New York). Then cold water was replaced by sterile water at room temperature and seeds were placed in a growth chamber at 24°C, 16 h light/8 h dark cycles. Seedlings grown for 3–4 d were used for the experiments. For each data point, ten plants of similar size were selected and treatments were carried out at least in duplicate. Cytokinins were added as microaliquots or predissolved in H_2O to the desired final concentration. The final assay volume was adjusted with water to 0.4 ml. The seedlings were immediately subjected to a mild vacuum infiltration for 30–40 s and then incubated for 5 h at 24°C in the dark. At the end of the incubation period solutions were removed from the wells and seedlings were washed once with 0.6 ml H_2O. Seedlings were then placed into microtubes with 0.1 ml GUS extraction buffer and kept at –70°C until fluorometric assay. Quantitative GUS activity determinations were performed according to common protocols with only minor modifications using 4-methylumbelliferyl glucuronide (MUG) as a substrate (Jefferson et al. 1987, Zvereva and Romanov 2000). Fluorescence was measured with an F2000 spectrofluorometer (Hitachi, Japan) at excitation and emission wavelengths of 365 and 450 nm. Protein quantification was performed according to Bradford (1976) using Roti-Q staining solution (Roth, Germany). GUS specific activity was expressed as nmol 4-methylumbelliferone (MU) × mg $protein^{-1}$ × h^{-1}. Mean values, standard deviations and significance of experimental differences were calculated using a t-test. Cytokinin metabolites were from Sigma (Deisenhofen, Germany), Calbiochem (Schwalbach, Germany), Duchefa (Haarlem, The Netherlands) and Olchemim (Olomouc, Czech Republic). Different zeatin glucosides were a kind gift of Olchemim (Olomouc, Czech Republic). Stock solutions were prepared in H_2O, DMSO, MeOH or EtOH and stored at –20°C.

same in the AHK3- and CRE1/AHK4-expressing strains. The ability of tZR to activate CRE1/AHK4 did not increase with prolonged incubation periods (data not shown). Therefore we conclude that ribosides have a genuine biological activity, at least with AHK3. Ribosides were also found to activate three different cytokinin receptors of maize (Yonekura-Sakakibara et al. 2004). Ribosides of isoprenoid-type cytokinins are, in seedlings of *Arabidopsis*, somewhat less abundant than the corresponding free bases, while the concentrations of ribotides, which are also active with AHK3, even exceeds the concentrations of the free bases (Werner et al. 2003). This indicates that the activity of these compounds could be significant in the physiological context.

The low activity of the aromatic cytokinins in the bacterial test system is inconsistent with the high activity they show in most bioassays, including the *Arabidopsis* assay employed in our study (Holub et al. 1998, Table 1). It is possible that this difference is due to their higher stability in plant tissues. Alternatively, AHK2, a cytokinin receptor that was not analysed in this study, may have a higher sensitivity towards these compounds or different response pathways may be activated by aromatic cytokinins.

cZ has long been considered a cytokinin with an unclear function. It has been argued that it may only become active after its metabolic conversion to tZ (Mok and Mok 2001). The results obtained in this study indicate that cZ itself has biological activity. cZ activates AHK3 about half as effectively as tZ, while CRE1/AHK4 is not responsive to cZ. This confirms an earlier result showing that cZ was not able to activate CRE1/AHK4 in a cytokinin-dependent yeast assay (Inoue et al. 2001) (Fig. 3). In some species or in specific organs cZ is the predominant cytokinin (Emery et al. 1998, Durand and Durand 1994, Veach et al. 2003). In *Arabidopsis* seedlings the cZ concentration is only 10% of tZ (O. Novak, personal communication). Similar to the results shown here, a recent study has reported that three different cytokinin receptors are activated by cZ (Yonekura-Sakakibara et al. 2004). Moreover, a cytokinin *O*-glucosyltransferase with cZ as the preferred substrate was cloned from maize (Martin et al. 2002) and maize was found to contain substantial amounts of cZ (Veach et al. 2003). Collectively, these and our data indicate strongly that cZ has a true hormonal function, at least in some species or organs.

It is intriguing to note that CRE1/AHK4 provokes a severalfold higher response in *E. coli* than AHK3. This could be due to differences in expression in *E. coli*, differences in the ability to couple to the *E. coli* signalling pathways or could reflect an intrinsic feature of the receptors. Hwang and Sheen found in a transient in planta assay system that transmission of a cytokinin signal through AHK3 (and AHK2) was severalfold lower than through CRE1/AHK4 (Hwang and Sheen 2001). It could be that plants have developed a highly sensitive and specific recognition system based on CRE1/AHK4 and a less sensitive response system with broader specificity based on AHK3. These distinct systems may reflect differences in the tissues in which they operate.

Taken together, results of this paper and of Yonekura-Sakakibara et al. (2004) show that a variety of cytokinin compounds may have signalling functions, but only with specific receptors. The different ligand specificities of the receptors may be a way of fine-tuning the various cytokinin responses and indicates that proper control of the metabolic system is very relevant in determining the final outcome of a given reaction. This is supported by the recent report of distinct origins of the isoprenoid side-chains in tZ and cZ (Kasahara et al. 2004), indicating parallel pathways and corroborating the relevance of the metabolic system.

Acknowledgments

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References

Anantharaman, V. and Aravind, L. (2001) The CHASE domain: a predicted ligand-binding module in plant cytokinin receptors and other eukaryotic and bacterial receptors. *Trends Biochem. Sci.* 10: 579–582.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.

Brandstatter, I. and Kieber, J.J. (1998) Two genes with similarity to bacterial response regulators are rapidly and specifically induced by cytokinin in *Arabidopsis*. *Plant Cell* 10: 1009–1019.

D'Agostino, I.B., Deruere, J. and Kieber, J.J. (2000) Characterization of the response of the *Arabidopsis* response regulator gene family to cytokinin. *Plant Physiol.* 124: 1706–1717.

Durand, R. and Durand, B. (1994) Cytokinins and reproductive organogenesis in *Mercurialis*. *In* Cytokinins. Chemistry, Activity and Function. Edited by Mok, D.W.S. and Mok, M.C. pp. 295–304, CRC Press, Boca Raton.

Emery, R.J.N., Leport, L., Barton, J.E., Turner, N.C. and Atkins, A. (1998) *cis*-isomers of cytokinins predominate in chickpea seeds throughout their development. *Plant Physiol.* 117: 1515–1523.

Heyl, A. and Schmülling, T. (2003) Cytokinin signal perception and transduction. *Curr. Opin. Plant Biol.* 6: 480–488.

Higuchi, M., Pischke, M.S., Mähönen, A.P., Miyawaki, K., Hashimoto, Y., Seki, M., Kobayashi, M., Shinozaki, K., Kato, T., Tabata, S., Helariutta, Y., Sussman, M.R. and Kakimoto, T. (2004) *In planta* functions of the *Arabidopsis* cytokinin receptor family. *Proc. Natl. Acad. Sci. USA* 101: 8821–8826.

Holub, J., Hanuš, J., Hanke, D.E., and Strnad, M. (1998) Biological activity of cytokinins derived from ortho- and meta-hydroxybenzyladenine. *Plant Growth Regul.* 26: 109–115.

Hwang, I. and Sheen, J. (2001) Two-component circuitry in *Arabidopsis* cytokinin signal transduction. *Nature* 413: 383–389.

Inoue, T., Higushi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki, K. and Kakimoto, T. (2001) Identification of CRE1 as a cytokinin receptor from *Arabidopsis*. *Nature* 409: 1060–1063.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6: 3901–3907.

Kakimoto, T. (2003) Perception and signal transduction of cytokinins. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 54: 605–627.

Kasahara, H., Takei, K., Ueda, N., Hishiyama, S., Yamaya, T., Kamiya, Y., Yamaguchi, S. and Sakakibara, H. (2004) Distinct isoprenoid origins of *cis*-

and *trans*-zeatin biosyntheses in *Arabidopsis*. *J. Biol. Chem.* Epub, PMID: 14726522.

Letham, D.S. (1967) Regulators of cell divison in plant tissues. V. A comparison of the activity of zeatin and other cytokinins in five bioassays. *Planta* 74: 228–242.

Martin, R.C., Mok, M.C., Habben, J.E. and Mok, D.W. (2002) A maize cytokinin gene encoding an O-glucosyltransferase specific to *cis*-zeatin. *Proc. Natl Acad. Sci. USA* 98: 5922–5926.

Mok, D.W.S. and Mok, M.C. (2001) Cytokinin metabolism and action. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52: 89–118.

Mok, M.C. (1994) Cytokinins and plant development – an overview. *In* Cytokinins. Chemistry, Activity and Function. Edited by Mok, D.W.S. and Mok, M.C. pp. 155–166. CRC Press, Boca Raton.

Mok, M.C., Mok, D.W.S. and Armstrong, D.J. (1978) Differential cytokinin structure-activity relationship in *Phaseolus*. *Plant Physiol.* 61: 72–75.

Mougel, C. and Zhulin, I.B. (2001) CHASE: an extracellular sensing domain common to transmembrane receptors from prokaryotes, lower eukaryots and plants. *Trends Biochem. Sci.* 26: 582–584.

Romanov, G.A., Kieber, J. and Schmülling, T. (2002) A rapid cytokinin response assay in *Arabidopsis* indicates a role for phospholipase D in cytokinin signalling. *FEBS Lett.* 515: 39–43.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Strnad, M. (1997) The aromatic cytokinins. *Physiol. Plant.* 101: 674–688.

Sujatha, M. and Reddy, T.P. (1998) Differential cytokinin effects on the stimulation of in vitro shoot proliferation from meristematic explants of castor (*Ricinus communis* L.). *Plant Cell Rep.* 17: 561–566.

Suzuki, T., Miwa, K., Ishikawa, K., Yamada, H., Aiba, H. and Mizuno, T. (2001) The *Arabidopsis* sensor His-kinase, AHK4, can respond to cytokinins. *Plant Cell Physiol.* 42: 107–113.

Ueguchi, C., Koizumi, H., Suzuki, T. and Mizuno, T. (2001) The AHK4 gene involved in the cytokinin-signaling pathway as a direct receptor molecule in *Arabidopsis thaliana*. *Plant Cell Physiol.* 42: 751–755.

Veach, Y.K., Martin, R.C., Mok, D.W., Malbeck, J., Vankova, R. and Mok, M.C. (2003) *O*-glucosylation of cis-zeatin in maize. Characterization of genes, enzymes, and endogenous cytokinins. *Plant Physiol.* 131: 1374–1380.

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H. and Schmülling, T. (2003) Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating opposite functions of cytokinins in regulating shoot and root meristem activity. *Plant Cell* 15: 2532–2550.

Yamada, H., Suzuki, T., Terada, K., Takei, K., Ishikawa, K., Miwa, K., Yamashino, T. and Mizuno, T. (2001) The *Arabidopsis* AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. *Plant Cell Physiol.* 42: 1017–1023.

Yonekura-Sakakibara, K., Kojima, M., Yamaya, T. and Sakakibara, H. (2004) Molecular characterization of cytokinin-responsive histidine kinases in maize. Differential ligand preferences and response to *cis*-zeatin. *Plant Physiol.* 134: 1654–1661.

Zvereva, S.D. and Romanov, G.A. (2000) Reporter genes for plant genetic engineering: characteristics and detection. *Russian J. Plant Physiol.* 47: 479–488.

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