PALACKÝ UNIVERSITY IN OLOMOUC
FACULTY OF SCIENCE
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DEPARTMENT OF CHEMICAL BIOLOGY AND GENETICS

# Study of the Ligand Specificity of Cytokinin Receptors

Ph.D. THESIS

BY
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IN PROGRAMME BIOLOGY-BOTANY
P1507

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2019

# DECLARATION

Hereby I declare that this Ph.D. thesis is my original work. I have include the complete list of cited and used literature.

25th March 2019

Prague, Czech Republic

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# ACKNOWLEDGEMENT

I would like to thank my supervisor, Msc. Lukáš Spíchal, Ph.D. for the opportunity to work on a project focused on cytokinin receptors. Further, I would like to thank my colleagues for their help and cooperation, mainly Zuzka, Markéta, Jarda, Mrs. Šubová, Jaruška, Jana N., Agáta, Vašek, Tomáš, Libor, Noemi and all people from the 4th floor. Great thanks belong to professor Miroslav Strnad, for his effective support to finish my Ph.D. study, associate professor Karel Berka for his prompt writing of our article, professor Peter Hedden for his English corrections and also to the people who supported me during long 8 years of study...mainly my family - parents, Tom, Borka and my friends - Iva, Markét, Terka, Eva, Majka, Jitka... to share my good and bad days with me.

# BIBLIOGRAPHICAL IDENTIFICATION

**Author´s first name and surname:** Lucia Hlusková

**Title:** Study of the ligand specificity of cytokinin receptors

**Type of thesis:** Ph.D. thesis

**Department:** Department of Botany

**Supervisor:** Msc. Lukáš Spíchal, Ph.D.

**The year of presentation:** 2019

**Keywords:** Cytokinins, Receptor Binding Domain, Active Site, *Arabidopsis thaliana*, *Brasica napus*, Histidine Kinases

**Number of pages:** 129

**Number of appendices:** 3

**Language:** English

# BIBLIOGRAFICKÁ IDENTIFIKÁCIA

**Meno a priezvisko autora:** Lucia Hlusková

**Názov:** Štúdium ligandovej špecifity cytokinínových receptorov

**Druh práce:** doktorská dizertačná

**Katedra:** Katedra botaniky

**Školiteľ:** Mgr. Lukáš Spíchal, Ph.D.

**Rok obhájenia práce:** 2019

**Kľúčové slová:** cytokiníny, viažuca doména recepora, aktívne miesto, arábkovka Thalova (*Arabidopsis thaliana*), repka olejná (*Brassica napus*), histidinkináza

**Počet strán:** 129

**Počet príloh:** 3

**Jazyk:** anglický

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1) Characterisation of ligand specifity of the *Brassica napus* cytokinin receptors
2) Testing of specifity of interactions of selected ligands and mutant forms of cytokinin receptors for better understanding of the plant signalling system using molecular biology and chemical biology approaches
    a. Studies of cytokinin receptor affinity and activation using a chemical biology approach.
    b. Analysis of the importance of the conserved residues in the binding pocket of AHK3 and AHK4

# ABBREVIATIONS

| | |
|---|---|
| 5'-UTR | 5' untranslated region |
| ABC | ATP-binding cassette |
| ABCG14 | ABC transporter subfamily G 14 |
| Ade | Adenine |
| ADP | Adenosine 5'-diphosphate |
| AHK, AtHK | *Arabidopsis* histidine kinase |
| AHP | Phosphotransfer protein |
| AMP | Adenosine 5'-monophosphate |
| ARR | Arabidopsis response regulator |
| AtIPT | *Arabidopsis thaliana* isopentenyl transferase |
| ATP | Adenosine 5'-triphosphate |
| BAP | 6-benzylaminopurine |
| BiFC | Bimolecular fluorescence complementation |
| CA | Catalytic domain |
| CHASE | Cyclases/Histidine kinases associated sensory extracellular |
| CHK | CHASE-containing His kinase |
| CKI | Cytokinin Independent |
| CKX | Cytokinin oxidase/dehydrogenase |
| CRE1/AHK4 | *Arabidopsis* histidine kinase 4 |
| CRF | Cytokinin response factor |
| *cZ* | *cis*-Zeatin |
| DAG | Days after germination |
| DHZ | Dihydrozeatin |
| DMAPP | Dimethylallyl pyrophosphate |
| DMSO | Dimethylsulfoxide |
| *E. coli* | *Escherichia coli* |
| ED | Extracellular putative input domain |
| ENT | Transporter for cytokinin nucleosides |
| ER | Endoplasmatic reticulum |
| GAF | motif presented in cGMP-regulated cyclic nucleotide PDEs, certainadenylyl cyclases and the bacterial transcription factor FhlA (domain) |
| GFP | Green fluorescent protein |
| H, His | Histidine |
| HATPase | Histidine kinase-like ATPase |
| HisKA | Catalytic histidine kinase A |
| HK | Histidine kinase |
| HMBDP | (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate |
| HPt | Histidine phosphotransfer |
| iP | $N^6$-($\Delta^2$-isopentenyl)adenine |
| iPR | $N^6$-($\Delta^2$-isopentenyl)adenine riboside |
| IPT | Isopentenyl transferase |
| $K_d$ | Dissociation constant |
| K | Kinetin |
| KD | Kinase domain |
| LB | Lysogeny broth |
| LOG | LONELY GUY |

| | |
|---|---|
| LUC | Luciferase |
| M9 | Minimal medium |
| MAP | Mitogen-activated protein (kinase) |
| Mcs4 | mitotic catastrophe suppressor (response regulator) |
| MEP | Methylerithritol phosphate |
| MS | Murashige skoog |
| *mi*T | *meta*-Topolin |
| MVA | Mevalonate |
| Myb | Myeloblastosis |
| Nu | Nucellus |
| OsCRL | *Oryza sativa* CHASE domain containing protein |
| P | Phosphate residue |
| PAS | Per-Arnt-Sim |
| PDC | PhoQ-DcuS-CitA |
| Phk | Phosphorylase kinase |
| PM | Plasma membrane |
| pTCS | Two-component output sensor |
| PUP | Purine permease |
| REC | Receiver |
| RR | Response regulator |
| SpyI | HPt factor |
| TCS | Two-component systems |
| TCSn | New two-component output sensor |
| TDZ | Thidiazuron |
| TM | Transmembrane domain |
| UGT | *N*-glucosyltransferase |
| WOL | WOODEN LEG |
| WT | Wild-type |
| *tZ* | *trans*-Zeatin |
| tZ7G | *trans*-Zeatin-7-glucoside |
| tZ9G | *trans*-Zeatin-9-glucoside |
| tZOG | *trans*-Zeatin-O-glucoside |
| *tZR* | *trans*-Zeatin riboside |
| ZmHK | *Zea mays* histidine kinase |

# INTRODUCTION

The interaction between individual cells at the organ and tissue levels is mediated by extracellular signal molecules that regulate growth and development of multicellular organisms (Davies, 1995). In plants, phytohormones are active at low concentrations (Neumann et al., 2009). The production of these small molecules, which are derived from secondary metabolism (Santner and Estelle, 2009) is mobilized in certain tissues at defined stages of development (Durán-Medina et al., 2017). They are distributed by the vascular system, thus often exerting functions at distant tissues (e.g. Takei et al., 2001b). Plant cells and tissues produce and respond to different groups of phytohormones (Chow and McCourt, 2006), which are the main regulators of growth and differentiation (Neumann et al., 2009). They have multiple functions and are involved in cognate physiological processes. The hormonal signaling system acts nonredundantly (Jaillais and Chory, 2010; Neumann et al., 2009), although often a specific reaction can be activated by several hormones (Neumann et al., 2009).

Cytokinins represent one of the major classes of phytohormones. They are represented by natural or synthetic compounds derived from adenine or phenylurea (Mok and Mok, 2001). They are associated with youth (prevention of senescence), growth (stimulation of cell division and differentiation) and health (flexible responses to changeable environment) during the plant life. There are two conditions that must be satisfied for cells to respond to the cytokinin stimulus (Zürcher and Müller, 2016):

1) proper place and time for the presence of the active ligands
2) ability of recipient cells to perceive and transduce the stimulus.

This thesis summarizes recent findings on the perception of cytokinin signal molecules. It focusses on the characterization of cytokinin receptors, features of their cytokinin binding domain, and the influence of point mutations on their function. The methods for testing cytokinin response are described.

## „That thing as a result of whose presence something first comes into being“

The history of cytokinin discovery goes back to ancient Greece, when the philosopher Aristotle predicted the existence of certain substance important for the origin of new plant forms. He called it “that thing as a result of whose presence something first comes into being“. This thesis was developed in the 19th century by German botanist Julius von Sachs and British naturalist Charles Darwin. They both presumed the existence of compounds responsible for phototropism. Sachs suggested there is a compound produced by the plant and transported into its parts to regulate growth and development. Darwin hypothesised that stimuli such as light or gravity may be received by one part of plant, but the response may occur in another part (Hwang et al., 2012). These conclusions led to the assumption that plants control their life cycle on hormonal level (Letham, 1969). Gottlieb Haberlandt and Julius Wiesner suggested in the beginning of the 20th century existence of soluble substances, which could support plant cell division and proliferation. Research of van Overbeek (1941) demonstrated a positive effect of coconut milk on plant tissue culture proliferation (Hwang et al., 2012; Kieber and Schaller, 2010).

The first compound with such properties was isolated by Carlos Miller in the laboratory of Folke Skoog and identified as $N^6$-furfuryladenine, named kinetin. It was obtained from old or autocalved DNA from herring sperm or calf thymus (Miller et al., 1955, 1956). A new group of compounds called cytokinins was established based on these results. They were defined as compounds promoting division of tobacco cultured cells in the presence of auxin. The first endogenous cytokinin to be identified, *trans*-zeatin, was isolated from immature sweet corn seeds (Letham, 1963). Naturally occurring cytokinins are $N^6$-derivatives of adenine with either an isoprenoid or aromatic side chain (Mok and Mok, 2001). Synthetic compounds with cytokinin activity were also prepared. They are either derivatives of adenine or of phenylurea, even though phenylurea is not structurally related to natural cytokinins (Mok et al., 2000).

Cytokinins are present throughout the plant, but they are most abbundant in highly dividing tissues, such as the root tip, shoot apical meristem and immature seeds. They exert their function at nanomolar concentration (Schmülling, 2004). Cytokinins influence virtually all physiological processes: among the most important ones belong stimulation of cell division, branching, vascular bundle differentiation, chloroplast differentiation and delay of chlorophyll degradation and hence of plant senescence (Schmülling, 2004). Other functions of cytokinins include regulation of seed germination, organogenesis, *de novo* bud

formation and release of buds from apical dominance, enlargement of leaves, increasing the number of lateral roots and enhancing nutrient uptake (Hwang et al., 2012; Kieber and Schaller, 2014). Cytokinins negatively influence root growth by decreasing the number of dividing cells in the root tip (Mok and Mok, 2001). They have both positive and negative roles in stress coping (Zwack and Rashotte, 2015). Cytokinins further participate in the interaction of plants with other organisms, including bacteria, fungi, insects, mites, molluscs, nematodes, protists and viruses (Kaiser et al., 2010; Zhang et al., 2016).

Different processes of cytokinin metabolism are subcellularly compartmentalised, probably contributing to the regulation of the amount of active hormones (Kiran et al., 2012).

## Cytokinin Biosynthesis

So far, only biosynthesis of the isoprenoid cytokinins has been explored. There are two pathways of isoprenoid cytokinin biosynthesis (Sakakibara, 2006), where the isoprenoid chain is transferred to:

- adenosine nucleotide (AMP, ADP, ATP)
- adenosine bound in tRNA

The units for synthesis of isoprenoids originate from two metabolic pathways. The mevalonate (MVA) pathway starts by joining two acetyl-CoA molecules. It is localised in cytosol and mitochondria. The first step of the methylerithritol phosphate (MEP) pathway (also non-mevalonate pathway), which occurs in plastids, is the conjugation of pyruvate and glyceraldehyde-3-phosphate. An intermediate of the MEP pathway, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBDP), and dimethylallyl pyrophosphate (DMAPP), which is the final product of both pathways, is can both serve as donors of the side chain for cytokinin biosynthesis (Sakakibara et al., 2005).

The catalyst of the $N^6$-prenylation of adenine is the enzyme isopentenyl transferase (IPT; Chen and Melitz, 1979; Kakimoto, 2001; Takei et al., 2001a). *IPT* genes are categorised into two groups: *adenylate IPTs* (EC 2.5.1.27 and EC 2.5.1.112; 7 genes in *Arabidopsis*) which isopentenylate the $N^6$-group of free adenine and *tRNA IPTs* (EC 2.5.1.75; 2 genes in *Arabidopsis*) which work analogously on adenine in tRNA (Frébort et al., 2011).

The levels of cytokinins are controlled by the equilibrium between biosynthesis, transport, interconversions and degradation. Cytokinin biosynthesis runs only in specific types of cells, with organ and tissue specificity of *IPT* expression described (Miyawaki et al., 2004). However, in Arabidopsis, *IPTs* have been shown to be expressed throughout the whole plant (Miyawaki et al., 2004).

*AtIPT1* is mostly expressed in the procabium of the main root, cotyledons, pollen, ovules and immature seeds, while expression of *AtIPT4* and *AtIPT8* co-locates to immature seeds. High cytokinin synthesis in the seed may strengthen its sink capacity and hence influence cell division in the endosperm. *AtIPT5* is expressed in the columella root cap, in lateral root primordia and in the fruit abscission zone. *AtIPT3* is expressed mostly in phloem tissues and *AtIPT7* in trichomes of young leaves, pollen tubes, phloem and in the endodermis of the root elongation zone. The *tRNA IPTs*, *AtIPT2* and *AtIPT9* are expressed mostly in proliferating tissues, such as roots, leaf primordia and the shoot apical meristem (Miyawaki et al., 2004).

The enzymes AtIPT1, AtIPT3, AtIPT5 and AtIPT8 localise to plastids (Kasahara et al., 2004). Thus, the donors of the isoprenoid side chain are provided primarily by the MEP pathway (Kasahara et al., 2004). AtIPT2, the tRNA isopentenyl transferase, is localised in the cytosol (Kasahara et al., 2004).

The primary products of cytokinin biosynthesis are cytokinin nucleotides, while the free bases are considered as the active forms (Lomin et al., 2015), which must be produced from the nucleus by hydrolysis. The cytokinin nucleotides may be activated in a single step in most of the plant body by the enzyme LONELY GUY (LOG), a cytokinin riboside 5'-monophosphate phosphoribohydrolase. This enzyme gives rise to cytokinin free base releasing ribose 5'-monophosphate (Kurakawa et al., 2007).

The only observed biosynthesis of dihydrozeatin to date is via reduction of *trans*-zeatin by a putative zeatin reductase (Gaudinová et al., 2005; Martin et al., 1989; Sondheimer and Tzou, 1971).

Another option for cytokinin biosynthesis is by degradation of the above mentioned isoprenylated tRNA. Cytokinin residues in tRNA may be considered as direct precursors of free cytokinins (Chen, 1997). The plant tRNAs contain mostly *cis*-zeatin ((*Z*)-$N^6$-(4-hydroxy-3-methylbut-2-enyl)adenine; Miyawaki et al., 2006). Since tRNA degradation may account for only up to 40% of total cytokinin (Barnes et al., 1980), it was assumed that cZ may originate through the action of a putative zeatin *cis-trans* isomerase (Bassil et al., 1993). However, the existence of the isomerase was disputed recently (Hluska et al., 2017) and thus, the indirect tRNA pathway is considered to be the only one for *c*Z biosynthesis (Miyawaki et al., 2006).

## Cytokinin Degradation

Cytokinins are irreversibly degraded by cytokinin oxidase/dehydrogenase (CKX; EC 1.5.99.12). CKX cleaves the cytokinin molecule at the $N^6$-position yielding adenine (or corresponding derivative) and an aldehyde derived from the side chain (Galuszka et al., 2001; Pačes and Kamínek, 1976; Whitty and Hall, 1974).

The family of N-glycosyltransferases is another group of enzymes inactivating both isoprenoid and aromatic cytokinins (Entsch et al., 1979). *N*-glucosyltransferase (UGT, EC 2.4.1.118) transfers glucose from uridine-5'-diphosphoglucose to the N7- and N9-atoms of the cytokinin. *N*-glucosides show only low activity in bioassays (Spíchal et al., 2004), although current data challenge this notion (Pokorná and Motyka, in unpublished).

Cytokinins with a hydroxyl group on the side chain may be inactivated also by *O*-glycosylation catalysed by *O*-glycosyltransferases. The *O*-glycosides are resistant towards degradation by CKX though (Zalabák et al., 2014). Zeatin-*O*-glucoside is stored in the vacuole (Kiran et al., 2012). In the case of cytokinin *O*-glucosides and kinetin N3-glucoside, maize β-glucosidase, localised in plastids/chloroplasts is capable of hydrolysing them to produce their active forms (Brzobohatý et al., 1993).

## Cytokinin Signaling and its Origins

A signal transduction system connects inputs from the outside with specific responses of the organism. In comparison with the wide range of possible inputs and resulting responses only a relatively small group of molecular strategies is used for signal transfer (West and Stock, 2001).

Bacteria had to accommodate quickly changing conditions of the environment. A rapid response to changes of temperature, nutrient availability, toxins, changes in humidity, pH, osmolality and other stress factors was crucial for survival of the organisms. Thus, an elaborate system was formed which monitors and subsequently responds to changes of external conditions. This system is based on a network of signal receivers and response regulators (Parkinson and Kofoid, 1992). The arrangement of the system depends on the needs of the particular signalling pathway. The functionality of the system may be extended by re-arrangement of conserved domains in proteins and incorporation of proteins into the signalling cascade (West and Stock, 2001).

The basis of the system is mostly phosphorylation (West and Stock, 2001). His-Asp phosphotransfer system is present mainly in bacteria, but it is rather rare in eukaryotes, which rely mostly on Ser, Thr and Tyr phosphorylations (Stock et al., 1995). However, the modular components of the signalling systems seem to be interconnected across several paths (as reviewed in Stock et al., 2000). For example, His-Asp phosphorylations are conjugated with MAP kinases or cAMP-dependent protein kinases (as reviewed in Stock et al., 2000).

Stock et al. (1990) defined two families of homologous proteins transferring signals among 11 prokaryote species. They included sensory histidine kinases (HKs, transmembrane proteins) and response regulators (RRs, cytoplasmic proteins; Parkinson

and Kofoid, 1992). The level of RR phosphorylation is further regulated by the activity of phosphatases (Stock et al., 1990).

The functionality of the system components is directed by three reactions of phosphate transfer (Stock et al., 1990):

1) autophosphorylation of conserved histidine on the transmitter domain of a receptor

$ATP + HK \leftrightarrow ADP + HK \sim P$

2) transfer of the phosphate onto a conserved aspartate in the receiver domain of a response regulator

$HK \sim P + RR \rightarrow HK + RR \sim P$

3) dephosphorylation of the response regulator

$RR \sim P \rightarrow RR + P$

The response regulators control a wide range of processes in the cell by transferring the signal from receptors to elements responsible for regulation of movement or gene expression (Stock et al., 1990).

Already Stock et al in 1990 asked the questions about the molecular mechanisms regulating activity of the kinases and phosphatases, the nature of the mechanism of RR switching and also about analogies of the two-component system in eukaryotic signal transduction systems.

## A. Molecular Mechanisms of the His-Asp Phosphorylation System

The two-component regulatory His-Asp system triggers response to changes of the external environment. It is present both in prokaryotes and eukaryotes (Mizuno, 1997). It is necessary to multiply the signal upon ligand detection, often before starting the metabolic reaction. In the end, the signal is often terminated through a feedback loop, which changes the sensitivity of the receptor (Alex and Simon, 1994).

There is a relatively small number of molecular strategies in comparison with the huge diversity of stimuli and responses. One of the basic strategies is phosphorylation of proteins by protein kinases. Apart from autophosphorylation, kinases activate also other protein substrates by phosphorylation at specific amino acids – serine, threonine or tyrosine – in eukaryotic signaling systems (Stock et al., 1990).

The first component of the two-component systems (TCS) is a detector that is capable of interaction with a ligand at appropriate concentration (Alex and Simon, 1994). Based on available sequences in databases, most proteins belonging to the family of histidine kinases are homodimeric membrane proteins containing sensory and kinase domains. The

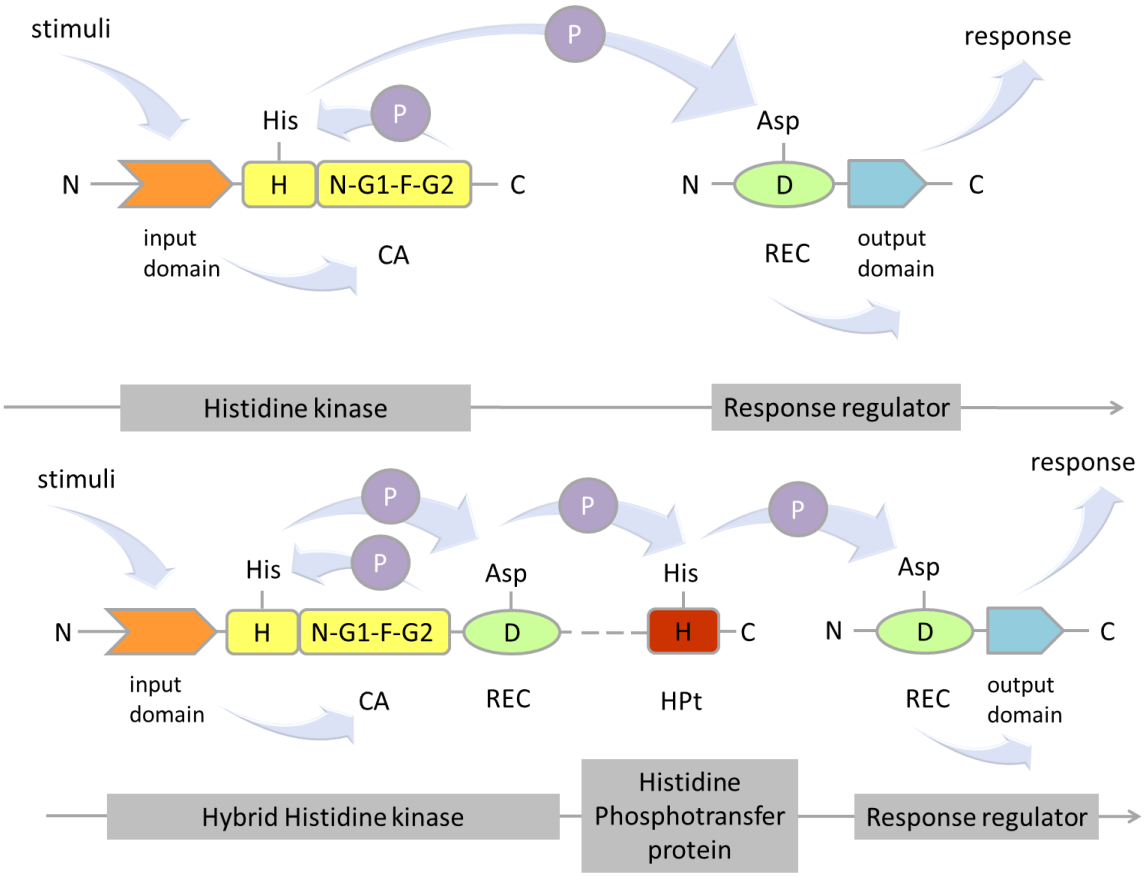



Figure 1: Scheme of two-component systems (TCS).
a) The prototype of the TCS signalling pathway has features as a conserved ph osphoryl transfer to link various input stimuli and output responses. A phosphate residue is transferred from the highly conserved kinase core (CA – catalytic domain with homology boxes H, N, G1, F, G2) to receiver (REC) domain.
b) The multi-step phosphorelay system uses a more complex pathway for multiplication of phosphotransfer events in comparison with TCS. It contains hybrid histidine kinase with additional REC and phosphotransfer (HPt) domains which can be linked to HK or independent (figures modified from Gao and Stock, 2009; Parkinson and Kofoid, 1992).

sensory domain is at the N-terminus and it has variable sequence depending on the perceived input. The kinase domain is at the cytoplasmic C-terminus and it is highly conserved. Primary conserved motives were described as H, N, G1, F and G2 blocks (Fig. 1). The designation is based on characteristic residues. Block H is variable, but it contains the histidine involved in the autophosphorylation. N and F blocks are represented by β-secondary structures. Blocks G1 and G2 are similar to glycine-rich parts of nucleotide-binding domains. Both, G1 and G2 blocks may contain a nucleotide-binding site because of the presence of glycine loops characteristic for many ATP-binding sites. Beside that, block G2 is involved in autokinase and phosphatase activity (Parkinson and Kofoid, 1992; Stock et al., 1989, 2000).

Similarly as a sensor, the RRs contain a receiver module at their N-terminus paired with one or more C-terminal transmitter domains. There are two ways by which the transmitters regulate the phosphorylation level of their receiver proteins. They may possess autokinase activity which transfers a phosphate group from ATP to a histidine residue. This reaction is reversible, due to the formation of a high energy product – phosphohistidine. Another mechanism by which the level of phosphorylation is regulated is through the transmitter's phosphatase activity towards its related receivers. In this way the flux of phosphoryl groups toward and back to cognate receivers is regulated (reviewed in Parkinson, 1993).

The cytokinin signal transduction pathway in the model plant *Arabidopsis thaliana* is based also on the TCS system. It is composed of four principal steps: first sensing and signalling by histidine protein kinase (AHK), next translocation of a phosphotransfer protein (AHP), Arabidopsis Response Regulator (ARR)-dependent trancriptional activation and a negative feedback loop *via* cytokinin-induced expression of some *ARR* genes (Fig. 2). The cytokinin signal is perceived by a membrane-bound CHASE domain, which is a component of a histidine kinase (Hwang and Sheen, 2001). Earlier, the histidine kinases were abbreviated as HK. However it was agreed at the cytokinin meeting (Cytokinin Metabolism, Signaling, and Function, July 8–10, 2012, in Berlin) that if the His kinase contains a CHASE domain, it should be designated as CHK (CHASE-containing His kinase; Heyl et al., 2013).

The function of cytokinin receptors include direct perception of adenine and diphenylurea derivatives and transduction of external cytokinin signals across the membrane (Yamada et al., 2001). After binding of the ligands, the receptor autophosphorylates its His residue and the phosphate residue is transferred to a Asp residue on the hybrid CHK (Hwang et al., 2002).

Cytoplasmic-to-nuclear transfer of the phosphate residues is mediated by *Arabidopsis* phosphotransfer (HPt) proteins (AHP1-5), that phosphorylate *Arabidopsis* type-B RRs (ARR-B 1,2,10-14,18-21) in the nucleus (Hwang et al., 2012; Hwang and Sheen, 2001). AHPs shuttle between the cytoplasm and nucleus by active nuclear import and export. There is experimental evidence that localisation of AHPs is not based on their level of phosphorylation. Rather, AHP proteins occur in the nucleus and cytosol, independently from cytokinin signaling (Punwani and Kieber, 2010).

AHP6, called pseudo-AHP, belongs also to the group of histidine phosphotransfer proteins. It carries asparagine, instead of the conserved His residue. Thus, it showed no ability to accept the phosphoryl group, indicating no phosphotransfer activity. The opposite situation was detected in the case of alternative splicing variant of AHP6b that had the aminoacid Asn83 replaced with His (Mähönen et al., 2006a). AHP6 plays a role as

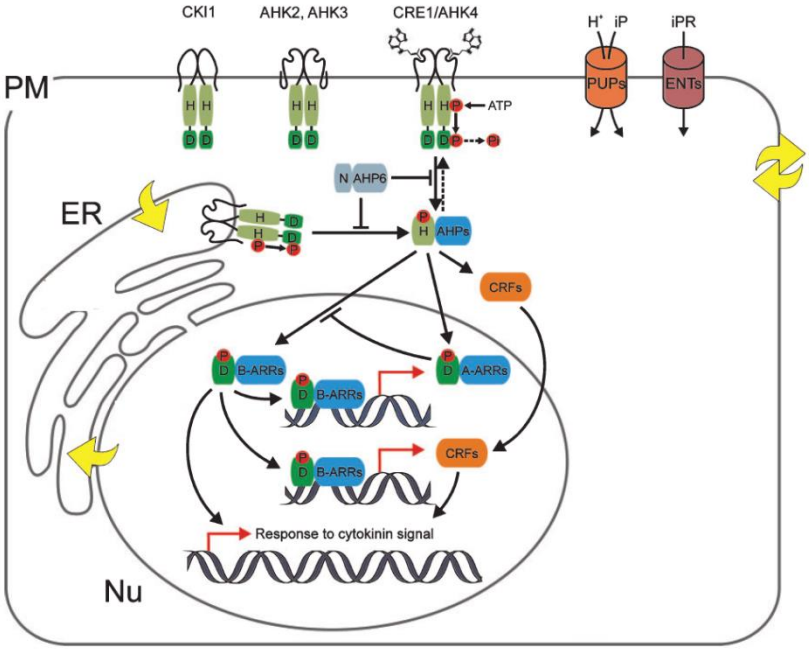



Figure 2: Model of cytokinin two-component signalling system in *Arabidopsis thaliana*. Cytokinin molecules are perceived by AHK2, AHK3, CRE1/AHK4 receptors in the plasma membrane (PM) and endoplasmatic reticulum (ER). Transduction of the signal starts by autophosphorylation of histidine kinases, transport of a phosphate residues (P) from His (H) to Asp (D) and then to a His residue of histidine phosphotransfer proteins (AHPs). Phosphate is deliveried to the nucelus (Nu) and it activates type-B response regulators (B-ARRs), which promote gene expression, including that of response regulators type-A (A-ARRs) serving as negative feedback. Many other factors are involved in the regulation of TCS: cytokinin response factors (CRFs), AHP6 (pseudo-AHP), CKI1 (cytokinin independent 1) histidine kinase, purine permease transport system for nucleobases (PUPs), transporters for cytokinin nucleosides (ENTs). The presumed pathways of cytokinin membrane transport are represented by yellow arrows. Figure modified from Spíchal (2012).

an inhibitor of cytokinin signaling in the phosphorelay cascade, usually by competition with AHP1-5 (Mähönen et al., 2006a).

ARRs are clasified into three types: type-A, type-B and type-C. The signal is transduced as phosphate from the HPt protein to transcription factors, represented by type-B ARRs. They contain both an RR domain and a myeloblastosis (Myb)-related DNA binding domain at the C-terminal region. Myb-related domains allows binding of target DNA sequences (Hosoda et al., 2002; Sakai et al., 2001). A second set of *Arabidopsis* response regulators are type-A ARRs (ARR-A 3-9, 15-17), which are shown by phylogenetic analysis to be separate from the type-B ARRs (D'Agostino et al., 2000). Expression of type-A ARRs is quickly up-regulated by exogenous cytokinin and therefore they belong to the target genes of type-B ARRs (Rashotte et al., 2003). ARR-As have a function as negative-feedback regulators of the cytokinin signalling pathway (To et al., 2004). The

interaction of ARR-As with AHPs, but not with ARR-Bs, predicts the mechanism of ARR-A activation (Dortay et al., 2006). When a type-A ARR is phosphorylated, it can negatively coordinate the cytokinin response, separately from phospho-competition with type-B ARRs. Negative feedback regulation of type-A ARRs seems to be directed through phospho-interactions with target proteins (To et al., 2007). Dephosphorylation of ARR-As occurs much faster than of ARR-Bs, so that ARR-As could function as phosphatases for the AHPs and thus reset the signalling pathway (Gruhn and Heyl, 2013). The last g roup of ARRs are type-C (ARR22 and ARR24), which contain receiver domain sequences, suggesting their role in the phosphorelay system. While they have a domain structure similar to the type-A ARRs, their transcription level is not activated by cytokinins (Kiba et al., 2004). Consequently the role of the ARR-Cs in the TCS is currently not clear (Kang et al., 2013).

Cytokinin response factors (CRFs) are also involved in regulating the transcriptional response to cytokinin. Interaction of CRF with members of the TCS has been shown (Cutcliffe et al., 2011). Expression level of three *CRF* genes (*CRF2*, *5*, *6*) is markedly up-regulated in response to cytokinin in *Arabidopsis thaliana*. *CRF1, CRF3* and *CRF4* showed no or only little change in expression level in response to cytokinin. However, after cytokinin application, all GFP-CRF fused proteins were accumulated in the nucleus. The CRFs relocalization is dependent on the CHKs and AHPs. The CRF proteins with B-type ARRs are probably involved in initial response to cytokinin (Rashotte et al., 2006).

## B. Histidine Kinases in *Arabidopsis thaliana*

*Arabidopsis* contains 6 histidine kinase receptors that do not belong to ethylene or phytochrome receptors (Tran et al., 2007). These are: AHK1 (AtHK1) – an osmosensor (Urao et al., 1999; Wohlbach et al., 2008); three cytokinin receptors AHK2, AHK3 and CRE1/AHK4 (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001b; Yamada et al., 2001); AHK5 (CKI2; Desikan et al., 2008; Iwama et al., 2007; Mira-Rodado et al., 2012) and CKI1 (Hwang et al., 2012; Kakimoto, 1996).

The first putative cytokinin receptor was reported by Kakimoto (1996). Transgenic calli formed shoots upon *CYTOKININ INDEPENDENT1* (*CKI1*) overexpression without external cytokinin. However, it showed constitutive activity in bacterial system (Yamada et al., 2001). Thus, CKI1 was estimated to be a constitutively active receptor and/or it could perceive endogenous signals (Hwang and Sheen, 2001). CKI1 interacts with several AHPs and it is essential for female gametophyte development (Liu et al., 2017).

*AHK5/CKI2* (*ARABIDOPSIS HISTIDINE KINASE5/CYTOKININ INDEPENDENT2*) participates in responses to biotic and abiotic stresses. Further, it regulates the equilibrium concentration of hydrogen peroxide in the guard cells in

*Arabidopsis* (Desikan et al., 2008) and stomatal closure in response to ethylene or hydrogen peroxide (Mira-Rodado et al., 2012). CKI2 should localise to the cytoplasm and also to the plasma membrane (Desikan et al., 2008). It does not contain the N-terminal transmembrane domain, but contains two coiled domains, which are probably involved in the interaction with other proteins in cytoplasm (Hwang et al., 2002). CKI2 interacts with AHP1, AHP2 and AHP5 (Mira-Rodado et al., 2012).

AHK1 (AtHK1 – *Arabidopsis thaliana HISTIDINE KINASE 1*) was annotated as a putative osmoreceptor. The structure of the protein is similar to that of other AHKs. It contains two transmembrane domains, and transfer and receiver domains at the C-terminus with conserved histidine and aspartate residues, respectively. Its localisation to the plasma membrane was demonstrated by fusion with GFP (Caesar et al., 2011). It operates as an osmosensor *in vivo* when is expressed in yeast. The expression of *AHK1* is regulated in response to external osmolarity (Urao et al., 1999) and it functions as positive regulator in osmotic stress in *Arabidopsis* (Tran et al., 2007). Further, it is involved in germination and growth.

The first cytokinin receptor was unambiguously identified only at the beginning of the 21st century (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001b; Yamada et al., 2001). Plants insensitive to cytokinin in a root bioassay were found to have a mutation in *cytokinin response1* (*cre1*) coding for a histidine kinase. On the other hand, CRE1 successfully complemented a dysfunctional histidine kinase receptor in a yeast system in a cytokinin-dependent manner (Inoue et al., 2001). Further, it was confirmed in the same year, that CRE1 (also called Arabidopsis HISTIDINE KINASE4 (AHK4) or WOODEN LEG (WOL)) binds cytokinins specifically in the extracellular domain and transfers the signal through the membrane (Suzuki et al., 2001; Ueguchi et al., 2001b; Yamada et al., 2001). CRE1/AHK4 exerts kinase activity upon cytokinin binding and it phosphorylates AHP proteins. However, if cytokinin is absent, CRE1/AHK4 acts as a phosphatase and removes phosphate from AHP proteins to decrease phosphate load in the system (Mähönen et al., 2006b).

In contrast to CRE1/AHK4, CKI1 contains only one conserved REC domain and it shares only 25% sequence identity with this receptor. Although CKI1 does not function as a cytokinin receptor (Hwang et al., 2002), it does influence cytokinin signalling independently of CRE1/AHK4 in specific organs of *Arabidopsis* (Hwang and Sheen, 2001). When the conserved residues His405 in the histidine kinase domain and Asp1050 in the receiver domain were mutated, expression of *ARR6-LUC* was observed to determine, whether the kinase activity or phosphate transfer are important for activation of a response regulator (Hwang and Sheen, 2001). Wild-type CKI1 showed high LUC/GUS activity regardless of *tZ* application, whereas CKI1 with both aforementioned mutations, H405Q and D1050N, induced higher activity in the presence of 100 nM *tZ*.

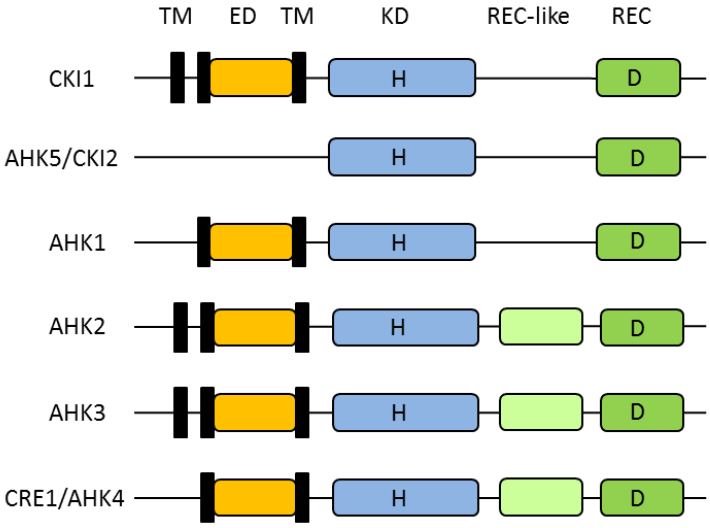



Figure 3: Schematic model of AHK family´s primary domain structures. CKI1 and CRE1/AHK4 are similiar in domain structure but divergent in amino acid sequence. AHK1 and CKI1 consist of 2 or 3 transmembrane helicles on the N-terminus surrounding an extracellular domain, followed by a C-terminal kinase and receiver domains (REC). Cytokinin receptors display a similar structure with an additional REC-like domain. TM – transmembrane domain, ED – extracellular putative input domain, KD – kinase domain, REC – receiver domain, REC-like – receiver-like domain, H – His, D – Asp (adapted from Hwang et al., 2002).

The activity of H405Q was lower than of D1050N. Thus, the histidine kinase activity and the ability to transfer phosphate are required for CKI1's functioning in cytokinin signalling (Hwang and Sheen, 2001).

Further, additional genes *AHK2* and *AHK3* were identified as cytokinin receptors in *Arabidopsis* (Higuchi et al., 2004; Hwang and Sheen, 2001; Nishimura et al., 2004). The key property which distinguishes the cytokinin receptors from other histidine kinases is the presence of CHASE (Cyclases/Histidine Kinases Associated Sensory Extracellular) domain (Anantharaman and Aravind, 2001; Mougel and Zhulin, 2001). There are 2 (CRE1/AHK4) or 3 (AHK2 and AHK3) transmembrane domains at the N-terminus of the receptors (Fig. 3). These surround the CHASE domain (Anantharaman and Aravind, 2001; Hwang et al., 2002) and are followed by an intracellular catalytic histidine kinase domain with a receiver (REC) domain at the C-terminus. The REC domain bears the phosphorylatable aspartate residue (Ueguchi et al., 2001b; Yamada et al., 2001).

All three receptors participate in the growth of the *Arabidopsis* shoot and exhibit a high level of redundancy. They take part in respective processes to various degrees (Riefler et al., 2006). Knock-out of a single receptor has little to no effect on the plant phenotype. However, the individual receptors mediate also specific responses in certain processes (Inoue et al., 2001; Riefler et al., 2006).

Expression of the *AHK2* receptor was detected in the root, leaves and some parts of the flower; *AHK3* is expressed in the root, leaves and stem (Ueguchi et al., 2001a). The double *ahk2 ahk3* mutant contained less chlorophyll and had fewer cells in the leaves. Especially AHK3 controls leaf development. Root branching was enhanced in all double mutants, but especially so in the *ahk2 ahk3* mutant. This is in accordance with cytokinins negatively regulating root growth. The function of these two receptors is further redundant namely in quantitative control of organ growth and root and shoot development (Riefler et al., 2006). Both AHK2 and AHK3 participate in the regulation of the onset of leaf senescence (Kim et al., 2006; Riefler et al., 2006).

The AHK3 receptor is involved through ARR1 and ARR12 in the induction of differentiation of cells in the transition zone of the root apical meristem (Dello Ioio et al., 2007). Further, it controls the asymmetric cell division in the vascular initials (Mähönen et al., 2000) as well as regulating the sensitivity to cytokinins during root elongation (Inoue et al., 2001; Riefler et al., 2006).

CRE1/AHK4 is active in leaf initiation, the timing of flowering (Riefler et al., 2006), vascular bundle morphogenesis and cell division in the procambium throughout embryogenesis (Mähönen et al., 2000). It is expressed mostly in the root, more specifically in the vascular cylinder and pericycle (Mähönen et al., 2000). AHK4 senses cytokinins primarily in the main root (Higuchi et al., 2004; Inoue et al., 2001; Nishimura et al., 2004; Riefler et al., 2006).

The rosette diameter was decreased in the single *ahk3* mutant, whereas the double mutant *ahk2 ahk3* and the triple mutant *ahk2 ahk3 cre1/ahk4* developed only a minuscule rosette. Knocking-out *AHK3* had the biggest effect on chlorophyll content. The triple mutant had three-times larger seeds in comparison with WT. Seed germination in the dark is probably suppressed by the combined activity of all three receptors with a significant contribution from CRE1/AHK4. The double mutant *ahk2 ahk3* had a greater number of lateral roots as did to a lesser extent the other double mutants, while single mutants did not exhibit this phenotype (Riefler et al., 2006).

The first model of *Arabidopsis* cytokinin receptor localisation was based on bioinformatic predictions and on homology with bacterial and yeast histidine kinases (Inoue et al., 2001; Ueguchi et al., 2001a). The first experimental evidence came from *Arabidopsis* protoplasts in which a fusion protein of AHK3 with green fluorescent protein (AHK3-GFP) was observed in the plasma membrane (Kim et al., 2006). In the same paper, authors also presented data on the dependence of the cytokinin-receptor interaction on pH, temperature and salt concentration. The optimal condition for AHK3 to bind *tZ* is a basic environment; for CRE1/AHK4, it is a basic to neutral environment (Romanov et al., 2006). These conditions are typical for the cytoplasm, nucleus, endoplasmic reticulum (ER) or mitochondria, while the apoplast is characterised by an acidic environment. Thus,

Romanov et al. (2006) postulated location of the CHASE domain inside of the cell, because the ability to bind cytokinin decreased with decreasing pH.

The localisation of the maize cytokinin receptor ZmHK1 was observed a few years later (Lomin et al., 2011). The signal of ZmHK1-GFP in maize protoplasts was detected in the ER and nuclear envelope. The authors used also monoclonal antibodies against the REC domain and observed receptor localisation in microsomal fractions from roots. Consequently, the authors concluded that ZmHK1 is localised mainly in the ER membrane. However, they were not able to confirm the presence of ZmHK1 also in microsomes from leaves, supposedly because of low expression (Lomin et al., 2011).

The group of prof. Harter with his colleagues used transient expression in epidermal cells of *Nicotiana benthamiana*, cotyledons of *Arabidopsis* and transgenic *Arabidopsis* plants with stabile expression to study localisation of AHK3 (Caesar et al., 2011). They showed ER localisation with an internal signal from N- and C-terminal fusions with GFP. Further experiments showed sensitivity of AHK3 to endoglycosidase H. Thus, AHK3 probably contains mannose structures, which are sensitive to endoglycosidase H, characteristic for glycoproteins in the ER (Caesar et al., 2011).

It is assumed that the CHASE domain is in the lumen of the ER and the C-terminal kinase domain is localised in the cytoplasm (Caesar et al., 2011). Other biochemical and biological techniques were used to specify the position of cytokinin receptors - membrane fractionation from Arabidopsis seedlings; display of AHK-GFP fusion proteins expressed in tobacco epidermal cells; BiFC analysis for the AHK2 receptor (Wulfetange et al., 2011). Based on results from membrane fractionation and subsequent cytokinin-binding assays, the localization of AHK2 and AHK3 receptors was confirmed mostly in the internal membrane system. In contrast, the CRE1/AHK4 receptor was detected also in the plasma membrane. Study of the localization of AHK3-GFP and GFP-CRE1/AHK4 in epidermal tobacco leaf cells showed occurrence of both receptors at the ER. The bimolecular fluorescence complementation analysis of the AHK2 receptor even supported the hypothesis that the receptor was localised mainly in the ER. Cytokinin receptors were detected also at low abundance on the plasma membrane (Wulfetange et al., 2011). Zurcher et al. (2016) characterised PUP14 (purine permease 14) cytokinin transporters, with plasma membrane localization, which import bioactive cytokinins into the cytosol. By taking up ligands from the apoplast, they suppressed the response to cytokinins. In this case, extracellular cytokinins bound to receptors located just in the plasma membrane to initiate a signal response process, while PUP14 functions in the plasma membrane as a cytokinin importer, thereby reducing the cytokinin signal (Zürcher et al., 2016). Thus, the localization of cytokinin receptors remains unclear.

## C. Characterisation of the CHASE Domain of CRE1/AHK4

By comparing the CRE1/AHK4 amino acid sequence with sequences from different bacterial membrane proteins, a conserved region called the CHASE domain was characterized. Sequence alignment included various receptor-like proteins with different nucleotide cyclase or histidine kinase domains. This mobile domain contains 200-230 amino acids conserved across evolution. The CHASE domain is always followed by other intracellular enzyme domains - adenylate cyclase, GGDEF-type of nuclease cyclase and EAL-type of phosphodiesterase domain (Anantharaman and Aravind, 2001). There are also present non-enzymatic domains (PAS, GAF), phosphohistidine and input domains (Han et al., 2004). A large number of CHASE domain-containing proteins were found in bacterial genomes. Some are histidine kinases, hybrid forms of histidine kinases or various types of receptors with other catalytic function, especially adenylate or guanylate cyclases (Mougel and Zhulin, 2001).

PAS domains were primarily recognised as intracellular sensor domain (Ponting and Aravind, 1997; Zhulin et al., 1997), If the sensory domains were extracellular, they were reffered to as PAS, PAS-like, PDC, PDC-like or PDC/PAS, depending on the particular protein databases (Upadhyay et al., 2016). Based on the MHH model of available PAS-like domains and their 3D structure comparisons, it was shown, that extracellular PAS (PDC)-like domains should have their own superfamily, which was named the Cache superfamily (Upadhyay et al., 2016). It is homologous to the original PAS superfamily (Anantharaman and Aravind, 2001; Upadhyay et al., 2016). The Cache superfamily is characterised by a long N-terminal α-helix and presence of connecting elements (between two globular domains). Cache transmits signal through its C-terminal transmembrane helix and the N-terminal helix is an extension of the first transmembrane helix, which converts the intracellular sensor to its extracellular form (Upadhyay et al., 2016). However, in this thesis, the old naming PAS and PAS-like will be used to distinguish the two parts of the Cache domain.

There are three main groups of receptor proteins in prokaryotes: the sensor-based histidine kinase in TCS, the chemotaxis-mediating proteins and the diguanyl cyclase. These receptor proteins correspond in several sensor domains, which are involved in the recognition of an intracellular or extracellular signal, e.g. a PAS or a GAF domain. Some of these domains have also been found in eukaryotic organisms. Histidine kinase VhK-1, containing the CHASE domain, was also found in the EsV-1 virus virion. It is assumed that the virus' CHASE domain originates from its host - the brown alga *Ectocarpus siliculosus*. (Mougel and Zhulin, 2001).

In higher plants, the CHASE domain is part of the hybrid histidine kinase. An exception is the predicted cytokinin receptor of rice, OsCRL4, where the binding domain is

combined with the Ser/Thr kinase domain and does not contain the receiver domain. OsCRL4 should not belong to the two-component signal system (Han et al., 2004).

There are 6 known splicing variants of CRE1/AHK4 (NCBI Gene ID 814714; At2g01830). They differ in the length of the 5'-UTR and/or in the position of the START codon (Hrtyan et al., 2015). The boundaries of the CHASE domain are recognised by at least two transmembrane segments. In comparison with the central part of the domain, the boundary regions are not very conserved. The conserved motives form α+β secondary structures forming hydrophobic core of leading strands indicative of an extracellular domain (Anantharaman and Aravind, 2001; Ueguchi et al., 2001a; Yamada et al., 2001). The extracellular CHASE domain is connected with an intracellular catalytic histidine kinase A (HisKA) domain, followed by a histidine kinase-like ATPase (HATPase) with the receiver (REC) domain at the C-end. The REC domain contains the aspartate capable of being phosphorylated (Fig. 3; Anantharaman and Aravind, 2001; Heyl et al., 2007).

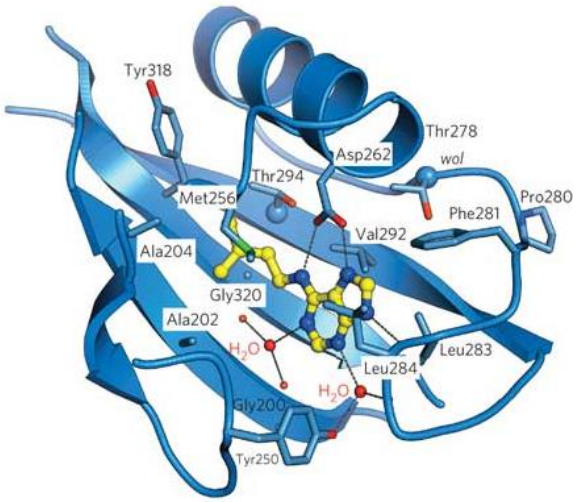



Figure 4: AHK4 binding pocket with ligand. The threedimensional structure shows complex of the membrane-distal PAS domain with iP inside of its binding cavity. Amino acid residues connected via hydrogen bonds to the ligand are displayed (from Hothorn et al., 2011).

Most of the plant CHASE domains contain 203 - 226 amino acids (Steklov et al., 2013). Part of the CHASE domain are two PAS (Per-Arnt-Sim) domains (Pas et al., 2004), membrane-proximal and membra-ne-distal (Steklov et al., 2013). However, the membrane-proximal PAS domain misses one β-strand and hence is called PAS-like or pseudo-PAS (Henry and Crosson, 2011; Steklov et al., 2013). In the cytokinin binding pocket, the N-terminal part of α1-helix is covalently bound with the β12 strand by disulfide bonds (Hothorn et al., 2011; Steklov et al., 2013). A hydrophobic top of the binding site is formed by two turns: β3-β4 and β4-α4. In the binding pocket the cytokinin adenine is bound by hydrogen bonds to Asp262 and Leu284 (Fig. 4). Other interactions are through 3 water molecules: the first water molecule forms hydrogen bond between the hydroxyl of Tyr 250, backbone of Leu284 and N3 of adenine. The second water molecule is near N1 of adenine without interaction with the protein, while the third molecule is near the cytokinin side chain and Asp262 and Thr294 (Steklov et al., 2013). The bottom part of the binding site is delimited by the central β-sheet of the PAS

subdomain (β7 a β8). It is lined by small hydrophobic amino acids, which if substituted with more bulky ones, do not allow cytokinin binding and the receptor is inactivated (Hothorn et al., 2011; Steklov et al., 2013).

Hydrogen bonds between Asp285 and the adenine ring are indispensable for receptor function. Active cytokinins are characterized by the presence of a planar adenine ring that is capable of forming hydrogen bonds with Asp285, and a planar aliphatic or aromatic side chain (Hothorn et al., 2011). These conclusions are based on the crystal structure of CRE1/AHK4 with various cytokinin ligands bound into the CHASE domain. There is a long helical stalk on the outside of the CHASE domain providing stability to the domain (Hothorn et al., 2011). About 20 amino acids come into contact with the ligand (Fig. 4). In addition to the above-mentioned hydrogen bonds, aminoacids are mainly involved in the formation of hydrophobic interactions with the adenine ring, but especially with the side chain of the cytokinin (Steklov et al., 2013).

## D. Cytokinin Receptors in Other Organisms

Since 2001, when cytokinin receptors were discovered in the model plant *Arabidopsis* (Inoue et al., 2001; Suzuki et al., 2001), their functional and biochemical properties have been studied. Cytokinin receptor genes have been identified in numerous plant species, initially in *Arabidopsis thaliana* (Ueguchi et al., 2001a), and then also in Madagascar periwinkle (*Catharanthus roseus*; Papon et al., 2002), corn (*Zea mays*; Lomin et al., 2011; Yonekura-Sakakibara et al., 2004), tomato (*Lycopersicon esculentum*; Kumar et al., 2004), potato (*Solanum sparsipilum*; Jolivet et al., 2007), rice (*Oryza sativa*; Choi et al., 2012; Du et al., 2007; Ito and Kurata, 2006; Pareek et al., 2006), barrel clover (*Medicago truncatula*; Gonzalez-Rizzo et al., 2006), *Lotus japonicus* (Murray et al., 2007; Tirichine et al., 2007), white lupin (*Lupinus albus*) and alfalfa (*Medicago sativa*; Coba de la Peña et al., 2008a, 2008b), birch (*Betula pendula*; Nieminen et al., 2008), poplar tree (*Populus trichocarpa*; Immanen et al., 2013; Nieminen et al., 2008), grape vine (*Vitis vinifera*; Fernandes et al., 2009), oilseed rape (*Brassica napus*; Kuderová et al., 2015), brown mustard (*Brassica juncea*; Srivastava et al., 2009), peach (*Prunus persica*; Immanen et al., 2013), coyote tobacco (*Nicotiana attenuata*; Schäfer et al., 2015), apple tree (*Malus domestica*; Daudu et al., 2017), potato (*Solanum tuberosum*; Lomin et al., 2018), lower plant *Marchantia polymorpha* (Gruhn et al., 2014, 2015) and also in mosses (*Physcomitrella patens*; Gruhn et al., 2014, 2015; Pils and Heyl, 2009) and lycophytes (*Selaginella moellendorfii*; Pils and Heyl, 2009). Based on phylogenetic analysis, CHASE-containing proteins have been found in eukaryotic green algae *Volvox caterei* and *Chlamydomonas reinhardtii*, the brown alga *Ectocarpus siliculosus* and diatom *Phaeodactylum tricolorum*. Also the genomes of free-living amoeba, *Dictyostelium discoideum* and *Naegleria gruberi* contain CHASE proteins (Steklov et al., 2013; Wang et al., 1999). Kieber and Schaller (2010) have suggested that different forms of cytokinin could

have distinct informational content due to binding to special subsets of cytokinin receptors mediating various outputs.

## Cytokinin Transport

Cytokinins are synthetized in both roots and shoots. Their transport can be local and also long-distance from both parts in the xylem and phloem (Hirose et al., 2008). Xylem sap transports acropetally mainly *t*Z ribosides by transpiration flow. However, iP- and *c*Z-type cytokinins are transported by phloem sap from shoot to root (Kudo et al., 2010; Takei et al., 2004). The content and flow rate of *t*ZR is directed by endogenous and environmental signals. Nitrate contcentration positively regulates the amount of *tZR* via transcripts of AtIPT3 and cytokinin-responsive genes (Sakakibara et al., 1998; Takei et al., 2001b, 2004). Basipetal transport of cytokinins controls vascular patterning in the root meristem and transport of auxin via PIN activity regulation (Bishopp et al., 2011).

The mechanisms by which cytokinins are transported through the plasma membrane, are not completely elucidated (Kieber and Schaller, 2018). Cytokinin efflux from roots to shoots is mediated by *Arabidopsis* ATP-binding cassette (ABC) transporter subfamily G14. AtABCG14 is responsible for loading of cytokinins info xylem for subsequent transfer to the shoots (Ko et al., 2014; Zhang et al., 2014). Influx transport is supported by PUP transporters (Bürkle et al., 2003; Gillissen et al., 2000) and nucleoside transporters from the family of equilibrative nucleoside transporters (ENT; Hirose et al., 2005; Sun et al., 2005). PUPs are small hydrophobic polytopic proteins, which are localised in the plasma membrane (Gillissen et al., 2000). A $H^+$-coupled high affinity purine transport system mediates the energy-dependent high-affinity uptake of adenine and cytokinins by AtPUP1 and AtPUP2 transporters when expressed in yeast (Bürkle et al., 2003; Gillissen et al., 2000). AtPUP1 was tested as a complementation adenine transporter in the yeast system, in which there was competition between adenine, *t*Z and kinetin (Gillissen et al., 2000). PUP proteins probably function as transporters of nucleic acid base derivatives, as cytokinins. They may also transport secondary metabolities, such as alkaloids that are structurally similiar to purines (Gillissen et al., 2000). Bürkle with colleagues showed transport of [$^3$H]*t*Z by AtPUP1 and inhibition of its transport by cytokinins such as iP and kinetin and as well as by adenine (Bürkle et al., 2003). The homologues AtPUP1, AtPUP2 and AtPUP3 showed different responses with respect to adenine uptake. AtPUP2 mediates adenine uptake and it recognises substances such as iP, kinetin, BAP, *trans*- and *cis*-zeatin as subtrates. On the another hand AtPUP3 did not exhibit transport activity in the yeast system (Bürkle et al., 2003). Results from Arabidopsis cell culture showed that the PUP transport system recognises adenine and cytokinins (*t*Z, iP, BAP, *t*ZR) as its substrates. In the case of *t*Z, two types of transport systems were described –

low-affinity transport, where [$^3$H]*t*Z transport required energy and acted at micromolar concentrations. High-affinity transport works at nanomolar cytokinin concentration in the phloem and xylem. *t*Z was perceived primarily, while adenine acted as an inhibitor and other cytokinins reduced the perception of *t*Z. It follows that adenine has a higher affinity than *t*Z in the PUP transport system (Cedzich et al., 2008). Transport activity of the other plasma membrane-localized purine permease AtPUP14 is dependent on the presence of ATP. AtPUP14 imports bioactive cytokinins from the apoplast and decreases their perception by cytokinin receptors. This creates a sink for active ligands, which leads to a suppresion of the cytokinin response (Zürcher et al., 2016).

A second type of potential cytokinin translocation is *via* equilibrative nucleoside transporters (AtENT1-AtENT8). Some of these transporters showed participation in transport of nucleosides - adenosine, iPR, *t*ZR (Hirose et al., 2008; Li et al., 2003).

## Cytokinin Physiological Bioassays

The essential attributes that determine the efficacy of a bioassay are as follows – it must be specific to cytokinins, highly sensitive, detecting minute quantities of the more active cytokinins (for detection from plant extracts) and it must be quantitative. It should have a short assay time, to avoid long delays in experiments and to obtain more accurate measurements of activity. The ideal bioassay would last only hours or even minutes as bioassays are not capable of distinguishing between direct cytokinin action and indirect effects due to metabolic conversions. Especially naturally occuring compounds are rapidly metabolised or broken down in plant tissues (Mok and Mok, 1994). There is a list and some characterisation of physiological bioassays given below.

The lettuce seed germination assay was based on the relationship between biochemical activity of kinetin together with red-light to promote germination of lettuce seeds (Miller, 1958). It turned out to be affected by a number of compounds – most notably the gibberellins (Mok and Mok, 1994). The *Funaria protonemata* bioassay (Hahn and Bopp, 1968), pea lateral buds assay and chlorophyll retention oat-leaf assay (Thimann and Sachs, 1966) were based on the ability of cytokinins to promote formation of new buds, to release lateral buds from apical dominance and to delay senescence, respectively. In the case of the pea lateral buds assay, it does not detect *t*ZR, but it is sensitive to *trans*- and *cis*-Z (Mok and Mok, 1994). The etiolated bean leaf disc (Miller, 1963), *Spirodela* frond expansion (Letham, 1967) and radish cotyledon (Letham, 1971) bioassays were based on the activity of cytokinins to promote cotyledon expansion. The cucumber cotyledons bioassay (Fletcher et al., 1982) depends on the formation of chlorophyll in 5-days old cotyledons, treated with KCl and various combinations of cytokinins. Chlorophyll content is measured after incubation in the dark followed by exposure to light.

The following assays are still used for testing of compounds with cytokinin activity. The cytokinin-dependent tobacco callus growth assay from *Nicotiana tabacum* L. cv. Wisconsin 38 is based on stimulation of cytokinin-dependent callus growth on medium with and without cytokinin. Biological activity is determined as the difference between fresh weight of treated and non-atreated callus. *tZ*-derived cytokinins showed the highest growth-stimulatory activity in this assay (Holub et al., 1998; Letham and Palni, 1983; Murashige and Skoog, 1962). The callus bioassay is specific, sensitive and quantitative, but it demands a long time for growing the callus (3-5 weeks; Mok and Mok, 1994). The senescence assay detects cytokinin-stimulated retention of chlorophyll in excised leaves of wheat. Winter wheat, *Triticum aestivum* cv. Hereward, is grown at standard conditions for one week and the basal end of the first leaf is placed into the cytokinin solution. After four days of incubation in the dark, chlorophyll from leaves is extracted and absorbance at 665 nm is measured (Holub et al., 1998).

The principle of the last assay is cytokinin-induced synthesis of betacyanin in Amaranthus cotyledons in the dark (Biddington and Thomas, 1973; Köhler et al., 1987). Two cotyledons and the hypocotyl from seedlings of *Amaranthus caudatus* var. *Atropurpurea* are incubated on medium with the test compounds. Extracted betacyanin is determined by comparing the absorbance at 537 nm and 620 nm (Holub et al., 1998). The formation of betacyanin is measured, but it responds to the presence of jasmonic acid (Mok and Mok, 1994).

To investigate the role of each cytokinin receptor, various cytokinin-regulated processes were studied in single and higher-order knock-out mutants of AHK2, AHK3 and/or CRE1/AHK4. Several assays were tested: callus and organ formation in hypocotyl explant, rosette diameter, shot development, time of flower induction, content of chlorophyll in leaves and ability to retard leaf senescence, seed size, germination time (dark-light conditions, red/far-red light), hypocotyl elongation assay (different dark-light regimes), root growth assay (measurement of root's length, counting of lateral roots and weighing the root system). In the root growth assay, seedlings are grown on vertical plates, the length of primary roots is determined after 7, 10 or 14 DAG (days after germination), number of lateral roots is counted at 14 DAG and root weight is established at 21 DAG (Riefler et al., 2006).

All bioassays have their intrinsic shortcomings, often other groups of hormones influence the same biological proceses (Mok and Mok, 1994) and many of the responses are caused by multiple cytokinin receptors in an additive manner. Therefore, it is relevant to study regulatory molecular mechanismus in greater details (Riefler et al., 2006). Previously described assays and all physiological bioassays may not distinguish between individual receptors because these assays integrate the responses of several putative cytokinin signalling pathways (Spíchal et al., 2004).

## Molecular-based Cytokinin Biotests

One of the *Arabidopsis* reporter gene assays is based on the ability of cytokinin to induce gene expression. Cytokinins increase the expression of type-A ARRs (D'Agostino et al., 2000). The induction of the type-A *ARR5* gene by exogenous cytokinin is characteristic for cytokinin primary-response genes. The induction is rapid and specific for cytokinins: elevation of the steady-state level of transcript occurs within 10 minutes and it is insensitive to the inhibitor of protein synthesis cycloheximide (Brandstatter and Kieber, 1998; D'Agostino et al., 2000). The *ARR5::GUS* assay depends on the activation of the promoter of this primary response gene by cytokinin (D'Agostino et al., 2000). The bioassay utilise the GUS (β-glucoronidase) reporter gene, which is localised downstream of the ARR5 promoter. This assay is rapid, sensitive, dose-dependent and highly specific for adenine and phenylurea-type cytokinins. However, the reporter gene assays are not sufficient to interpret early signaling events except in the case of combination with kinetic studies or studies on mRNA accumulation (Romanov et al., 2002).

In an alternative assay, a two-component output sensor (pTCS) was used to generate a universal cytokinin reporter system in *Arabidopsis*. The reporter system harbours conserved concatemeric 5´-(A/G)GAT(T/C)-3´ type-B ARR-binding motifs (Hosoda et al., 2002; Imamura et al., 2003; Sakai et al., 2000). The *pTCS::LUC* synthetic reporter system was designed for visualization of cytokinin output in an *in vivo Arabidopsis* mesophyll protoplast assay and it showed activation only with cytokinins. This synthetic reporter system can be used to report even low levels of phosphorelay output triggered by any of the three endogenous cytokinin receptors and relayed to any tested response regulator. Subsequently, a GFP reporter, controlled by the *pTCS* promoter, was generated to determine its expression patern *in planta*. It overcame the limitations as it is an immediate-early cytokinin target gene (Müller and Sheen, 2008), due to the promoter motif being enriched in the cis-regulatory region (Rashotte et al., 2003), which suggest its *in vivo* relevance. The *pTCS::GFP* system facilitated the discovery of novel cytokinin functions (roles of phosphorelay signalling *in vivo* during early embryogenesis, signalling components involved in embryonic phosphorelay activity – *ARR7::GFP*, *ARR15:GFP*) by reflecting the signal output pattern in transgenic *pTCS::GFP* plants (Müller and Sheen, 2008). In some cases, *pTCS*-induced expression showed some limitations - weak expression in certain developmental contexts (embryo sac, in the shoot, in the vasculature) and GFP expression showed progressive reduction with increasing generations (Zürcher et al., 2013), probably due to silencing effects triggered by the monotony of the repetitive sequence in *pTCS* (Chan et al., 2005). New version of *pTCS::GFP* was published (Zürcher et al., 2013). The *pTCS new* (*TCSn*)*::GFP* construct showed higher sensitivity to cytokinin and components of Multi-step phosphorelay

system in transient transfection assays, a brighter GFP signal and stable GFP expression during propagation (Zürcher et al., 2013). Multimerization of a consensus sequence fragment (four binding sites recognised by type-B ARRs, separated by arbitrarily selected flanking nucleotides) resulted in 12 sites, each slightly different from the other. They were combined in random order to produce a synthetic sequence fragment. This fragment was repeated twice to harbour 24 binding sites as a final design. Variations inside the *TCSn* sequence reflect the range of potential diversity among sites and avoid sequence monotony that could trigger the silencing of GFP in transgenic plants (Zürcher et al., 2013).

Recently the assay helped to unravel the importance of cytokinin localisation and transport for cytokinin signaling. When PUP14 was transiently transfected and localised to the plasma membrane, *TCSn::LUC* activity was reduced reflecting reduced cytokinin signalling. These data provide evidence that PUP14 plays a role in the apoplast-cytosol transport of cytokinins (Zürcher et al., 2016).

To investigate the role of each receptor and their combinations in *Arabidopsis*, loss-of-function mutants were prepared by T-DNA insertion. Riefler with colleagues (2006) created loss-of-function single, double and triple mutants of cytokinin receptors in *Arabidopsis*. They used several assays: shoot induction assay, where excised hypocotyls are grown on media with hormones and formation of new organs is scored; seed germination assay, where germination rate of seeds is counted at various time points under different wavelengths of light sources; hypocotyl elongation assay with growing seedlings on vertical plates under various light conditions; photomorphogenesis for monitoring the effect of different concentrations of BAP on plant phenotype; chlorophyll retention assay where detached seventh leaves from plants are treated with different concentrations of BAP for 10 days in the dark and chlorophyll content is measured; root growth assay where the lengths of primary roots are measured after 14 days after germination; and also they determined seed size and rosette diameter. In summary, the results showed some contribution of all three receptors to processes including cytokinin metabolism, fertility, seed size, timing of germination, plastochrone, influence of far-red sensitivity and dark on seed germination. The AHK3 receptor was shown to make a major contribution to chlorophyll retention and cytokinin-induced photomorphogenesis. CRE1/AHK4 plays a primary role in primary root elongation, root response to exogenous cytokinin and in shoot regeneration *in vitro*. Leaf cell formation and root branching are controlled mainly by the AHK2 and AHK3 receptors (Riefler et al., 2006). To test the repressive effect of cytokinin on root growth, *Arabidopsis* WT seedlings or combinations of double mutants are grown on media, containing the tested compounds, including a positive control (BAP) and negative control (DMSO). Root growth is scored 6 days after germination and

root branching test is evaluated 11 days after germination to see the response to exogenously applied compounds (Spíchal et al., 2009).

## A. Experimental Systems to Study Receptor Activation, Affinity and Ligand Specificity

The first system used to demonstrate that *Arabidopsis* His kinase is capable of functioning as a cytokinin-responsive sensor, was based on the introduction of the receptor into living cells of fission yeast, *Schizosaccharomyces pombe*. The yeast uses a multistep His→Asp phosphorelay system, consisting of three His-kinases (Phk1/2/3), HPt factor (SpyI) and response regulator (Mcs4) to regulate progression of the cell from G2 to mitosis (G2/M). Mutant cells ΔPhk1/2/3 exhibit a changed phenotype – ovoid shape and shorter cell morphology in comparison with WT *S. pombe*. After introducing the AHK4 gene, the phenotype defect of the ΔPhk1/2/3 cells was abolished, when cells were grown on medium with *tZ* (Suzuki et al., 2001). In the transformed organism, CRE1/AHK4 protein was also detected on the membrane by anti-AHK4 antiserum and by *in vitro* binding assay based on competition of non-radioactive competitor with [$^3$H]iP. In order to determine the dissociation constant, competition of [$^3$H]iP with 200-fold excess of non-labelled cytokinin was used. CRE1/AHK4 and AHK3 proteins were able to propagate the external cytokinin signal in a β-galactosidase assay in comparison with CKI1 protein. In the case of the AHK3 protein, the response was relatively low compared to CRE1/AHK4. Beside that, CKI1 showed constitutive activity in an *E. coli* assay and it did not bind [$^3$H]iP (Yamada et al., 2001).

Another system for detecting receptor function was *E. coli* transformed with the receptor gene. In this prokaryotic eubacterium, many processes are controlled by His→Asp phosphorelay signaling pathways (Suzuki et al., 2001). The Rcs system represents a major control element for remodeling of the enterobacterial surface in response to host infection and other changing cellular environments. It consist of two approximately 900 amino acids-long membrane-bound sensor kinases RcsC and RcsD (formerly YojN), the cytoplasmic transcriptional regulator RcsB and co-activator RcsA (Fig. 5). Signal perception initiates the autophosphorylation of the RcsC histidine kinase domain, subsequently transferring the phosphoryl group to the conserved residue in the C-terminally located RcsC phosphoreceiver domain. A phosphate residue is shuttled between RcsC and the receiver domain of the response regulator (RcsB) through RcsD (Rogov et al., 2006). A heterodimer formed by one copy of RcsA and RcsB binds at corresponding regions, containing the RcsAB box, to activate gene expression (Wehland and Bernhard, 2000). The Rcs-phosphorelay system in *E. coli* is the most similar pathway to the cytokinin signal transduction system in *A. thaliana* (Suzuki et al., 2001). The RcsC

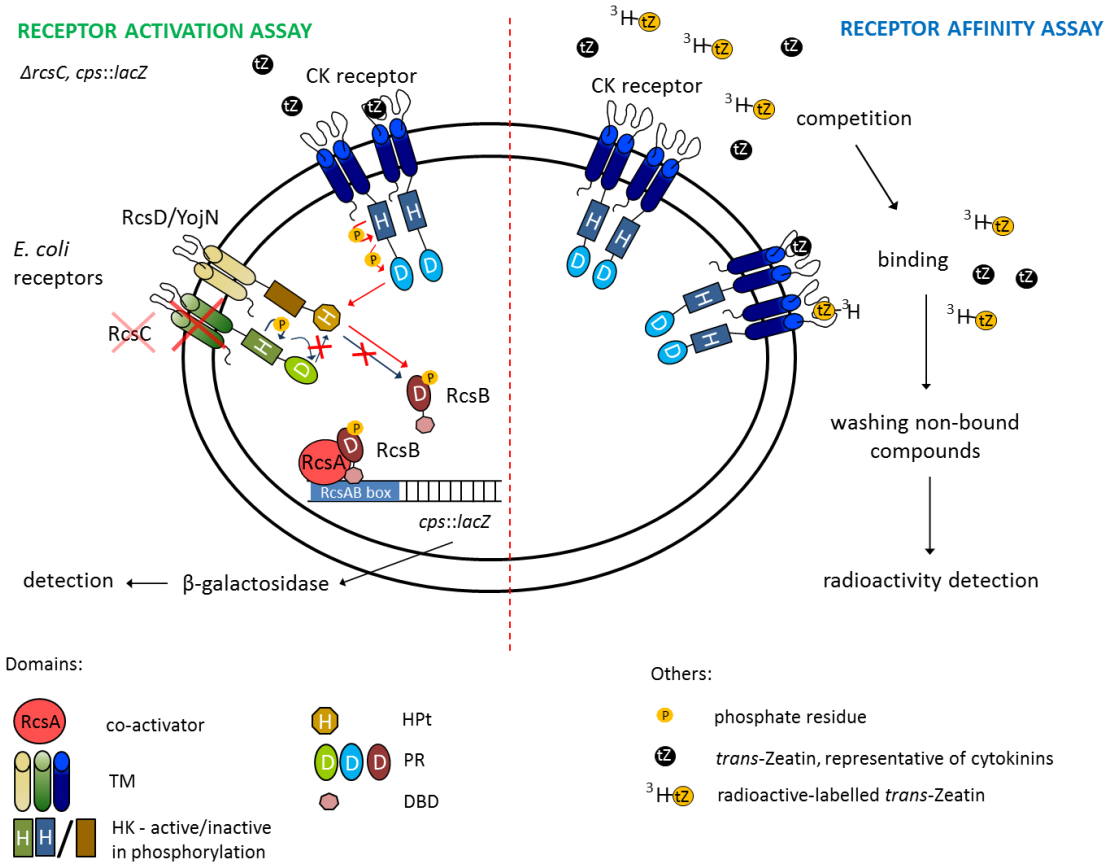



Figure 5: Depiction of two fundamental methods to determinate activity and affinity of cytokinin receptors in *E. coli* cells. Protein domains and other components, connected with both assays, are marked below the scheme (modified from Rogov et al., 2006; Spíchal, 2011; Takeda et al., 2001; Wehland and Bernhard, 2000).

hybrid sensor is structurally similar to AHK4, RcsD acts as an AHP factor and RcsB protein operates as a response regulator. The screening system was prepared by cloning *CRE1/AHK4* into the Δ*rcsC* and *cps::lacZ* genetic background, for blue/white test (Fig. 5). The effective cytokinin concentration was already 0.1 μM, which is comparable to the normal effective concentration in plants (Suzuki et al., 2001). Yamada et al. (2001) tested the AHK4 receptor as a cytokinin-responsive sensor in the *E. coli* mutant strain in a spot assay on agar plates and measured β-galactosidase activity upon treatment with three cytokinin representatives - *tZ*, iP, TDZ.

In the study of affinity of cytokinin receptors, Romanov et al. (2005) used a live-cell binding assay with the same transformed *E. coli* as were prepared by Suzuki´s research group (2001, Fig. 5). The affinity constant ($K_d$) was specified as the ligand concentration that displaced 50% of the bound [$^3$H]*tZ*. M9 medium with optimal pH 7 was used for binding experiments with CRE1/AHK4, measured in intact of *E. coli* cells. *tZ* had the highest affinity with an apparent affinity constant of approxiately 2.5 nM, DHZ and *cZ* displayed decreasing affinities and zeatin O-glucoside was not able to displace [$^3$H]*tZ*

(Romanov et al., 2005). For comparison, purified microsomes from *CRE1/AHK4*-expressing bacterial clone, were also used in a competition assay (Romanov et al., 2005). A $K_d$ of approximately 6 nM was obtained for *tZ*, and the order of binding affinities of the tested compounds was the same as for the intact-cell binding assay. The binding assay on microsomes is based on membrane proteins which are dialysed against buffer containing [$^3$H]*tZ* in the presence or absence of increasing concentrations of the studied compounds (Romanov et al., 2005).

In the following year, Romanov with colleagues (2006) published further results with cytokinin receptors from *Arabidopsis*. Binding of the hormone to the receptor complex was dependent on pH (maximum specific binding of *tZ* at pH 8.5-9), monovalent salt concentration (affinity for AHK4 was decreased by $Na^+$ and $K^+$, while the affinity of AHK3 was rather enhanced) and temperature (rapid and stable total binding of *tZ* at 0°C). The results showed difference between AHK3 and AHK4 in preference for *tZ*- and iP-type cytokinins - $K_d$ 1-2 nM for tZ and 150 nM for iP for AHK3 and $K_d$ 2-4 nM for *tZ* and 17 nM for iP for AHK4. The AHK3 receptor had a higher affinity for DHZ than did AHK4. On the another hand, the AHK4 receptor showed tight binding of iPR (130 nM) in comparison with AHK3 (2300 nM). Phenylurea derivatives, ribosides and *cZ* have higher affinities for AHK3 (Romanov et al., 2006).

The CHASE domains of AHK2 and AHK4 with 2 surrounding transmembrane domains were expressed in the same *E. coli* transgenic system (Stolz et al., 2011). Affinity constants $K_d$ were determined for a spectrum of ligands. Both receptors were the most sensitive to *tZ* (AHK2 0.4 ± 0.7 nM; AHK4 9.1 ± 1.5 nM) and iP (AHK2 1.43 ± 0.02 nM; AHK4 2 nM). Lower affinities were measured for their ribosides and the affinity constants were two orders of magnitude lower for *cZ* and DHZ in comparison with iP for both tested receptors (Stolz et al., 2011).

Plant assays with microsomes isolated from tobacco (*Nicotiana benthamiana*) leaves transiently expressing cytokinin receptor genes were preformed by Lomin et al. (2015). Receptor expression was controlled by means of a fluorescence signal. The dissociation constants for cytokinin receptors AHK2, AHK3 and ZmHK1 were determined using this system. The values determined for AHK2 were 1.93±0.26 nM for *tZ*, iP 1.42±0.52 nM and BAP 26.9±9.9 nM. In the case of AHK3 the measured dissociation constant for *tZ* was 4.26±1.69 nM, iP 42.0±13.5 nM and BAP 359±10 nM. The most effective ligands for AHK2 and AHK3 were iP and tZ, respectively. ZmHK1 had the highest affinity for iP (0.61±0.27 nM) and BAP (1.22±0.56 nM). Previous results showed that cytokinin ribosides are able to activate cytokinin receptors (Kuderová et al., 2015; Romanov et al., 2005, 2006; Spíchal et al., 2004; Stolz et al., 2011; Yamada et al., 2001; Yonekura-Sakakibara et al., 2004). However, recent research indicates that cytokinin receptors do not bind ribosides with high affinity but only free cytokinin bases. Authors have

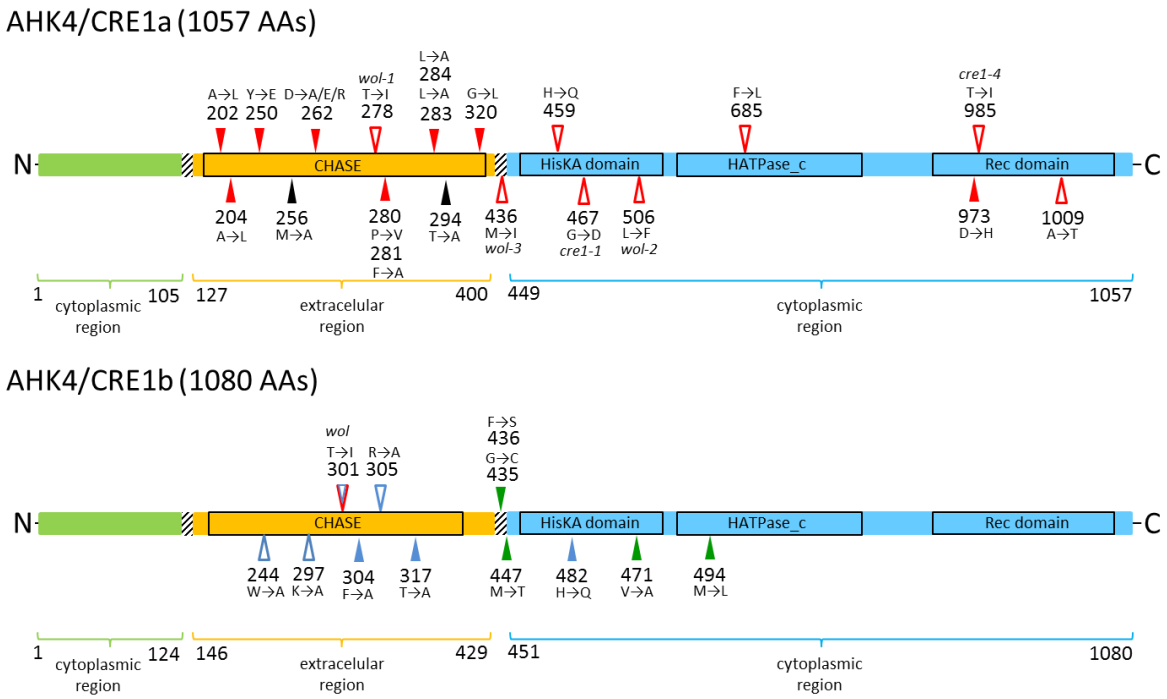
suggested that quick conversion of ribosides into their corresponding bases in *E. coli* cells is responsible for the observed activity of ribosides (Lomin et al., 2015). In accordance with crystallographic structure, ribosylated *tZ* does not fit into the binding cavity because of the riboside moiety in the N9-position of the adenine ring (Hothorn et al., 2011).

## B. Point Mutations in the Cytokinin Receptor Sequences

Several point mutations have been introduced into the sequence of the AHK4 cytokinin receptor and tested for their influence on receptor functions. Moreover, Inoue with colleagues (2001) defined two splice variants for the CRE1/AHK4 receptor – CRE1a and CRE1b. The predicted CRE1a protein has 1057 amino acids while CRE1b, contains 1080 amino acids. The extra 23 amino acids are located at the N-terminus. Numbering of the mutations is used throughout this work as it was described in the original reports.

In many cases, point mutations caused modification of activity or binding capacity of the CRE1/AHK4 cytokinin receptor (Fig. 6). Loss of activity occurred in the CRE1a variant with mutation inside the CHASE domain (A202L, A204L, Y250E, D262A/E/R, P280V, F281A, L283A, L284A, G320L; Hothorn et al., 2011) or in the Rec domain (D973H; Mähönen et al., 2006b). Loss of binding capacity was observed upon mutagenesis of the CHASE domain (F304A, T317A) and the HisKA domain (H482Q) in the CRE1b splicing variant (Heyl et al., 2007). In contrast, a constitutively activated receptor was created by single mutations inside the second transmembrane domain (F436S, G435C, M447T), in the HisKA domain (V471A) or the HATPase_c domain (M494L) in the CRE1b version (Miwa et al., 2007). Additionally, other mutations decreased or had no effect on activity or binding capacity of CRE1a (de León et al., 2004; Franco-Zorrilla et al., 2002; Hothorn et al., 2011; Kuroha et al., 2006; Mähönen et al., 2006b) or CRE1b (Heyl et al., 2007) Franco-Zorrilla et al., 2004.

A mutant of CRE1b/AHK4 named *wooden leg* (*wol*) contains a single point mutation T301I located in the extracelular binding domain (Fig. 6; Yamada et al., 2001). Its name was derived from its indeterminate root growth. While this protein directly associates with cytokinins, it is inactive in the *S. pombe* membrane binding assay and unable to propagate signal in *E. coli* assay. It was labeled as a "loss-of-function" mutation, with retarded growth of primary root, reduced cell number and aborted root vasculature system (Yamada et al., 2001). The *wol-1* (CRE1a/AHK4-T278I) mutation was later shown to be recessive (Mähönen et al., 2006b). It brings a dose-dependent negative activity on vascular cell proliferation during root development, but this negative activity can be eliminated by various nonsense supressor mutations. Normal procambial-cell proliferation and differentiation needs mutually necessary and sufficient function of *CRE1/AHK4*, *AHK2* and *AHK3*. CRE1a/AHK4-T278I negatively impacts cytokinin signalling initiated by the cytokinin receptors AHK2 and AHK3. This results in the negative activity on the *wol-1*




Loss of activity
Decrease of activity
Constitutively activated independently of cytokinin
Loss of binding capacity
Decrease of binding capacity
No effect on activity
Transmembrane domain AHK4/CRE1a – 106-126, 401-448
AHK4/CRE1b – 125-145, 430-450

CHASE domain (AHK4/CRE1a – 177-359; AHK4/CRE1b – 200-382)
HisKA domain (AHK4/CRE1a – 449-514; AHK4/CRE1b – 472-537)
HATPase_c domain (AHK4/CRE1a – 561-732; AHK4/CRE1b – 584-755)
Receiver domains (AHK4/CRE1a – 925-1046; AHK4/CRE1b – 948-1069)

Figure 6: Overview of point mutations that have been tested so far and their influence on physiological effects. Two variants of CRE1/AHK4 (https://www.ncbi.nlm.nih.gov) were tested: shorter protein version (CRE1a/AHK4 - 1057 amino acids; de León et al., 2004; Franco-Zorrilla et al., 2002; Hothorn et al., 2011; Inoue et al., 2001; Kuroha et al., 2006; Mähönen et al., 2000, 2006b) and the longer version (CRE1b/AHK4 - 1080 amino acids; Heyl et al., 2007; Miwa et al., 2007; Suzuki et al., 2001; Yamada et al., 2001).

phenotype caused by inhibition of vascular-cell proliferation and promotion of ectopic protoxylem differentiation (Mähönen et al., 2006b). Another mutation (CRE1a/AHK4-M436I) phenocopied the *wooden leg* mutant and thus it was labeled *wol-3* (Kuroha et al., 2006). This single nucleotide substitution is situated in the region between the transmembrane and histidine kinase domains, the affected residue being conserved

among all cytokinin receptors (Kuroha and Satoh, 2007). Other mutants with retarded growth of the primary root and aborted vascular system were mapped to the histidine kinase domain in *wol-2* (CRE1a/AHK4-L506F; de León et al., 2004) and in *cre1-1* (CRE1a/AHK4-G467D; Inoue et al., 2001). F304A and T317A result in complete abolition of ligand binding. Mutations at positions W244A and R305A strongly reduced binding capacity (Heyl et al., 2007). However, a single amino acid substitution of a histidine in the cytoplasmic histidine kinase domain, H482Q, does not modify cytokinin binding capacity in AHK4, but it abolishes capacity of the receptor to transduce the signal (Heyl et al., 2007). Mutations M279A and T317A have no effect on activity (Hothorn et al., 2011), but M279A showed only little decrease of binding capacity in comparison with WT CRE1b/AHK4 (Heyl et al., 2007). Five single amino acid substitutions were made in a narrow region extending from TM2 downstream of the phosphorylated His site of CRE1b/AHK4. G435C, F436S, M447T, V471A and M494L exhibited high levels of β-galactosidase activity independently of the presence of the cytokinin in β-galactosidase assay. This set of *AHK4* mutants showed constitutive His-kinase activity in the *E. coli* activation assay (Miwa et al., 2007).

Analogous substitutions to the *wol* mutation were created in homologous receptors AHK2-T418I and AHK3-T281I (Miwa et al., 2007), resulting in no activation even upon application of cytokinin. AHK2-I586A, AHK3-V449A and CRE1b/AHK4-V471A were constitutively active in the *E. coli* assay (Miwa et al., 2007). In *S. pombe* (Δ*phk1/2/3*) cells, lacking all three His-kinases, WT *AHK4 wol* mutation was able to complement the mutational lesion. Similiar results were obtained for G435C, W471A and F436S (Miwa et al., 2007), whereas, as expected, CRE1b/AHK4-T301I did not complement the deficiency in a cytokinin-dependent manner. In comparison, in a double mutant, the CRE1b/AHK4-F436S-T301I lesion was fully masked by a gain-of-function F436S mutation. Certain amino acid alterations in the second membrane-spanning segment and/or the flanking region for all three receptors cause a conformational change, which propagate cytokinin signal without external stimulus (Miwa et al., 2007).

A membrane fraction from an insect cell line, expressing *CRE1a/AHK4*, was also used to study the bidirectionality of the phosphorelay network. CRE1a/AHK4 phosphatase activity results in dephosphorylation of HPts, in the absence of cytokinin, while the kinase activity occurs when cytokinin is present (Mähönen et al., 2006b). When CRE1a/AHK4 was incubated in the presence of radioactive-labelled ATP and particular HPt protein, the phosphoryl goup was transported to the HPt protein only in the presence of cytokinin. Mutation of Asp973, which accepts the phosphate residue, abolished phosphorylation of the HPt protein. Phosphotransfer from ATP to His459 of CRE1a/AHK4 depends on cytokinins and the phosphate residue requires Asp973 in order to move from CRE1a/AHK4 to HPt. The phosphatase activity was slightly decreased by a H459Q

mutation but a D973N mutation completely abolished the phosphatase activity. The T278I mutant of CRE1a/AHK4 showed similiar phosphatase activity as WT but, double mutants T278I-H459Q decreased and T278I-D973N abolished this activity (Mähönen et al., 2006b).

## C. Chemical-biology Approaches to Study Cytokinin Response

To gain a better understanding of the principles of receptor activation, the structure-activity relationships of cytokinins were studied (Mok and Mok, 1994; Skoog and Armstrong, 1970). Cytokinin derivatives are tested in various assays as described above – monitoring their effect on the protein and gene level and their influence on cytokinin-dependent processes in the plant (Doležal et al., 2006; Spíchal et al., 2009).

Antagonist substances, which act as inhibitors of cytokinin action – are called anticytokinins (Nisler et al., 2010). They inhibit the activity of the cytokinin receptor (Arata et al., 2010). The basic criteria for a compound to be categorized as a cytokinin antagonist are: competition with the natural ligands for the receptor, blocking of cytokinin action *in vivo*, no intrinsic cytokinin activity in cytokinin bioassays, no activation of the cytokinin signaling pathway and also no toxicity or inhibition of cyclin dependent kinases. Anticytokinins have strong inhibitory effects on cytokinin-induced responses in different bioassays (Spíchal et al., 2009). In 1971, Hecht with colleagues searched for cytokinin antimetabolites derived from purine or pyrazolopyrimidine. They showed that modification of the heterocyclic adenine moiety of cytokinins strongly decreased their activity and proposed competition between antagonist and cytokinin for the binding sites in receptors. Alkylated heterocycles might be also useful as inhibitors of cytokinin activity (Hecht et al., 1971). Later it was shown that a 4-phenylquinazoline compound showed antagonistic effects on various cytokinin-regulated physiological responses in *Arabidopsis thaliana*. It binds to CRE1/AHK4 non-competitively in *in vitro* binding assay. It acts as a non-competitive inhibitor of CRE1 and it inhibits induction of the cytokinin primary response genes (Arata et al., 2010). Another anticytokinin, $N^6$-(benzyloxymethyl)adenosine, showed high specificity to cytokinin receptors of *Arabidopsis*. It inhibits the activation of CRE1/AHK4, but not of the AHK3 receptor (Krivosheev et al., 2012). Anticytokinins derived from pyrrolo[2,3-*d*]pyrimidine and pyrazolo[4,3-*d*]pyrimidine do not operate as competitors of cytokinins at the receptor level. They inhibit the cell cycle, cause disorganization of the microtubular cytoskeleton and apoptosis. The potent anticytokinin 3-methyl-7-pentylamino-pyrazolo[4,3-*d*]pyrimidine (ANCYT1) occupies the ATP-binding pocket of human cyclin-dependent kinase, specifically CDK2. These anticytokinins demonstrated their capability to kill human cancer cells consistent with responses of known CDK inhibitors (Spíchal et al., 2007).

Small structural variations, such as the presence and position of a hydroxyl group, also have influence on the activities in bioassays of compounds derived from isoprenoid cytokinins (Mok and Mok, 2001). PI-55, a derivative of BAP with substituents at the *o*- and *m*- positions of the aromatic side chain, caused physiological changes as in the case of receptor loss-of-function mutations in *Arabidopsis*. In addition PI-55 was able to compete with naturally occuring cytokinins for binding space in AHK3 and AHK4 receptors. PI-55 showed an antagonistic effect on root branching. It completely reversed suppression of lateral root formation by BAP, but it exerted a positive effect on root branching when applied without any cytokinin (Spíchal et al., 2009).

Another reported cytokinin antagonist, derived from PI-55 with substitution at the C2-, N7- and N9-position of the adenine ring, were tested in classical cytokinin biotests (Nisler et al., 2010). From 11 tested compounds, 6-(2,5-dihydroxybenzylamino)purine (LGR-991) was found to antagonize cytokinin activity most effectively in transgenic *ARR5::GUS Arabidopsis* plants. It also inhibited CRE1/AHK4 and AHK3 receptors, accelerated *Arabidopsis* seed germination, blocked inhibition of hypocotyl growth in the dark and did not show cytokinin activity in classical cytokinin bioassays. In addition, a methyl group at the position N9 of the adenine ring in derivtive of PI-55 prevented binding or activation of the receptors (Nisler et al., 2010), causing loss of activity in all three classical cytokinin bioassays (Nisler et al., 2010). A set of iP derivatives alkylated at the N9-position were also prepared and tested (Mik et al., 2011b). The results showed that the activity of the tested compounds depends on the type of substituent at the N9-position and the assay. Ethoxyethyl-, acetoxy-, azido-, 4-chlorobutyl- and 3-cyanopropyl-groups negatively influenced recognition by CRE1/AHK4, ZmHK1 and ZmHK3a, but they improved proliferation in the tobacco callus assay (Mik et al., 2011b). The activity was probably triggered by steric effects and the polarity of particular substituents (Corse et al., 1989). N9-derivatives were not recognised by CRE1/AHK4 and in the case of ZmHK3 the effect was observed only at high concentrations.

However, substitutions at the end of the alkyl group appear to be critically determinant during activation of the receptor, probably related to the dimensions of the terminal group. The polarity and length of the spacer in the N9-position of the iP derivatives had significant impact on the activity of compounds in the tobacco assay. Significant cytokinin activity of the N9-derivatives can by caused by the existence of other ways for transmitting the signal into the cell in tobacco callus and *Amaranthus* assays. N9-modified iP derivatives did not show antisenescence activity. 2-chloroethyl and 2-bromoethyl groups at the N9-position can prevent negative effects on cell proliferation (Mik et al., 2011b).

N9-substituted kinetin (Kin) derivatives with halogenoalkyl, aliphatic/cyclic ether and/or carboxylic chains enhanced the cytokinin activity of the default compound in the

bioassays (Mik et al., 2011a). The polarity and length of the N9-chain have significant impact on activity of the particular compound in the tobacco callus assay. The effects of the halogenoalkyl derivatives were affected by the specific halogen atom – chlorine showed more effectivity than bromine, probably because of its smaller size. High affinity was measured with Kin derivatives containing aliphatic and/or cyclic ether substituents. These derivatives displayed a negative effect on perception by AHK3 and CRE1/AHK4 receptors but they improved anti-senescence properties and prevented the negative effect on proliferation of cells due to high concentrations of exogenously applied cytokinins. The length of the alkyl chain and the type of halogen atom had influence on delayed degradation of chlorophyll in senescence bioassays. Kinetins with halogenoalkyl substitution at N9 were most effective in the senscence assay (Mik et al., 2011a).

There have been relatively few studies with 9-deaza derivatives. Pyrazolo[4,3-*d*]pyrimidine derivates with disubstitution at positions 3 and 7 were effective at inhibiting CDK1 (Moravcová et al., 2003). 3,5,7-trisubstituted pyrazolo[4,3-*d*]pyrimidines published by Řezníčková et al. (2015) showed possible therapeutic potential due to CDK inhibiton and antiproliferative activities. It showed dual mode of action – apoptosis induction in cancer cells and blocking of angiogenesis-like activity in endothelial cells. These compounds have not yet been tested in classical cytokinin assays or for their effect on the cytokinin receptors. Vymětalová et al. (2016) prepared a series of pyrazolo[4,3-*d*]pyrimidine derivates, which were tested as potential inhibitors of CDK2 and CDK5 and antiproliferative compounds. As in the case of the Moravcová et al. (2003) and Řezníčková et al. (2015) studies, compounds were tested only for medical purposes.

The methods listed below represent author´s contribution to performed experiments.

## Sequence Alignment

Sequences containing the CHASE domain were obtained from the Pfam database (PF03924, http://pfam.xfam.org). Alignment of 1721 domains was generated with ClustalOmega (version: 1.2.4; guidetreeout: false; dismatout: false; dealign: false; mbed: true; mbediteration: true; iterations: 0; gtiterations: -1, hmmiterations: -1; outfmt: fa; order: aligned) by EMBL-EBI (http://www.ebi.ac.uk;). The alignment was manually modified to 277 CHASE domains originating from 52 different organisms.

## Site-directed Mutagenesis

Site-directed mutagenesis was performed using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, USA). Bacterial strain *E. coli* TOP10 (Thermo Fisher Scientific, USA), containing plasmid pINIIIΔEH:AHK4 (Suzuki et al., 2001; Yamada et al., 2001) were grown overnight at 37°C with vigorous shaking. Plasmid DNA was isolated by QIAprep Spin Miniprep Kit (Qiagen, The Netherlands). The PCR reaction contained 25 ng plasmid DNA and 0,2 μM each primer concentration (Table 1). The initial denaturation temperature was 95°C for 1 minute, followed by 16 cycles of 95°C for 30 second, 65°C for 1 minute and 68°C for 10 minutes, with a final extension at 68°C for 10 minutes. Digestion was performed at 37°C for 2 hours with 4 units of DpnI (New England Biolabs, USA). Plasmid DNA was electroporated into competent *E. coli* TOP10 cells, which were grown on Lysogeny Broth plates (10 g tryptone, 5 g yeast extract, 10 g

Table 1: Primer sequences

| Changed amino acid position | | Sequence 5´- 3´ |
|---|---|---|
| Y250H | F: | GTT ATA TTC TCT CAA GAT AGT GTC TCT CAC CTT GAG TCA CTC |
| | R: | GAG TGA CTC AAG GTG AGA GAC ACT ATC TTG AGA GAA TAT AAC |
| L251V | F: | CTC AAG ATA GTG TCT CTT ACG TTG AGT CAC TCG ATA TGA TG |
| | R: | CAT CAT ATC GAG TGA CTC AAC GTA AGA GAC ACT ATC TTG AG |
| I226V | F: | GAG GAG GAT CGT GAG AAT GTT TTG CGA GCT AGA GAA A |
| | R: | TTT CTC TAG CTC GCA AAA CAT TCT CAC GAT CCT CCT C |
| A322V | F: | GGG TAC CTT GGT GGT GTG TTT GAT GTG GAG TCT |
| | R: | AGA CTC CAC ATC AAA CAC ACC ACC AAG GTA CCC |

NaCl) with Amp (100 μg/ml) at 37°C overnight. Isolated plasmid DNA was sequenced and transformed to KMI001 electrocompetent cells.

## Cytokinin Bioassays

The amaranthus assay, tobacco callus growth bioassay and wheat senescence assay were performed according to Holub et al. (1998) with slight modifications. In the tobacco callus growth bioassay, six-well plates were used for growing of calli. Each well contained 3 ml of Murashige-Skoog medium (MS; Murashige and Skoog, 1962), into which 0.1 g of callus was placed. The tested cytokinins and their 9-deaza derivatives were dissolved in dimethylsulfoxide (DMSO) and diluted with distilled water to $5 \times 10^{-2}$ M solutions. This stock solution was further diluted in the media appropriately for each biotest to concentrations from $10^{-9}$ to $10^{-4}$ M. The final concentration of DMSO in the media did not exceed 0.2% and thus did not affect the biological activity of the substance tested in the assays. Five replicates were prepared for each compound concentration and the entire tests were repeated at least twice.

## β-galactosidase Assay

The assay was performed in accordance to Spíchal (2011) with slight modifications. *E. coli* bacterial precultures were grown overnight at 25°C with shaking to $OD_{600}$~1. The cultivation was carried out in M9 medium containing 0.1% (w/v) casamino acids and appropriate antibiotic at 100 μg/mL. The next day, the preculture was diluted to a ratio of 1:600 using fresh M9 medium with casamino acids and antibiotic, and aliquoted at 200 μL per well into a 96-well microtiter plate. Cytokinin stock solution or the tested compound was added to the 96-well microtiter plate filled with diluted bacterial culture to obtain a final concentration of 50 μM. Subsequently, compounds in the cell culture were diluted to 0.01 μM. The microtiter plate was incubated at 25°C for 17 h under 450 rpm shaking in a thermomixer. After measuring the optical density at 600 nm, 50 μL of each sample of the bacterial culture with compound was incubated with 2 μL 25 mM 4-methylumbelliferyl galactoside substrate at 37°C for 30 min. The reaction was stopped by adding glycine carbonate buffer at pH 10.7. Quantification was performed based on the fluorescence signal using an excitation peak of 365 nm and emission wavelength of 460 nm (Synergy Multi-Mode Microplate Reader).

## Cytokinin Binding Assay

The cytokinin binding assay was performed according to the method described by Romanov et al. (2005, 2006) with slight modifications. *E. coli* cultures (strain KMI001),

expressing cytokinin receptor, were grown at 25°C overnight in modified M9 liquid medium, supplemented with casamino acids [0.1% (w/v)] and ampicillin (100 μg/ml) (Spíchal, 2011), to reach $OD_{600}$ ~1–1,4.

For assays, each sample contained 1 mL of the cell suspension, 3 pmol of 2-[$^3$H]*tZ* and various concentrations of unlabelled *tZ* or other tested competitors. As a control, 0.1% (v/v) DMSO was added instead of the unlabelled compound. After 30 min incubation at 4°C, the sample was centrifuged (8,000 rpm, 4 min, 4°C). The supernatant was removed and the bacterial pellet was resuspended in 20 μL dH2O and then 1 mL of scintillation cocktail (Beckman, Ramsey, USA) was added. Radioactivity was measured by a Hidex 300 SL scintillation counter (Hidex, Finland). To discriminate between specific and nonspecific binding, a high excess of unlabelled tZ (at least 3000-fold) was used for competition, as described by Romanov et al. (2006). IC50 values were determined as average values from three independent measurements. GraphPad Prism 5.1 was used for calculation of IC50 and preparation of the graphs (http://www.graphpad.com/scientific-software/prism/).

## *ARR5::GUS* Cytokinin Induction Assay

*ARR5::GUS* cytokinin induction assay was done according to Romanov et al. (2002). Transgenic *Arabidopsis ARR5::GUS* seeds were sterilized, placed in the wells of 6-well plates containing 1/2 MS medium and cultivated in darkness (4 °C) for 4 days. Then seeds were transferred to an orbital shaker (16 h light/8 h dark) for 3 days in a growth chamber (22 °C). Transgenic *ARR5::GUS Arabidopsis* seedlings were treated with cytokinins (*tZ*, iP, BAP, 6-AP) and their 9-deaza analogues. DMSO was used as a control (final concentration 0,1%). Seedlings were cultivated for another 17 h. GUS activity measurements were performed in accordance with previously published procedure (Romanov et al., 2002). Fluorescence was measured using a Fluoroscan Ascent microplate reader (Labsystems, Finland) at excitation and emission wavelengths of 365/450 nm.

## Characterisation of Ligand Specifity of the *Brassica napus* Cytokinin Receptors

Cytokinin receptors were studied first in the model plant *Arabidopsis thaliana* and research on these receptors still continues. Here we have characterised the first cytokinin receptors in the economically important dicotyledonous plant - *Brassica napus* var. Tapidor. Results were summarized in the *Journal of Experimental Botany* (Kuderová et al., 2015). Homologs of AHK2 (BnCHK1 and BnCHK3) and AHK3 (BnCHK5) cytokinin receptors were identified on the basis of phylogenetic analysis (Fig. 7).

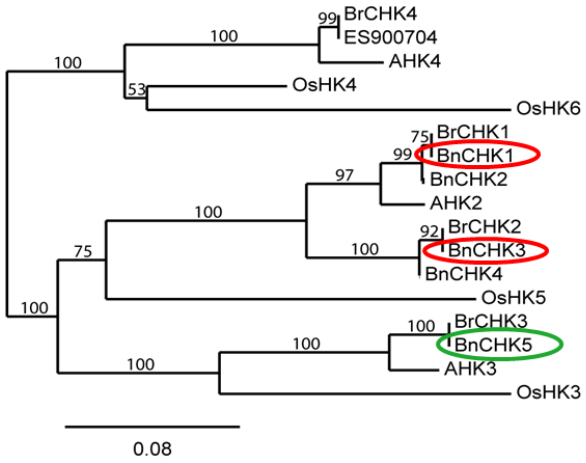



Figure 7. Phylogenetic relationship based on alignment of CHASE-containing His kinases from *Arabidopsis* (AHKs), *Brassica napus* (BnCHKs, ES900704), *Brassica rapa* (BrCHKs) and rice (OsHKs). Tested receptors are marked by elipses.

Cytokinin binding assay showed that they bind specifically cytokinins and it was confirmed that the three *Brassica napus* proteins, BnCHK1, BnCHK3 and BnCHK5, are functional cytokinin receptors. All three receptors showed the strongest recognition in the case of *tZ*, followed by phenylurea-derived cytokinin TDZ (Table 2). Differences in the ligand preference of BnCHK receptors were obtained for iP (Fig. 8) and *tZ*R (Table 2).

## Studies of Cytokinin Receptor Affinity and Activation Using a Chemical Biology Approach

The principle of cytokinin perception and activation of the receptor was recently described (Hlusková et al., submitted). Data were obtained based on the crystal structure of CRE1/AHK4 (3T4L; Hothorn et al., 2011) with combination of computer modelling and simulations. The cytokinin binding site, in the PAS domain, was defined as a beta-sheet "base" and flexible "lid" loop. Two entrances to the cavity were described – one is located between "hook" and "arm" with Asp262, the second entrance is "gated" by Tyr250 (Fig. 9A), which forms a hydrogen bond to water interacting

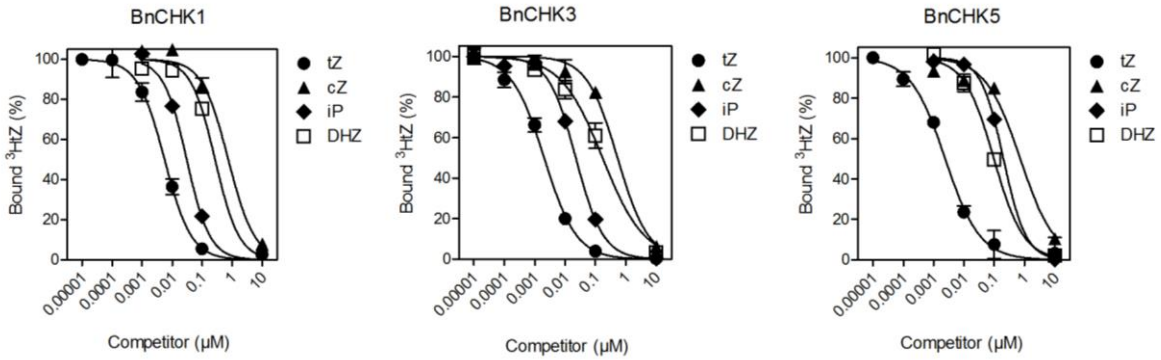



Figure 8: Functional analysis in *E. coli* reveals different cytokinin binding characteristics of BnCHK1, BnCHK3, and BnCHK5. Graphs display comparison of affinities of isoprenoids cytokinins among cytokinin receptors.
Although iP was strongly recognised by BnCHK1 and BnCHK3, *t*ZR showed very high affinity in the case of BnCHK1 and BnCHK5. *G*lucosides, metabolities of *t*Z were not bound effectively by all three *Brassica* receptors (Table 2). Among the aromatic cytokinins, BAP and *m*T were the most strongly recognized by BnCHK3, with *m*T having the highest affinity.

with N3 in the cytokinin. The cytokinin is held by Asp262 in the "arm" helix by hydrogen bonds to $N^6$ and N7 and by a hydrogen bond between backbone of Leu284 and N9. Stabilization of the ligand is primarily through the interaction between Asp262 and $N^6$ and N7 of the cytokinin, together with the cytokinin side chain (Hlusková et al., submitted). An additional hydrogen bond is formed between the tZ tail hydroxy group and Thr294 (Fig. 9A). This interaction is responsible for specific stereorecognition of

Table 2. Comparison of the cytokinin affinity to BnCHK1, BnCHK3, and BnCHK5. The apparent $K_d$ values were calculated as ligand concentrations that displaced 50% of the bound [$^3$H]tZ (results are presented as means and standard deviations obtained from two independent experiments, with three technical replicates per experiment).

| Cytokinin | Apparent Kd (nM) | | |
|---|---|---|---|
| | BnCHK1 | BnCHK3 | BnCHK5 |
| tZ | 4.6 ± 1.9 | 2.1 ± 0.5 | 2.4 ± 0.3 |
| tZR | 17.1 ± 1.6 | 27.1 ± 1.9 | 13.5 ± 1.0 |
| tZ7G | >10 000 | >10 000 | >10 000 |
| tZ9G | >10 000 | >10 000 | >10 000 |
| tZOG | >10 000 | >10 000 | >10 000 |
| cZ | 662 ± 85 | 440 ± 110 | 694 ± 28 |
| DHZ | 382 ± 70 | 174 ± 5 | 103 ± 5.1 |
| iP | 35.3 ± 4.6 | 22.8 ± 0.5 | 219 ± 18 |
| BAP | 855 ± 77 | 440 ± 70 | 4794 ± 382 |
| mT | 119 ± 1.5 | 44.7 ± 2.1 | 97.0 ± 13 |
| Kin | 895 ± 205 | 5456 ± 79 | 1695 ± 195 |
| TDZ | 11.1 ± 1.9 | 6.6 ± 0.1 | 13.9 ± 2.4 |
| Ade | >10 000 | >10 000 | >10 000 |

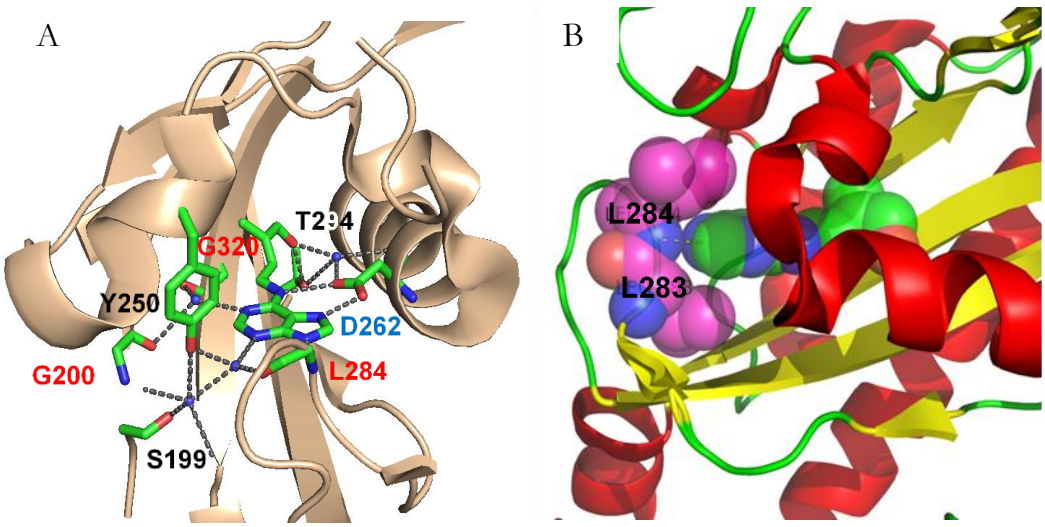



Figure 9. Structure of the AHK4 receptor. A) Interconnected system of hydrogen bonds stabilizing *tZ* inside the binding cavity of CRE1/AHK4 (3T4L). Blue balls – water; dashed lines – H-bonds; red residues - main chain H-bond forming; black residues - side chain H-bond forming; blue residues - both side chain and main chain H-bond forming. B) The structure of the "hook" loop. Leu284 and Leu283 (magenta spheres) form a symmetrical C-shaped docking platform stabilizing the ligand *tZ* (green spheres) in a position that enables hydrogen bond formation between *tZ* N9 and the Leu284 backbone amide (visualized by the yellow dashes).

zeatin in the *trans* form, the most preferred ligand for AHK4.

In CRE1/AHK4, a hydrogen bond donor is provided by the main chain amide of Leu284 and N9 serves as a hydrogen bond acceptor. Together with Leu283, these two amino acids form a symmetrical C-shaped docking platform, with the hydrogen bond in the centre and the plane of the cytokinin adenine ring perpendicular to it (Fig. 9B). This structure enables precise positioning of the interaction and fixes the "hook" loop to the rest of the PAS domain.

Further, comparison of 277 amino acid sequences from 52 organisms showed a conserved motif between 261 and 317 amino acids in the PAS domain. There is absolute conservation of key amino acidss – Asp262 and Leu283 and also high conservation of Leu284. This points to the importance of the C-shaped docking platform. The importance of hydrogen bond formation between N9 and Leu284 was confirmed by testing of 9-deaza cytokinin analogues. 9-deaza derivates of *tZ*, iP, BAP and 6-AP were tested in cytokinin assays.

Results from the *Amaranthus* assay and tobacco callus growth assay showed, that N-to-C substitution at position N9 of the adenine ring significantly decreases cytokinin activity. In addition, 9-deaza-cytokinins exibited no activity in the senescence assay.

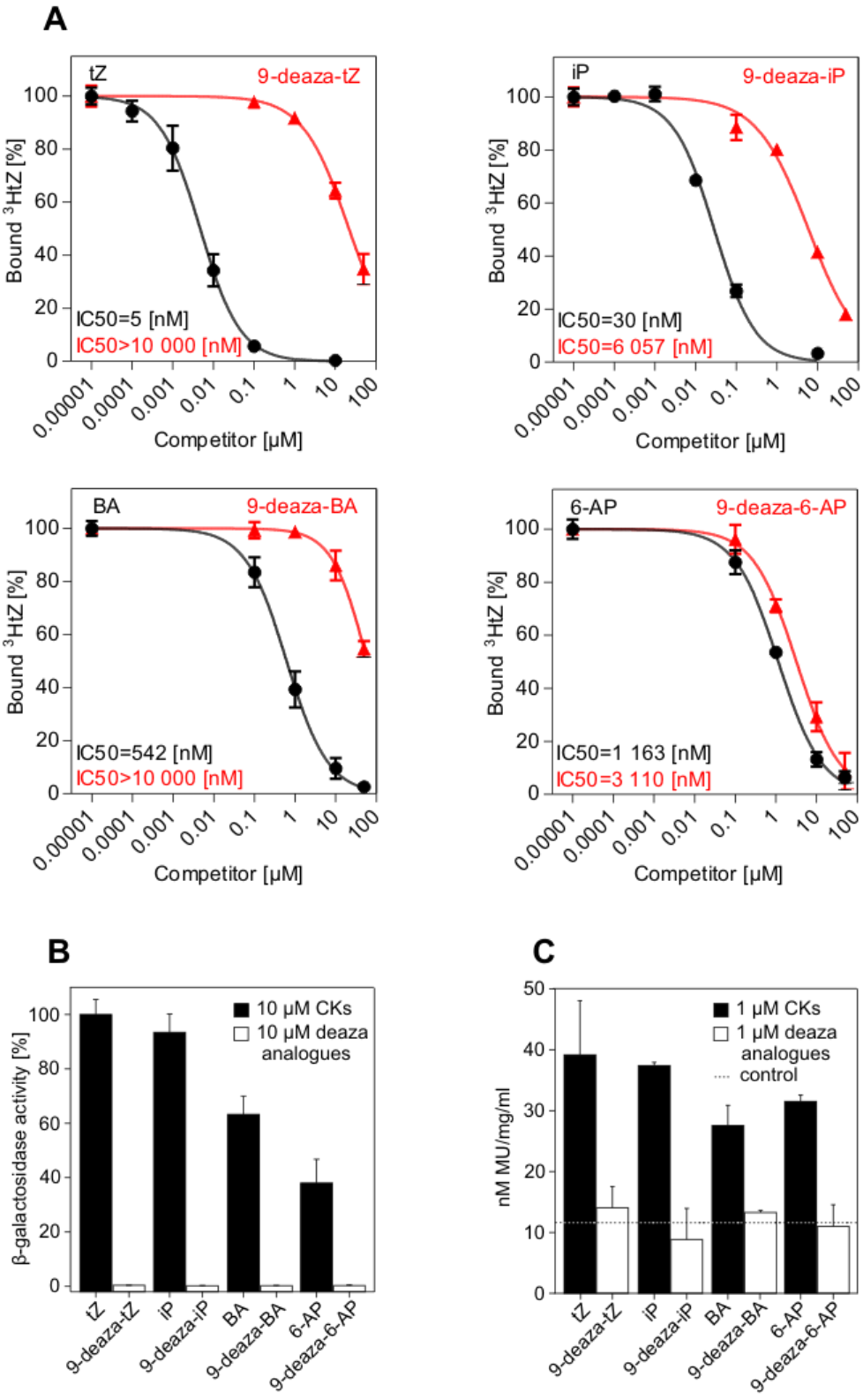



Figure 10. Interaction of 9-deaza-cytokinin analogues with the cytokinin signalling pathway. A) Cytokinin binding assay determined affinity of 9-deaza and aza-compounds towards the AHK4 cytokinin receptor expressed in *E. coli* cells. B) Activation of the AHK4 cytokinin receptor by cytokinins and their deaza-derivatives in the β-galactosidase activity assay. C) Induction of *ARR5::GUS* in transgenic *Arabidopsis* plants. The dashed line represents GUS activity in non-induced plants.

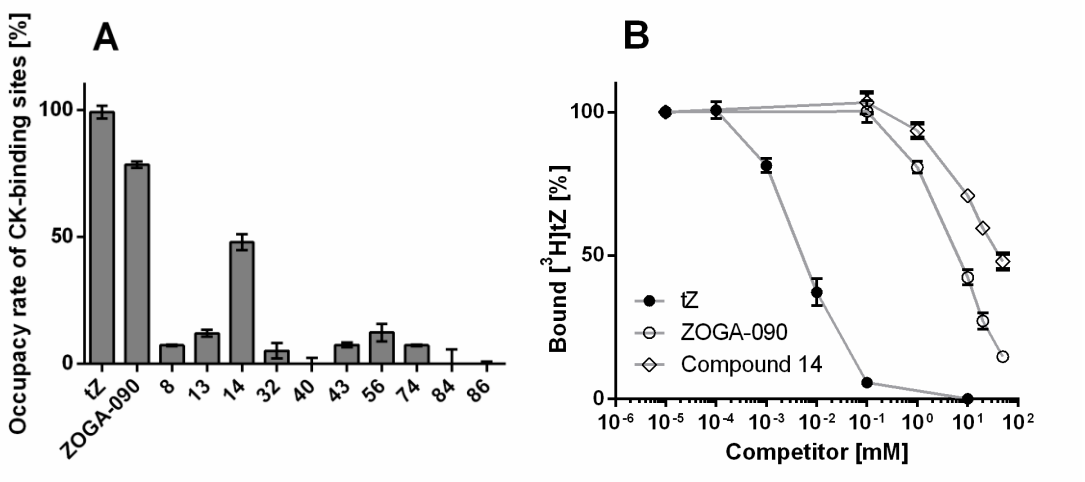



Figure 11. Hit validation. Live-cell cytokinin-binding assay with CRE1/AHK4 cytokinin receptor. Tested compounds (in 20 μM concentration) competed with radioactive-labeled [$^3$H]tZ at the receptor active site. ZOGA-090 is 9-deaza iP. Binding of 10 μM tZ was set as 100%.

Indeed, 9-deaza-cytokinins were able to bind to the CRE1/AHK4 receptor, although with lower affinity than their aza-counterparts (Fig. 10A). Nevertheless, the β-galactosidase activity assay did not show 9-deaza analogues were able to activate the cytokinin pathway (Fig. 10B). These results were confirmed also by the *ARR5::GUS* cytokinin induction assay. 9-deaza-cytokinin analogues did not induce the cytokinin signalling pathway even at the highest concentration of 10 μM (Fig. 10C).

A 9-deaza compound, 9-deaza-iP, was tested also in previous work by Klimeš et al. (2017). This work tested potential agronomically important substances by the β-galactosidase assay using high-throughput screening. The assay was done in 384-well plates to screen for cytokinin agonists and antagonists working at the receptor level. 93 compounds were tested for their capability to be agonist, neutral or antagonist with CRE1/AHK4. 19 potential agonists and 12 antagonists were identified. 10 potential agonists/antagonists were tested also in a cytokinin binding assay. Results are summarised in Figure 11A.

Cytokinin tZ (10 μM) and cytokinin antagonist ZOGA-090 (20 μM) were used as positive controls for agonist and antagonist, respectively. From the 10 selected hits one potential agonist (compound 14) showed effective displacement of the natural ligand from the CRE1/AHK4 active site (Fig. 11A). The detailed analyses confirmed that compound 14 competes for binding into the CRE1/AHK4 cytokinin binding site in a dose-dependent manner (Fig. 11B).

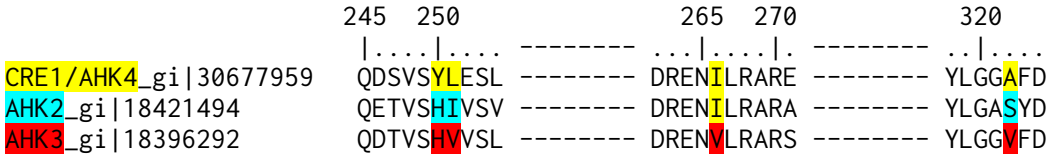



Figure 12. Part of an alignment of the CHASE domains of cytokinin receptors AHK2, AHK3 and AHK4. Inside conserved regions, four amino acids were selected that are conserved across yet described plant species, but differ between individual receptors. The sequence of the CRE1/AHK4 receptor was mutated to the corresponding AHK3 residue at the 4 labelled positions.

## Analysis of the Importance of the Conserved Residues in the Binding Pocket of AHK3 and CRE1/AHK4

The molecular basis of the ligand binding and recognition has been described using co-crystallisation of the CRE1/AHK4 sensor domain with cytokinins (Hothorn et al., 2011). To analyse the importance of conserved residues in the binding pocket, CHASE domains of AHK3 and the CRE1/AHK4 were used. These two receptors have widely contrasting ligand specificities. Protein sequences of available CHASE domains from different organisms were compared to see the most conserved regions within them. Based on this knowledge, four amino acid positions were selected as potentially responsible for the main differences in the ligand specificity of AHK3 and CRE1/AHK4 (Fig. 12).

Three of the selected amino acids are in the entrance to the binding pocket – Tyr250, Leu251 and Ala322 amino acid. The last amino acid, Ile266, is situated inside the binding pocket, near the end of the cytokinin side chain (Fig.13).

Further, the amino acid sequence alignment of all available CHASE domains showed their high conservation in several positions across all species (Fig. 14). It is clearly visible that the most conserved aminoacid is the isoleucine at position 266. Leucine at position 251 may be substituted by other hydrophobic amino acids (mainly alanine, valine, isoleucine and methionine). Hydrophobic amino acids, mostly alanine and to lesser extent valine, occur frequently at position 322, although proline and serine are also found that this position. There is more variability at position 250, tyrosine present in CRE1/AHK4 can be substituted by amino acids with a hydrophobic side chain and also by amino acids with a charged side chain (arginine, histidine, lysine). The importance of these four above mentioned amino acid positions was studied by means of mutagenesis in this work.

Point mutations within the CHASE domain of CRE1/AHK4 were introduced using side-directed mutagenesis. The aim was to change the ligand preference to a more AHK3-like profile. Receptor mutants were subsequently tested for their affinity in a competition

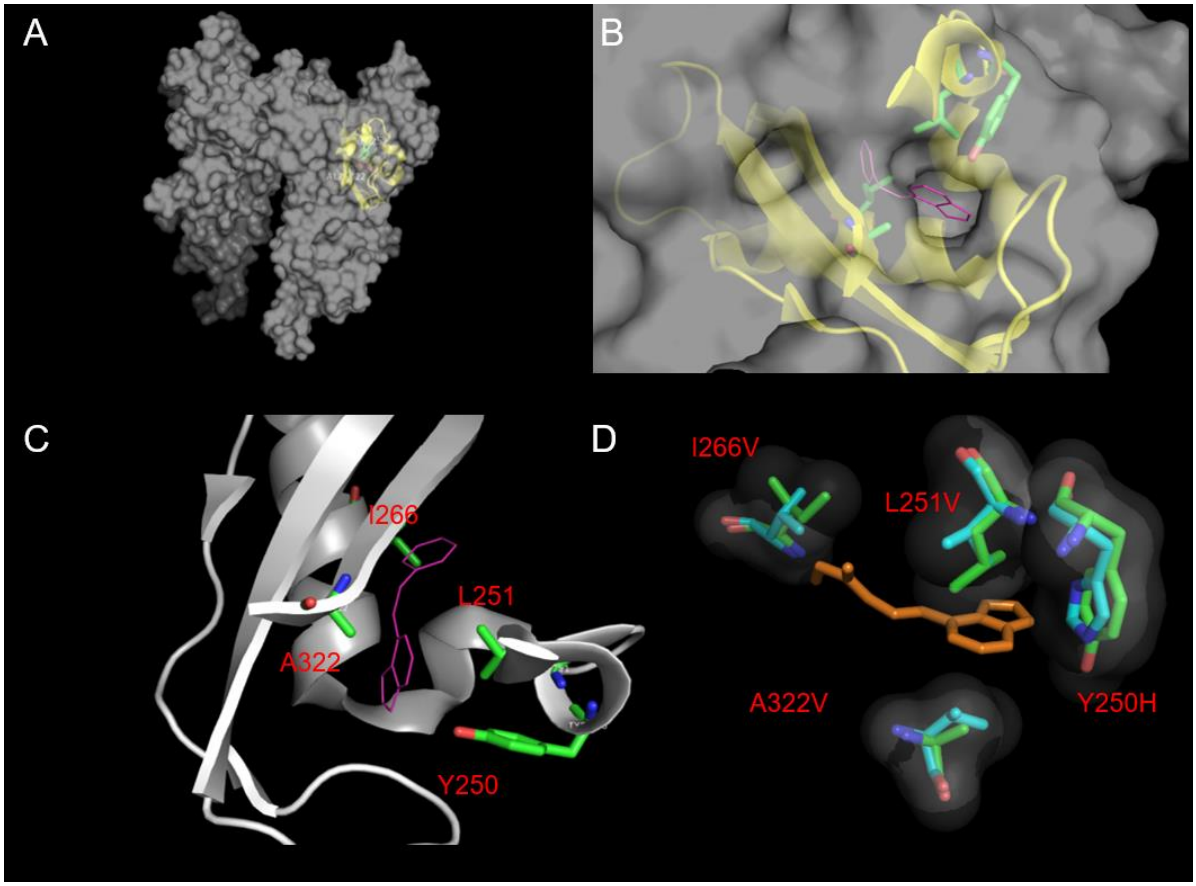



Figure 13. Binding site of the CRE1/AHK4 receptor. A) Protein model of the CHASE domain of CRE1/AHK4 with the cytokinin binding site in yellow. B) View of the tunnel into the binding pocket with BAP in violet. Residues of interest are shown in green. C) Detailed view of the binding pocket of CRE1/AHK4 with BAP (violet) and amino acid residues of interest (green). D) Inside of the binding pocket: BAP with residues of interests labelled in green (CRE1/AHK4) and blue (AHK3). All protein models were created by PyMOL, crystal structure with BAP was used (3T4K, PDB database; Hothorn et al., 2011).

assay using *E. coli* cells expressing the wild-type and mutated receptors. The results are summarised in Table 3.

The A322V mutation was predicted to influence CRE1/AHK4 affinity to cytokinin ribosides. The mutant CRE1/AHK4-A322V showed more than 3-times higher affinity for ribosides, namely for tZR and iPR, compared to that of AHK3. Substitution of isoleucine for valine at position 266 caused almost 3-times lower affinity for iP and *c*Z. Substitution of valine for L251 brought more than 4-times and almost 6-times lower affinity for iP and cZ, respectively, in comparison to the wild-type receptor. Both mutated forms, CRE1/AHK4-L251V and CRE1/AHK4-I266V, showed weak affinity for iP and cZ. The single mutation in CRE1/AHK4-Y250H did not influence binding of the tested cytokinins. Combination of mutations in double, triple and quadruple mutants were found to influence the binding capacity of the tested cytokinins. However, due to lack of

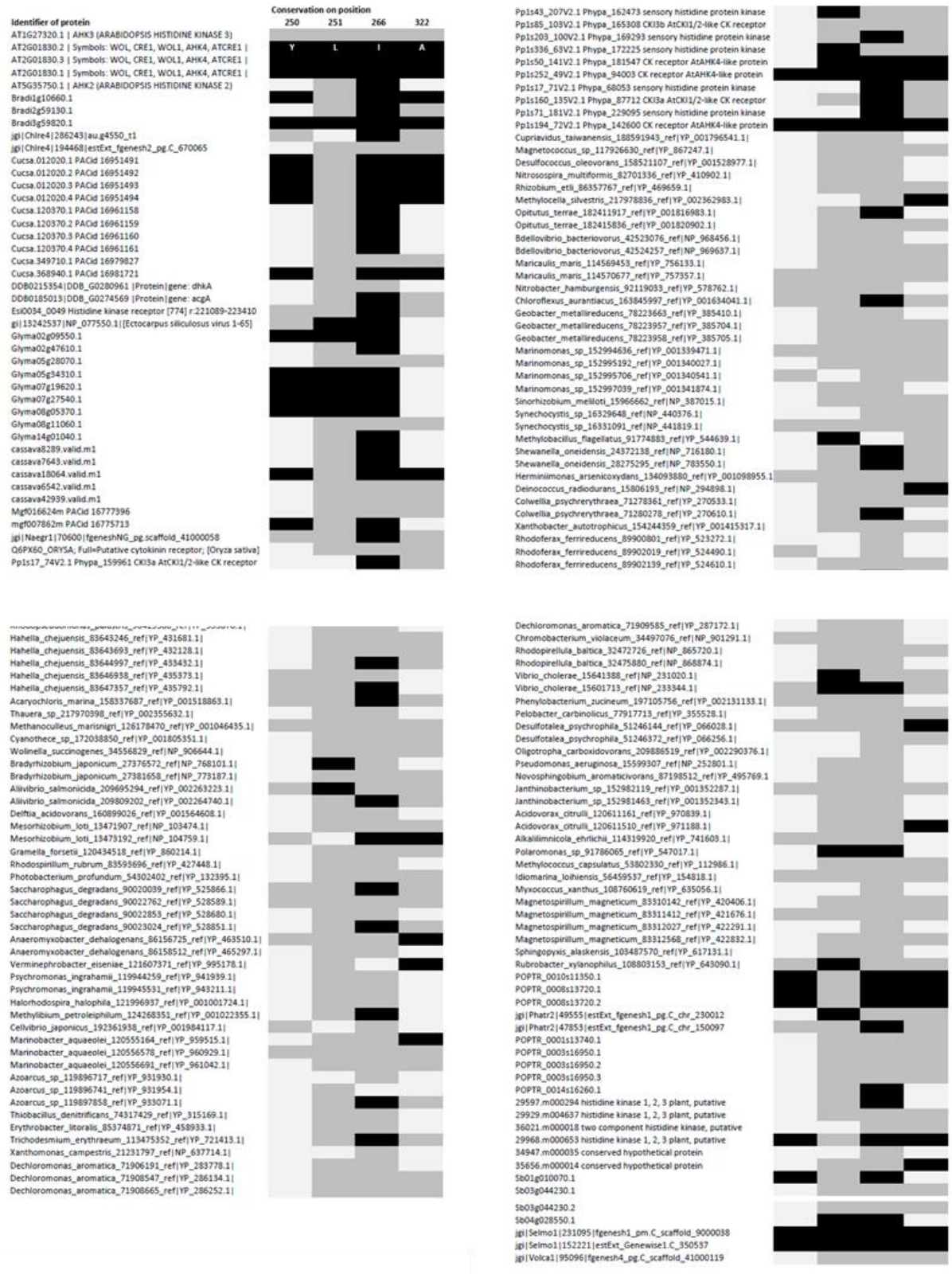



Figure 14. Comparison of all available seqences, containing CHASE domains (alignment was done by dr. Nijuscha Gruhn in October 2012). Only four position of interest are displayed. Black rectangles represent identity with amino acid in CRE1/AHK4, grey represent substitution within conserved group, white ones represent no similarity.

Table 3. IC50 values of selected ligands with WT cytokinin receptor CRE1/AHK4 and its mutated forms – single, double, triple and quadruple mutants. IC50 values highlighted in circles show main differences between WT CRE1/AHK4 receptor and its mutant forms. IC50 values were calculated using GraphPad Prism 5; n.d. – not detected.

| Receptor | Cytokinin<br>Apparent IC50 (nM) | | | | | |
|---|---|---|---|---|---|---|
| | tZ | iP | tZR | cZ | DHZ | iPR |
| CRE1/AHK4 | 4.9 ± 1.1 | 15.7 ± 7.8 | 40.4 ± 9.4 | 345 ± 75 | 232 ± 118 | 48.9 ± 17.5 |
| A322V | 6.1 ± 1.7 | 14.9 ± 4.3 | 11.4 ± 1.9 | 403 ± 120 | 402 | 13 |
| I266V | 4.6 ± 1.5 | 42.5 ± 10.6 | 37.0 ± 16.4 | 1002 ± 356 | 83 ± 30 | 118 ± 1.5 |
| Y250H | 5.1 ± 1.6 | 11.3 ± 4.7 | 54.0 ± 19.5 | 300 ± 84 | n.d. | n.d. |
| L251V | 3.5 | 71.2 | 111 | 2388 | n.d. | n.d. |
| I266V_A322V | 4.7 ± 0.6 | 56.0 ± 35.8 | 35.2 ± 22.3 | 1560 ± 237 | n.d. | n.d. |
| Y250H_I266V | 3.4 | 33.4 | 18.6 | 390 | n.d. | n.d. |
| Y250H_A345V | 4.7 ± 2.3 | 12.1 ± 5.6 | 42.8 ± 18.2 | 606 ± 231 | n.d. | n.d. |
| Y250H_L251V | 138 | 540 | n.d. | n.d. | n.d. | n.d. |
| Y250H_L251V_I266V | 1.2 | 13.7 | n.d. | n.d. | n.d. | n.d. |
| Y250H_L251V_A345V | 39.4 | 123 | n.d. | n.d. | n.d. | n.d. |
| Y250H_I266V_A345V | 45.3 | 169 | n.d. | n.d. | n.d. | n.d. |
| quadruple mutant | 56.9 | 201 | n.d. | n.d. | n.d. | n.d. |

time, these interesting preliminary results could not be followed up and need to be confirmed by repetition of the cytokinin binding assay.

# DISCUSSION

The results of the cytokinin binding assays validate the assumption that the newly described proteins in *Brassica napus* are true cytokinin receptors. They specifically recognize the tested adenine-type isoprenoid, aromatic and urea-type cytokinins. In the case of *tZ*, which was the best ligand among the naturally occuring compounds, $K_d$ values ($K_d$ 1–3 nM) are close to previously described values for CRE1/AHK4 - 2.5 nM (Romanov et al., 2005); 3.9 nM (Romanov et al., 2006) and 4.4 nM (Stolz et al., 2011). Two subgroups of cytokinin receptors, defined on the basis of cytokinin specificity, are known in *A. thaliana* – one subgroup including AHK2 and CRE1/AHK4, while the other contains AHK3. All of the receptors bind *tZ* with comparable affinity (Lomin et al., 2015; Romanov et al., 2006). But while AHK3 binds iP with lower affinity than *tZ*, AHK2 and CRE1/AHK4 bind iP with comparable affinity (Lomin et al., 2015; Stolz et al., 2011).

The binding data support the phylogenetic analysis, which indicates that BnCHK1 with BnCHK3 are orthologous to AHK2, while BnCHK5 is an orthologue of AHK3. Both BnCHK1 and BnCHK3 receptors have higher affinity for iP (~6.5- and 9.5-fold) than does BnCHK5; similarly, AHK2 has higher affinity for iP than does AHK3 (~10- to 100-fold; Lomin et al., 2015; Romanov et al., 2006; Stolz et al., 2011). *tZ*-glucosides did not show ability to bind into all tested receptors. The reason could be the modified core cytokinin structure. In the case of aromatic cytokinins, BAP and especially *m*T showed the highest affinity to BnCHK3, which corresponds to results from Lomin et al. (2015).

Two splice versions of the AHK2 orthologues, ZmHK3a and ZmHK3b, have been identified in *Zea mays*. However only the ZmHK3a isoform showed activity in the *E. coli* activity assay. ZmHK3b was not activated by cytokinins probably because part of the input and TM domains is missing (Yonekura-Sakakibara et al., 2004). The potato receptors recognize *tZ* with $K_d$ 2.5-4.7 nM, but there is no significant difference in preference for iP between them. Tetraploid potato, variety Désirée, has also two allelic forms for each type of receptor. However, $K_d$ values did not show any remarkable differences between each type of receptor - StHK2a, StHK3a and StHK4a/b. StHK3a showed only 2-times lower binding affinity for iP compared with the other tested receptors and 8-9-times higher affinity for DHZ. Both receptors, StHK3a and StHK2a showed better affinity for TDZ in comparison to StHK4a/b (Lomin et al., 2018). Based on phylogentic analysis, five forms of receptor were identified in *Malus domesticus* – MdCHK2, MdCHK3a/b, MdCHK4a/b, annotated according to their homology to AHK2, AHK3, CRE1/AHK4, respectively. All receptors showed activity and affinity to cytokinins in yeast complementation assays, but unfortunatelly $K_d$ values were not established (Daudu et al., 2017).

N-to-C substitution at N9 of the cytokinin molecule showed negative influence on cytokinin affinity and activity. Previous works from Doležal et al. (2007) and Holub et al. (1998) also demonstrated a decrease of biological activity by ribosylation and glycosylation at the N9 of adenine moiety. Alkylation on the N9-position of BAP also reduced its cytokinin activity (Savelieva et al., 2018). Further, substitution on N9 of cytokinin with a methyl group and reorganisation of heteroatoms in the adenine part to form pyrazolo[4,3-*d*]pyrimidine derivates resulted in a strong negative influence on cytokinin activity (Hecht et al., 1971). Results indicate that the N9-atom is important for accepting a hydrogen bond from Leu284 inside the binding site of the CRE1/AHK4 receptor. In addition, 9-deaza cytokinin analogues behave similarly to inhibitors of CRE1/AHK4: PI-55 (6-(2-hydroxy-3-methylbenzylamino)purine), LGR-991 (6-(2,5-dihydroxybenzylamino)purine), $N^6$-(benzyloxymethyl)adenosine (BOMA; Krivosheev et al., 2012; Nisler et al., 2010; Spíchal et al., 2009). Indeed, 9-deaza-cytokinins could be used as cytokinin antagonists (Klimeš et al., 2017). This possibility was established not only through their effect in classical cytokinin bioassays, but mainly *via* their direct interaction with receptors. Results confirmed the affinity of 9-deaza cytokinin analogues with the CRE1/AHK4 receptor, but without its activation (except very weak activity on the level of classical cytokinin bioassays).

Receptor CRE1/AHK4 with mutated A322V showed higher affinity to cytokinin ribosides (*t*ZR, iPR), with $K_d$ more than 3,5-times lower as for WT CRE1/AHK4. Results for *t*ZR are in agreement with Romanov et al. (2006), where similarly the AHK3 receptor prefers *t*ZR with 3,3-times higher affinity compared with CRE1/AHK4. However, $K_d$ values for iPR were completely different for AHK3 ($K_d$ 2300 nM) and CRE1/AHK4 ($K_d$ 130 nM). Single mutants CRE1/AHK4-I266V, CRE1/AHK4-L251V and double mutant CRE1/AHK4-I266V-A322V had lower affinity for iP and *c*Z than did the WT receptor, but a lower affinity was expected only for iP in the case of AHK3 (Romanov et al., 2006). Most double, triple and quadruple mutants of CRE1/AHK4 had changed affinity for *t*Z and iP, but there is still space for further experiments to confirm these preliminary results. Previously, some point mutations were characterised in CRE1/AHK4 receptor, but only one work was focused on modification of binding affinity. Heyl with colleagues (2007) focused on conserved amino acids, which are involved in binding of cytokinin. All mutations caused a decrease or loss of receptor activity. The F304A and T317A mutations caused a loss of binding capacity. K297A reached about 90% of WT relative binding capacity, W244A showed 60% and R305A had 40% of the WT binding capacity.

# CONCLUSIONS

This thesis wishes to shed more light on the cytokinin world, especially in the area of their first contact: ligand-receptor interaction. At first, we focused on identification and functional characterization of homologues of sensor histidine kinases homologous to *Arabidopsis* cytokinin receptors in agronomically important winter oilseed rape, *Brassica napus* var. Tapidor. There were identified five CHASE-containing His kinases (BnCHK1–BnCHK5). Four homologues of AHK2 (BnCHK1–BnCHK4) and one homologue of AHK3 (BnCHK5) were defined and their domain structures were determined. Tested receptors, BnCHK1, BnCHK3 and BnCHK5 displayed high affinity for *tZ* (1–3 nM), but not with other plant hormones as gibberelic acid, indole-3-acetic acid and abscisic acid, confirming the prediction that they are CK receptors. Implying potential functional divergence, the AHK2 homologues could be divided into two subfamilies (BnCHK1/BnCHK2 and BnCHK3/BnCHK4) that differ in putative transmembrane domain topology and CK binding specificity.

Structural details of cytokinin binding were previously described (Hothorn et al., 2011; Steklov et al., 2013). However, mechanism of receptor activation needed to be elucidated. Computer simulations indicated the importance of hydrogen bond formation between the N9-atom of the adenine ring and peptide backbone of the CRE1/AHK4 binding site. Possible involvement of this interaction in AHK4 active site structural rearrangement and its subsequent influence on receptor activation were investigated *in silico* and tested *in vitro* with 9-deaza cytokinin analogues. Lack of ligand binding at N9-position decreased affinity to AHK4 receptor and completely blocked its activation. Results indicate importance of the cytokinin N9-hydrogen bonding in the mechanism of AHK4 activation. It is likely that missing hydrogen bond in N9-position has influence on destabilization of the dimerization interface of the receptor. Another approach involved mutagenesis of the CRE1/AHK4 receptor and testing of ligand specificity of the mutants. The results suggest that the mutated amino acids are indeed involved in binding specificity as they generally accept cytokinin ribosides with better affinity than WT receptor. However, more testing would be required to confirm these results.

The knowledge of cytokinins and their derivatives' mechanism of action can be also used in agriculture. Compounds that specifically interact with plant hormone pathways have high utility in research and potential agronomic applications for improving crop yields or other aspects of crops' performance. A high throughput method for screening compounds with agonist or antagonist properties toward the CRE1/AHK4 cytokinin receptor was developed. Potential ligands were screened in *E. coli* by β-galactosidase assay. The presented method enables robust, automated screening of large libraries of

compounds for ability to activate or inhibit the *Arabidopsis thaliana* cytokinin receptor CRE1/AHK4 using the Nanodrop II liquid handling system in 384-well plate format.

Thus, the cytokinin receptors in the model plant *Arabidopsis thaliana* and its agrnomically important relative *Brassica napus* were studied. The obtained results moved us further forward in understanding of the interaction between cytokinin receptors and their ligands and of the selectivity of the receptors.

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# APPENDIX

Papers published in scientific journals:

A. Identification of AHK2- and AHK3-like cytokinin receptors in *Brassica napus* reveals two subfamilies of AHK2 orthologues

   Published as:

   Kuderová, A., Gallová, L., Kuricová, K., Nejedlá, E., Čurdová, A., Micenková, L., et al. (2015). Identification of AHK2- and AHK3-like cytokinin receptors in *Brassica napus* reveals two subfamilies of AHK2 orthologues. *J. Exp. Bot.* 66, 339–353. doi:10.1093/jxb/eru422.

B. 9-deaza cytokinins reveal molecular basis of cytokinin receptor ARABIDOPSIS HISTIDINE KINASE 4 signal transmission through membrane

   Published as:

   Hlusková, L., Gándara Barreiro, Z., Berka, K., Bazgier, V., Mik, V., Mazura, P., et al. 9-deaza cytokinins reveal molecular basis of cytokinin receptor ARABIDOPSIS HISTIDINE KINASE 4 signal transmission through membrane. *Phytochemistry* submitted.

C. High Throughput Screening Method for Identifying Potential Agonists and Antagonists of Arabidopsis thaliana Cytokinin Receptor CRE1/AHK4

   Published as:

   Klimeš, P., Turek, D., Mazura, P., Gallová, L., Spíchal, L., and Brzobohatý, B. (2017). High Throughput Screening Method for Identifying Potential Agonists and Antagonists of *Arabidopsis thaliana* Cytokinin Receptor CRE1/AHK4. *Front. Plant Sci.* 8, 947. doi:10.3389/fpls.2017.00947.

*Journal of Experimental Botany*, Vol. 66, No. 1 pp. 339–353, 2015
doi:10.1093/jxb/eru422 Advance Access publication 21 October, 2014

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

# Identification of AHK2- and AHK3-like cytokinin receptors in *Brassica napus* reveals two subfamilies of AHK2 orthologues

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Received 25 July 2014; Revised 18 September 2014; Accepted 19 September 2014

## Abstract

**Cytokinin (CK) signalling is known to play key roles in the regulation of plant growth and development, crop yields, and tolerance to both abiotic stress and pathogen defences, but the mechanisms involved are poorly characterized in dicotyledonous crops. Here the identification and functional characterization of sensor histidine kinases homologous to *Arabidopsis* CK receptors AHK2 and AHK3 in winter oilseed rape are presented. Five CHASE-containing His kinases were identified in *Brassica napus* var. Tapidor (BnCHK1–BnCHK5) by heterologous hybridization of its genomic library with gene-specific probes from *Arabidopsis*. The identified bacterial artificial chromosome (BAC) clones were fingerprinted and representative clones in five distinct groups were sequenced. Using a bioinformatic approach and cDNA cloning, the precise gene and putative protein domain structures were determined. Based on phylogenetic analysis, four *AHK2* (*BnCHK1–BnCHK4*) homologues and one *AHK3* (*BnCHK5*) homologue were defined. It is further suggested that *BnCHK1* and *BnCHK3*, and *BnCHK5* are orthologues of *AHK2* and *AHK3*, originally from the *B. rapa* A genome, respectively. BnCHK1, BnCHK3, and BnCHK5 displayed high affinity for *trans*-zeatin (1–3 nM) in a live-cell competitive receptor assay, but not with other plant hormones (indole acetic acid, $GA_3$, and abscisic acid), confirming the prediction that they are genuine CK receptors. It is shown that BnCHK1 and BnCHK3, and BnCHK5 display distinct preferences for various CK bases and metabolites, characteristic of their AHK counterparts, AHK2 and AHK3, respectively. Interestingly, the AHK2 homologues could be divided into two subfamilies (BnCHK1/BnCK2 and BnCHK3/BnCHK4) that differ in putative transmembrane domain topology and CK binding specificity, thus implying potential functional divergence.**

**Key words:** CHASE-containing His kinase, *E. coli*-based live-cell competitive receptor assay, gene structure, JBnB library, modular protein architecture, phylogenetic analysis

## Introduction

Cytokinin (CK) phytohormones regulate or participate in complex hormonal interactions involved in the control over numerous physiological and developmental processes such as cell division and differentiation, chloroplast maturation, leaf senescence, gametophyte formation, vascular tissue development, clock-related responses, responses to light, stresses (biotic and abiotic), and availability of macronutrients (for reviews, see Argueso *et al.*, 2009; Werner and Schmülling, 2009). CK signals are perceived and transduced through a multistep histidyl-aspartyl (His–Asp)

phosphorelay (MSP). The MSP pathway is intrinsic to ~20% of bacteria and is exclusively used by yeast and plants but not animals (Schaller *et al.*, 2011). According to the current model of CK signalling, CK molecules are perceived by a membrane-bound hybrid His-kinase (HK) receptor. Upon CK binding, the receptor autophosphorylates at a conserved histidine in the HK domain and the phosphate residue is transferred to the receptor's receiver (REC) domain. The phosphate is then relayed to a His-containing phosphotransfer protein (HPt), which translocates to the nucleus and activates type-B response regulators (RRs). The type-B RRs act as Myb-type transcription factors, inducing expression of primary response genes. The CK primary response genes include abundant type-A RRs, acting as negative feedback regulators of the CK signalling pathway. Generally, the CK receptors represent a small proportion of a plant's complements of sensor HKs, through which (*inter alia*) they also perceive ethylene (Chang *et al.*, 1993) and changes in osmotic conditions (Urao *et al.*, 1999). A feature that distinguishes CK receptors from other sensor HKs is their N-terminal ligand-binding cyclase/histidine kinase-associated sensory extracellular (CHASE) domain (Anantharaman and Aravind, 2001; Mougel and Zhulin, 2001). The CHASE domain is flanked by transmembrane domains and associated with a cytoplasmic HK domain and a C-terminal REC domain (Ueguchi *et al.*, 2001).

The properties and functions of CK receptors are best described in the model dicot *Arabidopsis thaliana.* Among eight identified HKs of this species, three transmembrane ARABIDOPSIS HIS KINASES (AHKs), designated AHK2, AHK3, and AHK4, have been shown to act as genuine CK receptors (Inoue *et al*, 2001; Ueguchi *et al.*, 2001). Studies of single and higher order *ahk* mutants (Higuchi *et al.*, 2004; Nishimura *et al.*, 2004; Riefler *et al.* 2006) have revealed partially redundant but differentiated functions for the individual receptors and prominent roles for the AHK2/AHK3 receptor combination in quantitative control of organ growth, with opposite regulatory functions in roots and shoots (Riefler *et al.*, 2006). Specific roles have been identified for single receptors. Notably, AHK4 and AHK3, respectively, play key roles in early vascular development (Mähönen *et al.*, 2000) and cell differentiation in the cell division/cell differentiation zone of the root meristem (Dello Ioio *et al.*, 2007). *Arabidopsis* loss-of-function mutants of CK receptors have also shown strong tolerance to drought and salt stress (Tran *et al.*, 2007, 2010), increased freezing tolerance (Jeon *et al.*, 2010), and resistance to clubroot disease (Galfe *et al.*, 2009). Using heterologous expression systems, it has been shown that various CK compounds have signalling functions, but only via specific receptors, and that CK binding activities are in the nanomolar range, in agreement with CK concentrations *in planta* (Spíchal *et al.*, 2004; Romanov *et al.*, 2005, 2006). AHK3 and AHK4 show different ligand preferences (Spíchal *et al.*, 2004; Romanov *et al*, 2006), while AHK2 functionally resembles AHK4 (Stoltz *et al.*, 2011). The structural basis for CK recognition by AHK4 was recently unravelled and the crucial amino acids in its CHASE domain for the receptor function were identified (Hothorn *et al.*, 2011).

Owing to the implications of CK signalling in economically important traits, such as nodulation, wood formation, drought, salt, and pathogen resistance (Tran *et al.*, 2007; Nieminen *et al.*, 2008; Choi *et al.*, 2010; Argueso *et al.*, 2012), detailed knowledge of CK signalling components in cultivated plant species including crops has valuable potential applications. However, current knowledge of CK signalling mechanisms in crops is mostly limited to monocotyledonous plants, particularly maize (Asakura *et al.*, 2003; Giulini *et al.*, 2004; Yonekura-Sakakibara *et al.*, 2004) and rice (Ito and Kurata, 2006; Jain *et al.*, 2006; Pareek *et al.*, 2006; Du *et al.*, 2007; Choi *et al.*, 2012; Tsai *et al.*, 2012). Thus, there is a clear need to characterize the receptors and downstream signalling components in dicotyledonous crop species. The most important of these species globally include various members of the genus *Brassica*. Oilseed rape, *Brassica napus*, a recently formed allotetraploid containing *B. rapa* (A) and *B. oleracea* (C) genomes, is a valuable crop that is widely used in food and feed industries. It also plays a significant role in arable rotations by improving yields of subsequent cereal crops. However, there is little or no knowledge of CK perception and signalling mechanisms in *B. napus* and other cultivated *Brassica* species. Here, the first identification of five members of the CK receptor gene family homologous to AHK2 and AHK3 and detailed CK binding studies of the encoded proteins from *Brassica napus* (var. Tapidor) are presented. Exploiting the phylogenetically close relationship of *B. napus* to *Arabidopsis thaliana*, five *Arabidopsis* homologues of *B. napus* CHASE-containing His kinase (*BnCHK*) genes were identified. Based on genomic DNA and cDNA sequencing results and bioinformatic predictions, it is shown that all five BnCHKs share typical molecular characteristics of CK receptors and they are phylogenetically compared with *B. rapa* counterparts annotated to date. Detailed binding and recognition characteristics of CK metabolites by BnCHK1, BnCHK3 (homologues of AHK2), and BnCHK5 (homologue of AHK3) are also presented.

## Materials and methods

### *Probe preparation*

Hybridization probes, used for identification of JBnB clones (and subsequently subcloned restriction fragments), carrying characteristic sequences of *BnCHK* genes, were prepared by PCR amplification of cDNA regions of *AHK2*, using the following primers: 5′-ACTGAGAGAACAAACTTTGAGAGG-3′ and 5′-CATGGT TCCTTGATGGATCAC-3′ (CHASE probe), 5′-GAATGC TGAAAATGCTGA-3′ and 5′-TGCCAGTTCCACCATAAG-3′ (HK probe), 5′-GGTTGTGGATGATAATCTTGTG-3′, and 5′-CTTGCTACCGCTGTGTAGAG-3′ (REC probe).

### *Filter hybridizations*

The JBnB library and individual bacterial artificial chromosome (BAC) clones were purchased from the John Innes Centre (JIC) Genome Laboratory, Norwich, UK. Filters were pre-processed by

soaking for 2 h at 42 °C in 5× SSC, 0.5% SDS, 1 mM EDTA (pH 8). Bacterial debris was removed using a paper towel; membranes were then rinsed in 2× SSC and hybridized with 100 ng of $^{32}$P-labelled probes under low stringency conditions, as described by O'Neill and Bancroft (2000). A DecaLabel™ DNA Labelling Kit (Fermentas) was used to prepare [α-$^{32}$P]dCTP random-primed labelled probes according to the manufacturer's instructions. Before the next hybridization step with another of the three probes, filters were stripped for re-use by soaking twice in 0.4 M NaOH at 50 °C for 30 min, then neutralized in 0.1× SSC, 0.1% SDS, 0.2 M TRIS (pH7.5) at 50 °C for at least 20 min according to JIC protocols. Restricted DNA, isolated from BAC clones, was Southern-blotted under alkaline conditions to HyBondTM-N+ membrane (GE Healthcare) and hybridized under low stringency conditions as outlined above. Autoradiography was performed using a STORM 840 Phospho-imager (GE Healthcare).

### *DNA preparation, vectors, and bacterial strains*

BAC clones from the JBnB library, constructed in the pBAC/SACB1 vector (Bendahmane, 1999), were propagated in LB medium supplemented with 12.5 μg ml$^{-1}$ chloramphenicol. BAC DNA was isolated using QIAGEN®Plasmid Midi Kit columns according to the manufacturer's protocol. Restricted DNA isolated from BAC clones was subcloned in the pBluescript SK vector and propagated in *Escherichia coli* strain DH10B.

### *cDNA cloning*

RNA was isolated from shoots, hypocotyls, and roots of 6-day-old *B. napus* var. Tapidor seedlings using an RNeasy Plant Mini Kit (QIAGEN) or Trizol reagent (Invitrogen) and treated with RNase-free DNase I (QIAGEN) prior to reverese transcription–PCR (RT–PCR). RNA template (3 μg, consisting of 1 μg from each of the shoot, hypocotyl, and root RNA samples) was used in first-strand cDNA synthesis, catalysed by SuperScript™ III Reverse Transcriptase following the manufacturer's instructions (Invitrogen, Life Technologies, Czech Republic). PCR was performed using Phusion High-Fidelity DNA Polymerase (Finnzymes). 5' and 3' cDNA ends were cloned using a GeneRacer™ (RLM-RACE) Kit, TOPO TA Cloning® Kit for Sequencing, or Zero Blunt® TOPO® PCR Cloning Kit and One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen) according to the manufacturers' protocols. Gene-specific primers used for cDNA cloning are listed in Supplementary Table S1 available at *JXB* online.

### *Sequence and phylogenetic analysis*

Raw sequencing data were obtained from Macrogen (http://dna.macrogen.com). Next-generation sequencing (NGS) was performed using the GS Junior system from ROCHE with the company's assistance. Most analytical steps, including primer walking, contig assembly, reading frame definition, sequence alignments, and database searches were performed using DNASTAR software (http://www.dnastar.com). Coding sequences of the defined genomic DNAs were predicted by GENSCAN (http://genes.mit.edu/GENSCAN.html; Burge and Karlin, 1997), Eucaryotic GeneMark.hmm (http://exon.gatech.edu; Lomsadze *et al.*, 2005), and FGENESH (http://linux1.softberry.com/all.htm; Yao *et al.*, 2005; Solovyev *et al.*, 2006). Gene structures were visualized in FancyGene (http://bio.ieo.eu/fancygene; Rambaldi and Ciccarelli, 2009). To confirm the presence of conserved domains, motifs were identified by PROSITE (http://prosite.expasy.org; Sigrist *et al.*, 2012) and SMART (http://smart.embl.de/; Schultz *et al.*, 2000). Transmembrane (TM) segments were predicted by SMART, TMAP (Persson and Argos, 1994) and TMHMM (Eddy, 1998; Sonnhammer *et al.*, 1998) algorithms at http://workbench.sdsc.edu/, TMpred (http://www.ch.embnet.org/software/TMPRED_form.html; Hofmann and Stoffel, 1993), TopPred 0.01 (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::toppred; von Heijne, 1992; Claros and von Heijne, 1994), SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html; Hirokawa *et al.*, 1998), and PHYRE2 (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index; Kelley and Sternberg, 2009). Phylogenetic analysis of the sequences was carried out by Phylogeny.fr (http://www.phylogeny.fr; Dereeper *et al.*, 2008), using the following 'a la Carte' applications: (i) multiple alignment by MUSCLE (Edgar, 2004) under default mode; (ii) Gblocks treatment of alignments (Castresana, 2000); and (iii) phylogenetic tree construction by the Neighbor–Joining method (Saitou and Nei, 1987) with 500 bootstrap replicates to obtain branch-support values (Felsenstein, 1989).

### *CK binding assay*

Non-labelled phytohormones used in the binding assays were obtained from OlChemIm Ltd. (Olomouc, Czech Republic). Radiolabelled *trans*-zeatin ([2-$^{3}$H]zeatin, 592 GBq/mmol) was obtained from the Isotope Laboratory, Institute of Experimental Botany, AS CR, Prague, Czech Republic. Live-cell CK-binding assays were performed with intact *E. coli* strain KMI001 carrying the pINIIIΔEH vector (Suzuki *et al.*, 2001; Yamada *et al.*, 2001). BnCHK1 and BnCHK5 coding sequences were obtained by gene synthesis at Life Technologies and subcloned in pINIIIΔEH using *Bam*HI and *Sal*I restriction sites. *BnCHK3* was obtained from pBluescript vector by PCR introducing an *Eco*RI restriction site and subcloned to the same restriction site of the empty vector pINIIIdEH. The binding assay was performed according to the method described by Romanov *et al.* (2005, 2006) with slight modifications. Bacterial cultures were grown in liquid M9 medium supplemented with 100 μg ml$^{-1}$ ampicillin and 0.1% (w/v) casamino acids at 25 °C overnight, with shaking (200 rpm), to $OD_{600}$ ~0.7–0.8. The culture density was then increased to $OD_{600}$ ~0.9–1.2 by centrifugation (1000 *g*, 7 min, 4 °C). For assays with each probe, 1 ml portions of the cell suspension were transferred to Eppendorf tubes, then 3 pmol of [$^{3}$H] tZ, with or without unlabelled tZ or other tested competitors at various concentrations, and 0.1% (v/v) dimethylsulphoxide (DMSO; solvent) was added. After at least 30 min incubation at 4 °C, the sample was centrifuged (6000 *g*, 6 min, 4 °C), the supernatant was carefully removed, and the bacterial pellet was resuspended in 1 ml of scintillation cocktail (Beckman, Ramsey, MN, USA) in an ultrasonic bath. Radioactivity was measured by a Hidex 300 SL scintillation counter (Hidex, Finland). To discriminate between specific and non-specific binding, a high excess of unlabelled tZ (at least 3000-fold) was used for competition, as described by Romanov *et al.* (2006). $K_d$ values were determined as average values from three independent Scatchard analyses (Scatchard, 1949), using GraphPad Prism 5.1 (http://www.graphpad.com/scientific-software/prism/).

## Results

### *Redundant numbers of BAC clones carrying putative CHASE-containing His-kinases were identified by screening the* B. napus *genomic library*

To obtain genomic DNA encoding potential CK receptors, a BAC library prepared from genomic DNA of *B. napus* var. Tapidor (JBnB) was experimentally screened (Rana *et al.*, 2004). The choice of hybridization probes was based on a preliminary assessment of frequencies of identical nucleotides between aligned *AHK2*/*AHK3*, *AHK2*/*AHK4*, and *AHK3*/*AHK4* pairs of genes (differing by not more than 2%, Supplementary Fig. S1 at *JXB* online) and knowledge of the three characteristic domains of AHK proteins (Ueguchi *et al.*, 2001). The PCR-generated probes were thus derived from nucleotide sequences corresponding to the phylogenetically conserved CHASE, HK, and REC domains of one of the three *AHK* genes, namely *AHK2*.

In total, 58, 41, and 115 BAC clones that hybridized with the CHASE, HK, and REC probes, respectively, were identified. Of all the BAC clones giving positive signals with at least one probe, 39 hybridized with each probe (Supplementary Table S2 at *JXB* online). In further experiments, most clones, which hybridized weakly with only the CHASE or REC probe, were omitted to avoid sequencing BAC inserts carrying incomplete HK genes. The final list of JBnB clones detected in the hybridization experiments and chosen for further analyses is presented in Supplementary Table S3.

### *Fingerprinting sorted overlapping BAC clones into five distinct groups*

The JBnB library contains 73 728 clones, of which 88% are recombinant, with a mean insert size of 145 kb. Thus, it should represent the 1200 Mb genome of *B. napus* with 7.8-fold redundancy (Rana *et al.*, 2004). To sort out BAC clones carrying overlapping genome fragments, restriction analysis, followed by hybridization of Southern blots of the restriction digests with the CHASE probe, was used. *Sal*I, *Bam*HI, and *Hin*dIII restriction digests and their hybridization patterns with the CHASE probe are presented in Supplementary Fig. S2 at *JXB* online.

In total, five distinct groups were defined among the identified BAC clones. This experimental outcome supported the theoretical estimate of 5.5 *BnCHK* genes among the 43 analysed BAC clones, based on the reported 7.8-fold redundancy in the JBnB library. However, five clones could not be assigned to any of the defined groups and the results of their analysis were ambiguous, mainly because they yielded very weak or no hybridization signals (Supplementary Table S3, Fig. S1 at *JXB* online).

### *Genomic DNA sequencing and analysis of the candidate BAC clones defined five distinct genes predicted to encode cytokinin receptors*

A BAC clone representing each group was chosen for sequencing (Supplementary Table S3 at *JXB* online). Sequencing by primer walking and NGS was used to acquire information about genomic DNA sequences carrying putative genes encoding CK receptors in the identified BAC clones (see the Materials and methods). The first round of sequencing by primer walking of DNA templates, isolated from JBnB025B19, JBnB162F14, JBnB041A21, and JBnB047D2, was successfully initiated by primers derived from *AHK2*. As mispriming of JBnB002E4 occurred, it was finally sequenced by the NGS approach.

In addition to 'BAC walking', in which the whole BAC inserts served as templates, their subcloned restriction fragments giving positive signals with CHASE, HK, and REC probes were also sequenced by primer walking. The subcloned genomic DNA regions of JBnB002E4, JBnB025B19, and JBnB162F14 were stable in *E. coli* (Supplementary Fig. S3 at *JXB* online), and these templates helped verify the outcome of BAC sequencing. Based on the sequencing results, five distinct genomic sequences were defined (accession nos KF621024, KF621025, KF621028, KF621026, and KF621027) with lengths ranging from 8.8 kb to 13.9 kb.

Within these genomic sequences, uninterrupted open reading frames, 3.0–3.6 kb long, were predicted by *ab initio* gene structure prediction software (see also the following section). In addition, preliminary analysis by PROSITE (Sigrist *et al.*, 2012) revealed the presence of three domain profiles typical of TM sensor HKs (CHASE, HK, and REC) within each translated reading frame (not shown). Thus, it was concluded that the five distinct genomic DNA sequences identified by the experimental approach used here encoded hybrid CHASE-containing HKs (CHKs) (Heyl *et al.*, 2013), members of a multistep phosphorelay system and putative CK receptors, which were designated *BnCHK1*–*BnCHK5* (Table 1).

### *Determination of the structure of the* BnCHK *genes by cDNA cloning confirmed bioinformatic predictions*

cDNAs of all identified *BnCHK* genes were cloned by total RNA isolation from *B. napus* seedlings followed by RT–PCR (see the Materials and methods for more details). Coding sequence (CDS) predictions (Supplementary Table S4 at *JXB* online) were confirmed or corrected by sequencing the respective cDNA clones (accession nos KF621029, KF621030, KF621031, KF621032, and KF621033), followed by alignment of the cDNA sequencing output with the corresponding genomic DNA sequences and manual curation of donor and acceptor splice sites. Numbers, positions, and lengths of exons and introns in the genes, together with 5'- and 3'-untranslated regions (UTRs), where identified, are schematically presented in Fig. 1. Both *BnCHK1* and *BnCHK2* have 13 exons (of almost identical sizes) and 12 introns. *BnCHK3* and *BnCHK4* are also very similar, both having 11 exons and 10 introns. However, the 11th exon of *BnCHK3* is much shorter (70 bp) than the corresponding exon of *BnCHK4* (214 bp) due to single nucleotide polymorphism (SNP; G for A substitution) at position 3225 in the *BnCHK3* CDS (corresponding to position 3231 in the *BnCHK4* CDS), introducing a 'premature' termination TGA codon in *BnCHK3*. The CDS close to the 3' end and the 3'-UTR of *BnCHK4* is otherwise almost identical to the genomic sequence flanking the 3' end of *BnCHK3* (not shown). Complete CDS regions of both couples of homologous HKs *BnCHK1*/*BnCHK2* and *BnCHK3*/*BnCHK4* share a high level of identity (97.7% and 96.6%, respectively) (Supplementary Fig. S4 at *JXB* online). The cDNA of *BnCHK5* is shorter than those of any of the other four *BnCHK* genes. It contains only 10 exons and nine introns, and the ninth exon is significantly longer than the other exons of this gene. The pairwise identities of aligned cDNA regions corresponding to the conserved CHASE, HK and REC domains are also presented in Supplementary Fig. S4.

In summary, five distinct genomic sequences of putative *BnCHK* genes, each representing one of the defined groups of BAC clones, carrying overlapping inserts of genomic DNA and encoding putative homologues of *Arabidopsis* CK receptors, have been identified. The five genes form two highly similar pairs BnCHK1/BnCHK2 and BnCHK3/BnCHK4 and one distinct sequence of BnCHK5.

**Table 1.** *List of sequenced JBnB clones and assigned* BnCHK *genes, identified within their inserts*

| BAC identification | | Genomic DNA sequencing | | Predicted (FGENESH) | | | Cloned | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Clone ID | Group | Method | No. of bp | No. of exons | CDS length (bp) | Protein length (no. of amino acids) | No. of exons | CDS length (bp) | Protein length (no. of amino acids) | Name |
| JBnB002E4 | I | NGS | 13 944 | 11 | 3225 | 1074 | 11 | 3225 | 1074 | BnCHK3 |
| JBnB025B19 | II | Primer walking | 9767 | 11 | 3375 | 1124 | 11 | 3375 | 1124 | BnCHK4 |
| JBnB162F14 | III | primer walking | 8819 | 10 | 3054 | 1017 | 10 | 3054 | 1017 | BnCHK5 |
| JBnB041A21 | IV | Primer walking | 9776 | 14 | 3,450 | 1197 | 13 | 3450 | 1149 | BnCHK2 |
| JBnB047D2 | V | Primer walking | 9013 | 13 | 3,450 | 1149 | 13 | 3450 | 1149 | BnCHK1 |

### *Multiple alignments of BnCHK proteins revealed modular architecture typical of cytokinin receptors*

Reading frames of the cloned *BnCHK* genes were subjected to computational analysis (see the Materials and methods) to examine the modular architecture of the encoded BnCHK proteins. All five proteins have three or four TM domains (Supplementary Fig. S6 at *JXB* online) and three basic domains of conserved tertiary structure: CHASE, HK, and REC domains (Fig. 2).

Multiple sequence alignments of BnCHK and AHK proteins revealed high homology and determined consensual amino acid motifs at conserved positions within the identified domains (Fig. 3). Within the CHASE domain (Anantharaman and Aravind, 2001; Mougel and Zhulin, 2001), there are four amino acid positions at which mutations reportedly cause a complete loss of function (Mähönen *et al.*, 2000), or either complete loss or decrease of CK binding to the AHK4 CHASE domain, and thus are essential for CK sensing (Heyl *et al.*, 2007; Hothorn *et al.*, 2011). With respect to amino acid numbering in the longer AHK4 isoform CRE1b (Q9C5U0-1), these are Trp244, Asp285, Thr301, Phe304, and Thr317. It was found that all these amino acids are conserved in all the identified BnCHKs (Fig. 3A).

Furthermore, each BnCHK possesses an H, N, G1, F, G2 motif within the HK domain (Fig. 3B) and a DD, D, K motif within the REC domain (Fig. 3C) (phosphorelay-mediating histidine and aspartate are underlined). Both of these motifs are characteristic of canonical HKs (West and Stock, 2001). More detailed analysis of the BnCHK protein sequences by the SMART program (Schultz *et al.*, 2000) defined two separate modules within the HK domain: an HK dimerization and phosphoacceptor domain (HisKA) and an HK catalytic domain, called the HK-like ATPase domain (HATPase) (Fig. 2).

CHASE, HK, and REC domains were predicted with high confidence for all proteins except BnCHK3, for which the reliability of the REC domain's prediction was rather low (Table 2). This may reflect the shorter terminal exon of *BnCHK3* described above and although the REC domain of BnCHK3 contains the complete DD, D, K AA motif, it may eventually prove to be a paralogue with a changed or limited HK function.

The amino acid region from the HK to REC domain, previously called the REC-like domain in CK-recognizing AHKs (Ueguchi *et al.*, 2001; Heyl and Schmülling, 2003), was also examined. Prediction programs defined this amino acid region of all BnCHKs as a REC domain with significantly higher than threshold e-values (Table 2). BnCHK2, 3, and 4 all have DS, D, K motifs within this region, while BnCHK1 and BnCHK5 contain DT, D, K and DH, E, K motifs, respectively (Figs 2, 3C). Notably, the e-value was lowest for the REC-like domain of BnCHK2 (Table 2).

TM domains of the BnCHK proteins deserve closer attention. Seven different programs were used to predict α-helixes in BnCHKs (and in AHKs in comparison) potentially spanning through the plasma membrane and flanking the CHASE domain (Supplementary Table S5, Fig. S6 at *JXB* online).

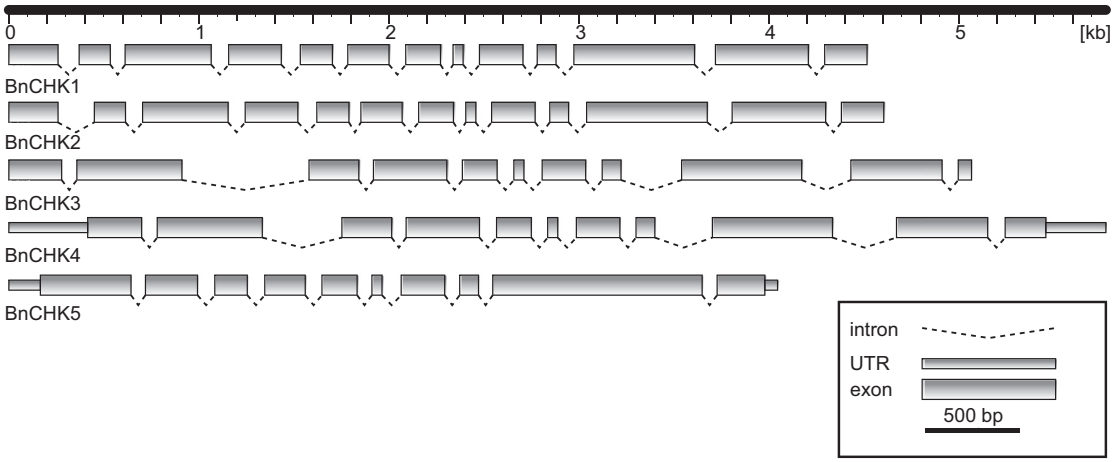


**Fig. 1.** Schematic structures of the five identified *BnCHK* genes, based on comparison of cloned and sequenced genomic DNA and cDNA sequences. See also Supplementary Fig. S5 at *JXB* online.

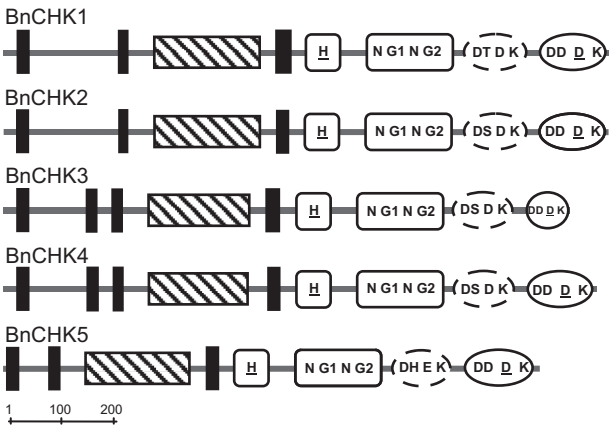


**Fig. 2.** Domain structure of the five identified *B. napus* CHASE-containing hybrid sensor His kinases. Transmembrane domain (filled rectangle), CHASE domain (striped rectangle), His kinase domain, consisting of HisKA (H) and HATPase (N G1 N G2) modules (open rectangle), putative receiver-like domain (open dotted oval), and receiver domain (open oval).

This analysis again defines two AHK2-like BnCHK pairs: BnCHK1/BnCHK2 and BnCHK3/BnCHK4. Interestingly, these two pairs differ in the putative TM domain topology. All used programs reliably identified three TM segments in BnCHK1 and BnCHK2, which resembles AHK2 (with respect to both the number and the relative position of putative TM segments). In contrast, BnCHK3 and BnCHK4 have four predicted TM domains, with very high probability of forming TM segments at three putative locations upstream of the CHASE domain. BnCHK5 strictly resembles AHK3, again in both the number and relative position of predicted TM domains (Supplementary Table S5, Fig. S6).

### *Designation of annotated* B. rapa *CHASE-containing His kinase genes and* in silico *identification of EST sequences homologous to* BnCHK *genes*

To incorporate CK receptors of *B. rapa*, contributing to the A-part of the allotetraploid genome of *B. napus* (not available when this study was begun) in the phylogenetic analyses (see the next section), sequences of all (four) CHASE-containing sensor HK genes of *B. rapa* listed in the http://brassicadb.org/brad/geneFamily.php?fam=Histidine%20Kinase database, which compiles all sequences of the *B. rapa* genome released and annotated to date (Wang *et al.*, 2011; http://www.gramene.org/genome_browser/index.html), were acquired. Based on the information in the *B. rapa* database and the present alignment with sequences of *Arabidopsis* CK receptors (see below), the listed AHK2-related receptor genes were designated as BrCHK1 (Bra035381) and BrCHK2 (Bra013186), and those related to AHK3 and AHK4 as BrCHK3 (Bra030037) and BrCHK4 (Bra024849), respectively (Table 3).

After identifying the *BnCHK* genes, a BLAST search of expressed sequence tag (EST) databases was performed, using the *BnCHK* genes and *BrCHK4* as queries. A total of 31 significant hits among the identified EST sequences were obtained, and are listed in Supplementary Table S6 at *JXB* online. All the EST sequences show significant similarity to the *BnCHK* and *BrCHK4* genes and are expressed in various plant tissues under various conditions (Suplementary Table S6). It is difficult to assign the identified ESTs to the respective *BnCHK* genes, because of the high similarity among *BnCHK* genes (see Supplementary Fig. S4) and numerous SNPs in *BnCHK* orthologues of different varieties compiled in the EST databases. However, links can be at least partially deduced from the score values (Supplementary Table S6). For instance, the EST yielding the highest BLAST score for *BrCHK4*, homologous to *AHK4*, was ES900704 from *B. napus*. This EST represents the HK domain and shares the same contig (#5, Supplementaary Table S6) as ES904730, the only identified *B. napus* EST carrying the CHASE domain. This indicates that ES900704 may represent a CHASE-containing HK homologous to AHK4. However, the cloning of genuine *BnCHK* homologous to AHK4 and its detailed sequence analysis remains to be done.

### *Phylogenetic analysis of the isolated* BnCHK *genes identified four homologues of* AHK2 *and one homologue of* AHK3

Phylogenetic analysis was performed primarily to distinguish between *BnCHK* genes related to *AHK2*, *AHK3*, and *AHK4*. However, not only well-studied sequences from *Arabidopsis*,

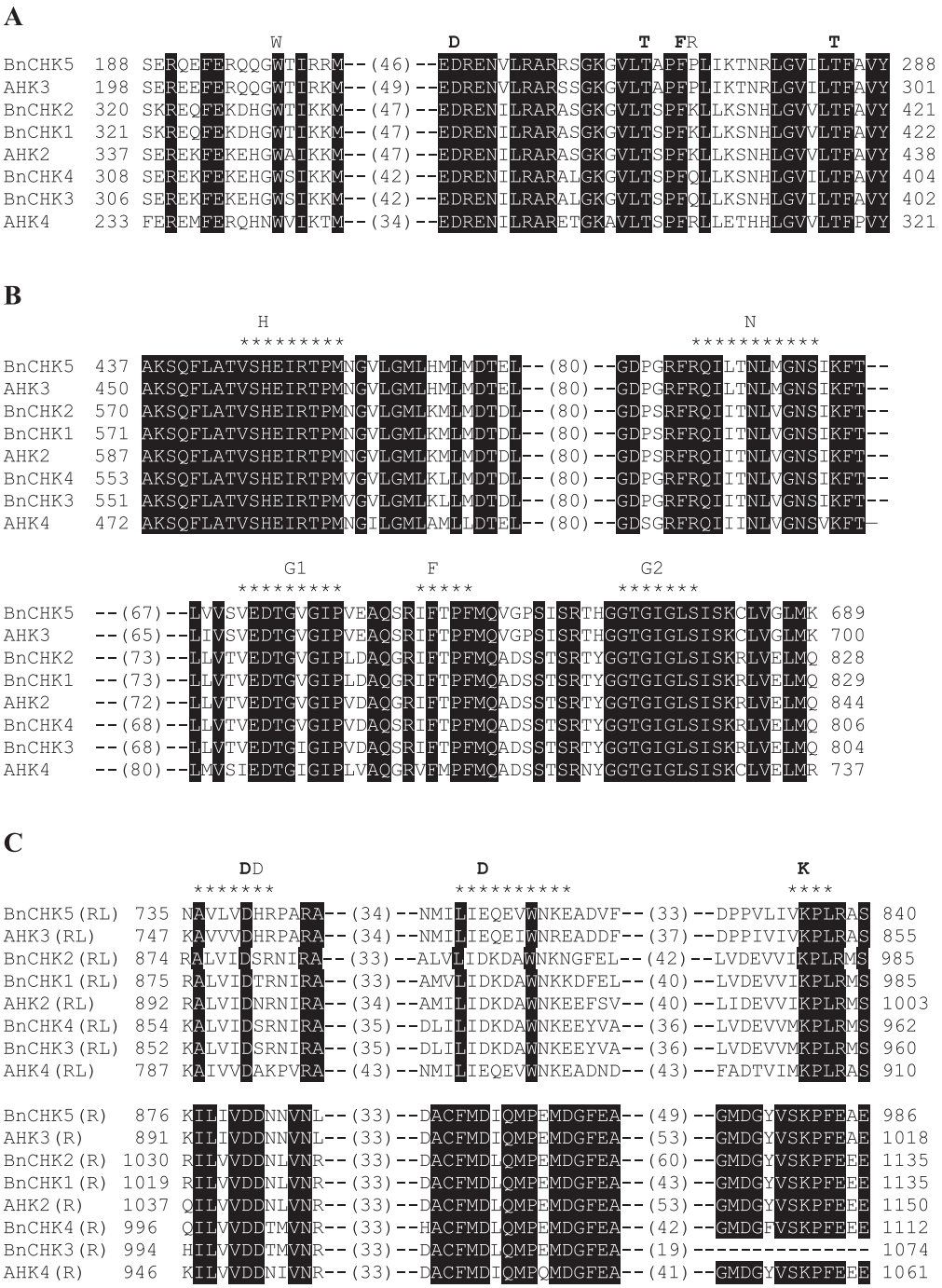


**Fig. 3.** Consensus amino acid motifs within conserved domains of the five identified *B. napus* CHASE-containing hybrid His kinases, based on multiple alignment with CHASE (A), His kinase (B), receiver-like (RL), and receiver (R) (C) domain sequences of related AHKs by ClustalW (Ueguchi *et al.*, 2001).

but also well-classified sequences from rice representing monocotyledonous CK receptors (Choi *et al.*, 2012; Tsai *et al.*, 2012; Heyl *et al.*, 2013), the four recently annotated sequences encoding putative CK receptors in *B. rapa* and designated here as BrCHK1–BrCHK4, two EST sequences of *B. napus* homologous to *BrCHK4*, and one EST sequence of *B. oleracea* (see the previous section) were incorporated.

First, phylogenetic analysis was performed based on alignments of conserved HK domains. The alignment report, displaying all the sequences used in the analysis, is presented in Supplementary Fig. S7 at *JXB* online. Based on the analysis and constructed phylogenetic tree (Fig. 4), it was determined that the five identified *BnCHK* genes include four *AHK2* homologues (*BnCHK1*, *2*, *3*, and *4*) and one *AHK3* homologue (*BnCHK5*). The ES900704 sequence represents a *B. napus* homologue of AHK4. Amino acid identities for putative HK domains of BnCHK1/BnCHK2 and BnCHK3/BnCHK4 pairs are 99.4% and 98.9%, respectively, pointing to very high similarity between these pairs (see the distance matrix in Supplementary Fig. S7D). Furthermore, pairwise amino acid identities between sequences of each of the BnCHK1/BrCHK1, BnCHK3/BrCHK2, BnCHK5/BrCHK3, and

**Table 2.** *Probabilities, expressed by e-values, of conserved protein domains identified in BnCHK sequences by SMART (http://smart.embl.de)*

| Gene | CHASE | HisKA | HATPase | REC-like | REC |
|---|---|---|---|---|---|
| BnCHK1 | 2.17e-29 | 1.86e-22 | 1.91e-33 | 0.363 | 1.24e-33 |
| BnCHK2 | 1.46e-30 | 1.86e-22 | 1.1e-33 | 0.0723 | 8.76e-34 |
| BnCHK3 | 1.25e-29 | 1.19e-20 | 6.17e-37 | 0.23 | 0.00549 |
| BnCHK4 | 1.25e-29 | 1.19e-20 | 4.67e-37 | 0.243 | 1.54e-29 |
| BnCHK5 | 3.34e-30 | 7.55e-23 | 4.79e-34 | 91.1 | 1.38e-31 |

**Table 3.** *Database accessions of* BnCHK *genes identified by screening the JBnB library, and* BrCHK *genes listed in the http://www.gramene.org/genome_browser/index.html, http://brassicadb.org/brad/geneFamily.php?fam=Histidine%20Kinase database*

| Gene name | *AHK* homologue | Gene ID | Transcript ID | Protein ID |
|---|---|---|---|---|
| *BnCHK1* | *AHK2* | KF621024 | KF621029 | |
| *BnCHK2* | *AHK2* | KF621025 | KF621030 | |
| *BnCHK3* | *AHK2* | KF621028 | KF621031 | |
| *BnCHK4* | *AHK2* | KF621026 | KF621032 | |
| *BnCHK5* | *AHK3* | KF621027 | KF621033 | |
| *BrCHK1* (s) | *AHK2* | Bra035381 | Bra035381.1 | Bra035381.1-P |
| *BrCHK2* | *AHK2* | Bra013186 | Bra013186.1 | Bra013186.1-P |
| *BrCHK3* (s) | *AHK3* | Bra030037 | Bra030037.1 | Bra030037.1-P |
| *BrCHK4* (s) | *AHK4* | Bra024849 | Bra024849.1 | Bra024849.1-P |

s, mapped syntenic to the *AHK* chromosome location; syntenic orthologue of *AHK*.

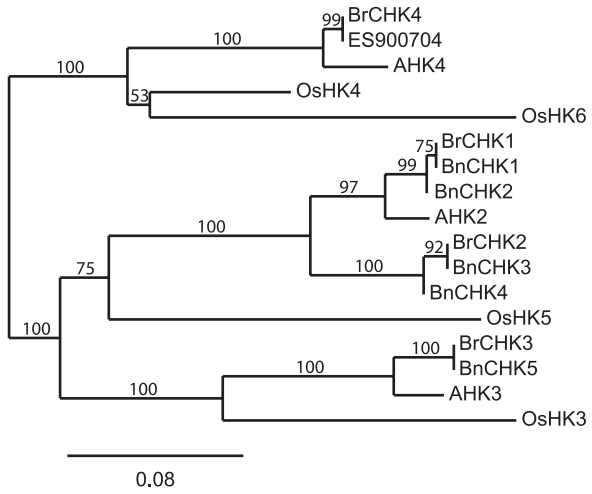


**Fig. 4.** Phylogenetic relationships of CHASE-containing His kinases from *Brassica napus* (BnCHKs—for gene IDs see Table 3—and gi|150870246|gb|ES900704), *Brassica rapa* (BrCHKs—for gene IDs see Table 3), *Arabidopsis* (AHKs—At1g27320, At2g01830, and At5g35750), and rice (OsHKs—Os01g69920, Os02g50480, Os03g50860, and Os10g21810). Alignments are based on His kinase domains.

ES900704/BrCHK4 pairs are 100%, indicating that the corresponding *B. napus* and *B. rapa* CHKs are very closely related.

A multiple sequence alignment of conserved CHASE domains (Supplementary Fig. S8 at *JXB* online) was also performed, in which two more EST hits from the database search described in the previous section were included: ES904730 from *B. napus* and asmbl48579 from *B. oleracea*, both representing novel putative CHASE domains, as predicted by SMART (not shown) (Supplementary Table S6). Here, three groups with 100% amino acid identity have been defined: (i) BnCHK1/BrCHK1; (ii) BnCHK3/BnCHK4/BrCHK2/ES904730/asmbl48579; and (iii) BrCHK3/BnCHK5 (Supplementary Fig. S8D), suggesting that the CHASE domains within each group might have similar CK recognition characteristics (Supplementary Fig. S8E). Notably, BnCHK3 has the same CHASE domain as BnCHK4, but both proteins differ in their HK domains (see above). The CHASE alignment further confirms the conclusion from the HK alignment that BrCHK1 and BrCHK5 are homologues of AHK2 and AHK3, respectively (Supplementary Fig. S8E).

Taken together, the phylogenetic analyses indicate that most of the identified *BnCHK* genes are homologues of *AHK2* and *AHK3*, but at least one homologue of *AHK4*, missed by the sequence hybridization screen, also seems to be present in the *B. napus* genome.

### *Functional analysis in* E. coli *reveals different CK binding characteristics of BnCHK1, BnCHK3, and BnCHK5*

To confirm that the cloned BnCHK protein-encoding genes with conserved CHASE domains can function as genuine CK receptors and specifically bind CKs, functional analyses was performed. BnCHK1 and BnCHK3, as representatives of the two groups of the identified BnCHKs homologous to AHK2, and BnCHK5 homologous to AHK3 were tested for their ligand specificity and affinity using an *E. coli*-based direct binding assay (Romanov *et al.*, 2005). First, the CK-binding capacity of the proteins was determined in a dose-dependent assay, using a range of concentrations of tritium-labelled

*trans*-zeatin ([$^3$H]tZ), followed by Scatchard analysis. As shown in Fig. 5A, in all cases classical saturation curves were obtained. Analysis of the data gave apparent affinity constants ($K_d$) in nanomolar orders, indicating high affinity binding typical of hormone receptors. Estimated $K_d$ values for BnCHK1, BnCHK3, and BnCHK5 were 2.9 ± 1.8, 1.9 ± 0.4, and 1.5 ± 0.8 nM, respectively. These values correspond well to dissociation constants published for *Arabidopsis* and maize receptors (Romanov *et al*., 2005, 2006; Lomin *et al*, 2011; Stolz *et al.*, 2011). The capacity of representatives of various other low molecular weight phytohormones to compete with [$^3$H]tZ for binding in the assays, including auxin (IAA), gibberellin ($GA_3$), abscisic acid (ABA), and adenine (Ade) was also tested (Fig. 5B). All three BnCHKs showed high specificity for the CK: unlabelled tZ (but not IAA, $GA_3$, ABA, or Ade) effectively reduced binding of [$^3$H]tZ (Fig. 5B). Based on these findings, it is concluded that BnCHK1, BnCHK3, and BnCHK5 can specifically recognize CKs.

As a next step, the ligand specificities of all three receptors were investigated in a series of binding experiments with several isoprenoid and aromatic Ade-type CK bases and the synthetic phenylurea-derived CK thidiazuron (TDZ). The apparent affinity constants of the active compounds obtained ranged from 6.6 nM to 5.5 µM (Table 4). The strongest recognized CK for all receptors was tZ, followed by TDZ (Fig. 5D; Table 4). All BnCHKs had much weaker affinity for the *cis*-isoform of zeatin (cZ), dihydrozeatin (DHZ), and kinetin (Kin), and bound the non-substituted aromatic CK $N^6$-benzyladenine (BA) only weakly. Notably, by far the weakest affinity for kinetin was observed for BnCHK3. On the other hand, BnCHK3 had the highest affinity of all the tested BnCHKs for the aromatic CK BA, which was almost not recognized by BnCHK5 (Fig. 5C, D; Table 4). Interestingly, the hydroxylated BA, *meta*-topolin (mT), was effectively recognized, again particularly by BnCHK3 ($K_d$=45 nM) but also by BnCHK1 and BnCHK5 ($K_d$ ~100 nM). This indicates that the presence of the OH-group at the *meta*-position of the aromatic side chain (a feature resembling tZ) is important for binding to the receptor. However, the most striking difference in the ligand specificity of the BnCHK receptors was in the binding of $N^6$-isopenteyladenine (iP), which was strongly recognized by BnCHK1 and BnCHK3 ($K_d$ only 8-fold and 12-fold higher than that for tZ, respectively), but not by BnCHK5 ($K_d$ 90-fold higher than that for tZ; Fig. 5C; Table 4). No significant differences were found in the binding capacities of BnCHKs for various CK metabolites, with the only exception of tZ riboside (tZR) (Fig. 5E; Table 4). While BnCHK1 and BnCHK5 showed very high affinity for tZR with similar $K_d$ values to those of TDZ (Table 4), BnCHK3 sensed tZR significantly less well (Fig. 5E; Table 4). As expected, the other tested tZ metabolites ($N^9$-glucoside, $N^7$-glucoside, and *O*-glucoside) were not effectively recognized by either receptor, indicating that modifications of the CK core structure negatively influence binding to the receptor active site. In summary, the tested BnCHKs reveal specificity in terms of their ability to recognize individual CK types and their metabolites. The order of CK preferences obtained by comparison of the relative binding affinities is tZ>TDZ>tZR>iP>mT>DHZ>cZ>BA~Kin>>Ade~tZ7G~tZ9G~tZOG for BnCHK1; tZ>TDZ>iP>tZR>mT>DHZ>cZ~BA>Kin>>Ade~tZ7G~tZ9G~tZOG for BnCHK3; and tZ>tZR~TDZ>mT~DHZ>iP>cZ>Kin>BA>>Ade~tZ7G~tZ9G~tZOG for BnCHK5.

## Discussion

### *Most of the identified BnCHKs are homologous to AHK2 and originate from both* B. oleracea *and* B. rapa *genomes*

Phylogenetic analysis groups the *Brassica* species into two, *nigra* and *rapa*/*oleracea*, lineages (Warwick and Black, 1991), which apparently diverged ~8 million years agio (Lysak *et al.*, 2005), while *B. rapa* and *B. oleracea* diverged ~4 million years ago (Inaba and Nishio, 2002). The *B. napus* genome seems to have resulted from the hybridization of *B. rapa* (A) and *B. oleracea* (C) genomes ~10 000 years ago (Nagaharu, 1935).

The phylogenetic analysis presented here is based on multiple alignment of amino acid sequences corresponding to the conserved HK domains from *Arabidopsis*, *B. napus*, *B. rapa*, and rice, and its outcome is consistent with the present gene and protein domain structure findings (see also next section): two pairs of very closely related *BnCHK* genes, *BnCHK1*/*BnCHK2* and *BnCHK3*/*BnCHK4*, are homologues of *AHK2*, while the structurally more distinct *BnCHK5* is a homologue of *AHK3*. As there is 100% amino acid identity between aligned conserved HK domains within the BnCHK1/BrCHK1, BnCHK3/BrCHK2, and BnCHK5/BrCHK3 pairs (Fig. 4; Supplementary Fig. S7D at *JXB* online), it is concluded that *BnCHK1*, *BnCHK3*, and *BnCHK5* originated from the *B. rapa* A genome. As *BrCHK1* and *BrCHK3* have been mapped to syntenic chromosomal regions of *AHK2* and *AHK3*, respectively (http://brassicadb.org/brad/geneFamily.php?fam=Histidine%20Kinase) (Table 3), it is concluded that *BnCHK1* and *BnCHK5* are orthologues of *AHK2* and *AHK3*, respectively. The lower similarity of BrCHK1 to BnCHK2 (99.4%) and BrCHK2 to BnCHK4 (98.9%), respectively, indicates that *BnCHK2* and *BnCHK4* may be inter-homoeologues that originated from the *B. oleracea* C genome.

Comparative physical mapping of the recently sequenced and annotated *B. rapa* genome (Wang *et al*., 2011; http://www.gramene.org/genome_browser/index.html) has indicated that it underwent a whole-genome triplication after divergence of the *Arabidopsis* and *Brassica* lineages (Lysak *et al.*, 2005; Wang *et al*., 2011). The reported final number of identified *B. rapa* protein-coding genes is 41 174, lower than theoretically expected for the triplicated genome (90 000), confirming previous observations of substantial gene losses, which typically occur following polyploidization events (Town *et al.*, 2006). The finding of four predicted *BrCHK* genes, out of a theoretical nine (in analogy to three *AHK* genes), is consistent with this phenomenon. Thus, the number of *BnCHK* genes identified might be close to the true number, particularly the number of identified *BnCHK* homologues of *AHK2*. However, the experimental approach used here will have missed putative *B. napus* orthologues of *AHK4*, as indicated by the position of the ES900704 EST sequence of another *B. napus* line in the

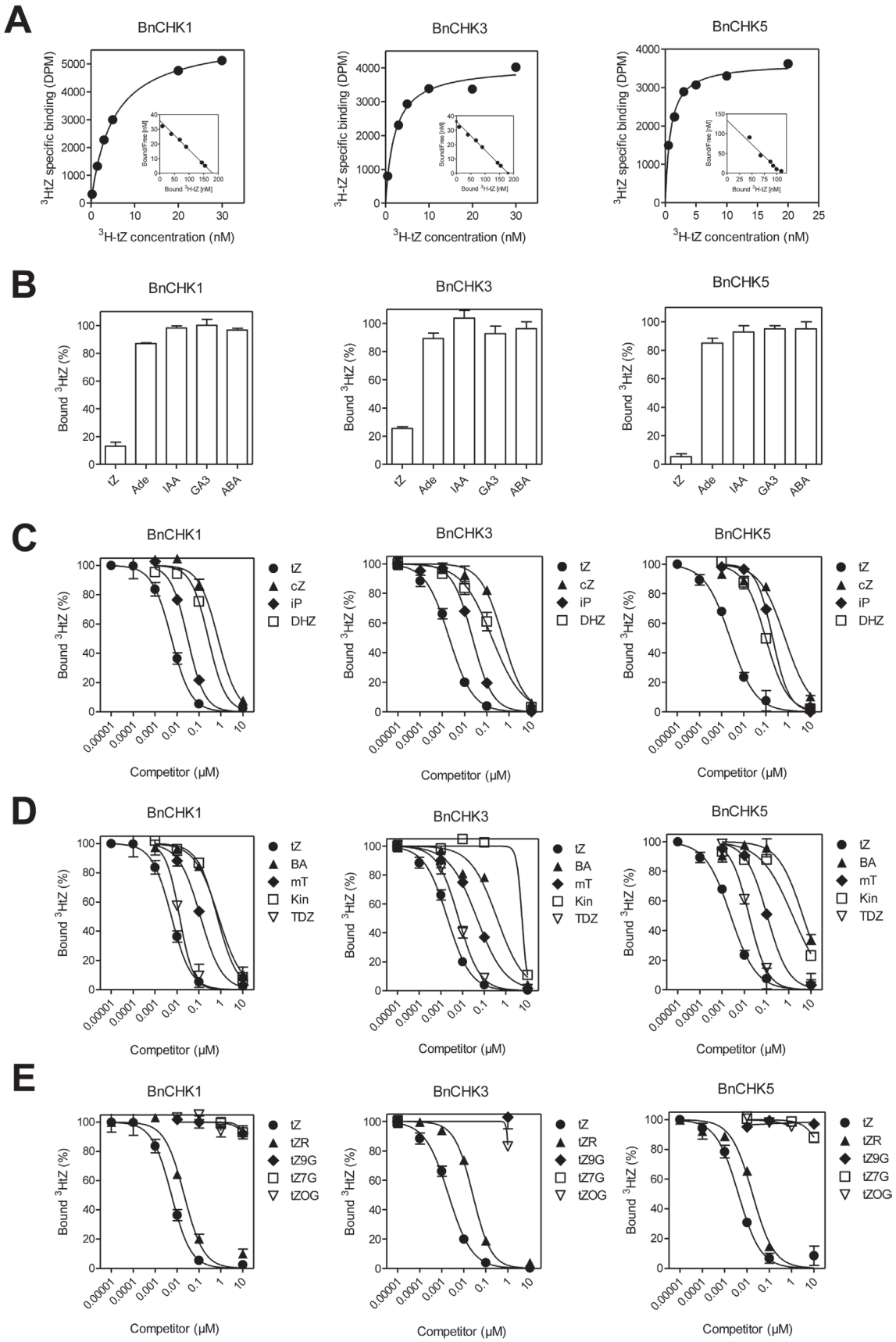


**Fig. 5.** Dose-dependent binding of [$^3$H]tZ to cytokinin (CK) receptor-expressing *E. coli* clones. Original data from specific binding and Scatchard plots (insets) are shown (A). Competition by non-CK compounds (B), CK metabolites (C, D), and various tZ derivatives (E) with [$^3$H]tZ for binding to the CK receptor-expressing *E. coli* clones. The bound radioactivity corresponding to 100% was 3338, 2383, and 4076 dpm in the case of binding experiments with BnCHK1, BnCHK3, and BnCHK5, respectively.

**Table 4.** Comparison of the cytokinin affinity of BnCHK1, BnCHK3, and BnCHK5

The apparent $K_d$ values were calculated as ligand concentrations that displaced 50% of the bound [$^3$H]tZ (means and standard deviations obtained from two independent experiments, with three technical replicates per experiment).

| Cytokinin | Abbreviation | Apparent $K_d$ (nM) | | |
|---|---|---|---|---|
| | | BnCHK1 | BnCHK3 | BnCHK5 |
| *trans*-Zeatin | tZ | 4.6±1.9 | 2.1±0.5 | 2.42±0.3 |
| *trans*-Zeatin riboside | tZR | 17.1±1.6 | 27.1±1.4 | 13.5±1.0 |
| *trans*-Zeatin-7-glucoside | tZ7G | >10 000 | >10 000 | >10 000 |
| *trans*-Zeatin-9-glucoside | tZ9G | >10 000 | >10 000 | >10 000 |
| *trans*-Zeatin-*O*-glucoside | tZOG | >10 000 | >10 000 | >10 000 |
| *cis*-Zeatin | cZ | 662±85 | 440±110 | 694±28 |
| Dihydrozeatin | DHZ | 382±70 | 174±5 | 103±5.1 |
| $N^6$-Isopenteyladenine | iP | 35.3±4.6 | 22.8±0.5 | 219±18 |
| $N^6$-Benzyladenine | BA | 855±77 | 440±70 | 4794±382 |
| *meta*-Tpoline | mT | 119±1.5 | 44.7±2.0 | 97.0±13 |
| Kinetin | Kin | 895±205 | 5456±79 | 1695±195 |
| Thidiazuron | TDZ | 11.1±1.9 | 6.6±0.1 | 13.9±2.4 |
| Adenine | Ade | >10 000 | >10 000 | >10 000 |

phylogram (Fig. 4). The possibility cannot be excluded that the 1–2% difference in nucleotide identity between the *AHK2* and *AHK4* probes (Supplementary Fig. S1 at *JXB* online) may have contributed to a lower $T_m$ of the hybrid molecules between the *AHK2* probe and potential *BnCHK* homologues of *AHK4* during the hybridization experiment, explaining why *BnCHK* homologues of *AHK4* were not retrieved. Further systematic analyses of the completely sequenced *B. napus* and *B. oleracea* genomes, when released, are required to determine definitively whether or not there is more than one *BnCHK* homologue of *AHK3* and *AHK4*.

The characterization of the first five *BnCHK* sequences presented here also confirms previous findings from comparative analyses of genome segments from *B. rapa*, *B. oleracea*, *B. napus*, and *A. thaliana* (O'Neill and Bancroft, 2000; Rana *et al.*, 2004) and comparative analysis of rice and *Arabidopsis* (Liu *et al.*, 2001). Although extensive divergence of gene contents was observed in the studied species, the examined genes showed highly conserved collinearity with their putative orthologues. This high degree of conservation is further corroborated by the detection of very few, or no, SNPs at the nucleotide level between aligned CDS pairs of *BnCHK1*/*BrCHK1* (100% identity), *BnCHK5*/*BrCHK3* (99.7% identity), and *ES900704*/*BrCHK4* (99.9% identity) (not shown), and reflects the recent polyploidization event of *B. rapa* and *B. oleracea* genomes ~10 000 years ago (Nagaharu, 1935).

### *Predicted conserved protein domains of the encoded BnCHK proteins reveal conserved motifs of two pairs of closely related CK receptors and another more distinct putative CK receptor*

All five identified BnCHKs contain the typical conserved functional domains and motifs of CK receptors in *Arabidopsis*: CHASE, HK, REC-like, and REC domains. All five CHASE domains contain identical amino acids at conserved positions that are reportedly crucial for CK binding and signalling of AHK4 (Mähönen *et al.*, 2000; Heyl *et al.*, 2007; Hothorn *et al.*, 2011). In addition, all five HK domains contain the conserved H, N, G1, F, G2 motif and all five REC domains contain the DD, D, K motif, characteristic of canonical HKs (West and Stock, 2001). The most pronounced difference in the conserved structure of functional domains among the BnCHKs is in the REC-like domains. BnCHK1, 2, 3, and 4 have highly similar amino acid motifs within their REC-like domains, with the typical DD, D, K motif of a regular REC domain, as they only have single amino acid substitutions at the second position of the motifs (threonine in DT, D, K of BnCHK1 and serine in DS, D, K, of BnCHK2, 3, and 4, both amino acids with polar, uncharged side chains, Figs 2, 3C, variable residues underlined). In contrast, BnCHK5 displays two substitutions in its REC-like motif: a positively charged histidine at the variable second position and a negatively charged glutamate replacing the conserved aspartate at the third position (DH, E, K, Figs 2, 3C). The latter could be functionally significant, as aspartate to glutamate mutation reportedly abolishes phosphorylation of the REC domain in the *Arabidopsis* response regulator ARR2 (Hass *et al.*, 2004). However, at the moment it can only be speculated whether the REC-like domains of BnCHKs meet the requirements of potentially functional cytoplasmic modules and their specific catalytic functions. Nevertheless, compared with the REC-like amino acid motifs of AHKs, BnCHK1, 2, 3, and 4 resemble AHK2, containing a polar uncharged asparagine at the second position of its DN, D, K motif, while the DH, E, K motif of the REC-like domain of BnCHK5 is identical to the same motif of the REC-like domain of AHK3. This again is in good agreement with the observed similarity between *BnCHK* and *AHK* genes at the nucleotide level and further implies that BnCHK1 and BnCHK5 could be orthologous to AHK2 and AHK3, respectively.

### *BnCHK1, BnCHK3, and BnCHK5 proteins specifically bind CKs and differ in their ligand specificity*

As mentioned above, all five identified BnCHKs contain identical amino acids at conserved positions of their CHASE domains that are known to be crucial for receptor function. These include: Asp285, which forms hydrogen bonds with the adenine ring; Thr301, which restricts the overall size of the binding pocket; and Thr317, which contributes to high-affinity recognition of tZ (Hothorn *et al.*, 2011) (numbering according to the longer AHK4 isoform CRE1b, Q9C5U0-1, used in this study). Results of the functional direct binding assay presented here confirm the predictions that BnCHK1, BnCHK3, and BnCHK5 are CK receptors, as they indeed specifically recognize known adenine-type isoprenoid and aromatic CKs, as well as urea-derived TDZ, with binding affinities similar to those of other, previously described CK-binding HKs (Fig. 5). $K_d$ values of ~1–3 nM obtained in this study with the most active natural CK, tZ, are consistent with reported values for CRE1/AHK4 [2.5 nM, Romanov *et al.* (2005); 3.9 nM, Romanov *et al.* (2006); 4.4 nM, Stolz *et al.* (2011)]. Both receptors also effectively bind tZR, whereas other metabolites, including *O*- and *N*-glucosides, are not recognized (Tab. 4).

CK receptors in *Arabidopsis* form two subgroups in terms of their specificity towards individual CK types. The subgroup formed by AHK2/AHK4 recognizes iP with high affinity; in the case of AHK2, even comparably with tZ. In contrast, AHK3 reveals affinity for tZ similar to that of AHK2/AHK4, but iP is recognized much less efficiently (Romanov *et al.*, 2006; Stolz *et al.*, 2011). The quantitative binding data obtained in this study support the presented phylogenetic data, which suggest that *BnCHK1* and *BnCHK3* are orthologous to *AHK2*, and *BnCHK5* to *AHK3*. Both BnCHK1 and BnCHK3 have ~6.5- and 9.5-fold, respectively, higher affinity for iP than BnCHK5 (Table 4), just as AHK2 has ~10- to 100-fold higher affinity for iP than AHK3 (Romanov *et al.*, 2006; Stolz *et al.*, 2011). In the relative comparison with tZ, BnCHK1 and BnCHK3 bind iP ~8-fold and 12-fold more weakly than tZ, respectively, compared with BnCHK5, which binds iP 90-fold less well than tZ (Table 4). In comparison, AHK2 binds iP even better than tZ (Stolz *et al.*, 2011) while AHK3 recognizes iP with about 100-fold lower affinity then tZ (Romanov *et al.*, 2006). The relatively weak preference of BnCHK5 for iP versus tZ may reflect a specific role in root-to-shoot communication, similar to that of AHK3 (Romanov *et al.*, 2006). Like AHK3, BnCHK5 may therefore be tuned to respond to long-distance signals transported from the roots via the xylem (Romanov *et al.*, 2006), in which tZ is the main CK (Takei *et al.*, 2001). To verify this hypothesis, demonstration that BnCHK5 is predominantly expressed in shoots is required.

### *Two subfamilies of AHK2 orthologues in* B. napus *differ in the number of putative transmembrane regions and CK binding specificity*

AHK2 was originally shown to form two TM domains upstream of the CHASE domain (U-TM) (Ueguchi *et al.*, 2001). Some prediction programs used in this work defined another putative α-helix in this region, although with lower probability (Supplementary Fig. S6 at *JXB* online). In a more phylogenetically distant subgroup of AHK2 orthologues BnCHK3 and BnCHK4, this TM domain was identified by each of the programs used in this study and with probability comparable with that of other TM domains. This result presenting four TM cytokinin receptors, three U-TM and one downstream TM, in *B. napus* is in good agreement with recently published data. Steklov *et al.* (2013) accomplished an analysis of the number and positions of TM domains based on structures of 100 putative CK receptors from different species. In their study, Steklov *et al.* demonstrate that all CREI/AHK4 orthologues have only one U-TM domain, whereas AHK2 orthologues possess three or four TM helices in this possition. In this respect, the 2–3 U-TM domains in BnCHK1 - BnCHK4 identified in the present study could be considered as additional evidence that these HKs are genuine AHK2 orthologues.

Whether there is any functional importance of the putative structural heterogenity, however, remains unclear. AHK2 and AHK3 possessing two and AHK4 possessing one U-TM domain have been shown to be dominantly located in membranes of the endoplasmic reticulum (ER) (Caesar *et al.*, 2011; Wulfetange *et al.*, 2011). The individual AHKs of *Arabidopsis* seedlings have nevertheless been observed to be present at endomembranes or in the plasma membrane (PM) to a slightly different extent with a relatively higher outer-to-endo membrane ratio for AHK4 and a lower ratio for AHK2 (Wulfetange *et al.*, 2013). This might imply that the higher number of U-TM domains the less probable is the receptor occurrence in the outer membrane. However, the potential impact of the structure of the respective HK on its subcellular localization as well as the functional importance of both localization types (PM versus ER) remains to be shown.

The functional analyses also revealed that representatives of both groups of AHK2 orthologues are truly functional CK receptors. In maize, two splice versions of the AHK2 orthologue have been identified, termed ZmHK3a and ZmHK3b (Yonekura-Sakakibara *et al.*, 2004). Compared with ZmHK3a which responded to cytokinins, the shorter isoform ZmHK3b lacking a part of the input domain including one TM domain did not respond to any CK tested (Yonekura-Sakakibara *et al.*, 2004). In comparison, both BnCHK1 and BnCHK3 representing two subgroups of AHK2 homologues (BnCHK1 most probably being a syntenic AHK2 orthologue, and BnCHK3 being a duplicated paralogue) bind CKs and reveal binding specificity (Table 4), which is in agreement with differences in the amino acid composition of their CHASE domains Supplementary Fig. S8D at *JXB* online). The most pronounced difference is the higher affinity of BnCHK3 for tZ (the highest affinity for tZ of all BnCHKs tested). Interestingly, compared with both BnCHK1 and BnCHK5, BnCHK3 also reveals higher affinity for aromatic CKs (both BA and mT). On the other hand, BnCHK3 shows lower affinity for tZR and almost no affinity for kinetin. What the physiological meaning of the presence of these two functional versions with certain ligand preference can be and whether

different TM domain topology could contribute to that specificity remains a matter for further studies.

### *Conclusion*

Where complete sets of CK receptor genes can be identified *in silico*, because genomes have been completely sequenced, comprehensive genomic and proteomic studies can be carried out, which greatly facilitates molecular and genetic identifications of members of gene families. However, annotated transcripts and translated reading frames are bioinformatic predictions, and specific regions hosting genes of interest must be confirmed by sequencing. To the authors' knowledge, no cloned full-length *B. napus* CK receptor gene has been previously described and characterized. Here, high-quality sequences (with accompanying database annotations), gene structure determinations of five CHASE-containing HK subfamily genes, and functional ligand-binding analyses of three encoded proteins from *B. napus* are presented. These data extend our knowledge of CK receptors in dicotyledonous crops and provide foundations for more detailed studies on CK perception and signalling pathways in *B. napus* and other *Brassica* species. As CK receptors play major roles in diverse developmental and physiological processes that govern crop yields, including germination, root formation, tolerance to abiotic stress, and pathogen defences, the findings of such studies should have valuable potential applications. Furthermore, the specific sequences of several members of the gene family obtained should aid further phylogenetic studies of this evolutionarily interesting polyploid species.

## Supplementary data

Supplementary data are available from *JXB* online.

Figure S1. Calculation of percentage identity and divergence of aligned CDS of *AHK* genes or their nucleotide regions corresponding to conserved CHASE, His kinase, and receiver protein domains.

Figure S2. Grouping of overlapping BAC clones.

Figure S3. Subcloning of restriction fragments carrying putative *BnCHK* genes.

Figure S4. Calculation of percentage identity and divergence of aligned cDNAs of *BnCHK* genes or their nucleotide regions corresponding to the conserved CHASE, His kinase, and receiver protein domains.

Figure S5. Gene structure analysis of *BnCHK* genes.

Figure S6. Predicted transmembrane segments within the BnCHK amino acid sequences.

Figure S7. FASTA format, multiple alignment in Clustal format, cured alignment in Phylip format, and sequence distances of His kinase protein domains from *Brassica napus* (BnCHK and ES900704), *Brassica rapa* (BrCHK), Arabidopsis (AHK), and rice (OsHK) used in phylogenetic analysis.

Figure S8. FASTA format, multiple sequence alignment in Clustal format, cured alignment in Phylip format, sequence distances, and phylogram of CHASE protein domains from *Brassica napus* (BnCHK and ES904730), *Brassica rapa* (BrCHK), and *Brassica oleracea* (asmbl48579).

Table S1. Primers used for cDNA cloning of *BnCHK* genes.

Table S2. Four filters, each containing six fields with individual clones of the BAC library of *Brassica napus*, var. Tapidor (JBnB library), giving positive signals with DNA probes, derived from sequences of the *AHK2* gene and corresponding to the conserved CHASE, His kinase (HK), and receiver (REC) protein domains.

Table S3. Fingerprinted JBnB clones.

Table S4. Exon prediction in *BnCHK* genes by GENSCAN.

Table S5. Positions of transmembrane (TM) segments predicted in BnCHK and AHK proteins by seven different programs.

Table S6. EST database search outcome of BLASTN using CDS of *BnCHK* genes and *BrCHK4* as query.

## Acknowledgements

We would like to thank Ian Bancroft and Colin Morgan for their gift of seeds of *B. napus* var. Tapidor. We thank Jiří Fajkus, Aleš Kovařík, and Viktor Brabec for technical support during hybridization experiments, and Jakub Horák, Matej Lexa, and Martin Lysák for their consultation and advice. This work was supported by the European Regional Development Fund (Central European Institute of Technology project no. CZ.1.05/1.1.00/02.0068), the European Social Fund (CZ.1.07/2.3.00/20.0189), CZ.1.07/2.3.00/20.0043 Plant GPPS, the Czech Science Foundation (13-25280S, P305/11/0756, and 13-39982S), and the Ministry of Education Youth and Sports, Czech Republic (grant LO1204 from the National Program of Sustainability I). OP was supported by the Operational Program Education for Competitiveness-European Social Fund (project CZ.1.07/2.3.00/20.0165) and LG from project IGA PrF 2014006.

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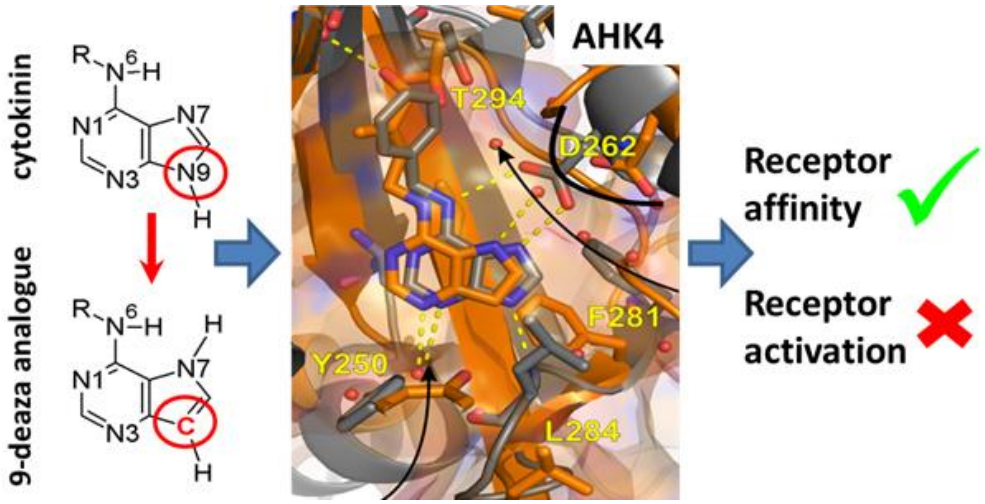
cytokinin
9-deaza analogue
AHK4
T294
D262
F281
Y250
L284
Receptor affinity
Receptor activation

**9-deaza cytokinins reveal molecular basis of cytokinin receptor ARABIDOPSIS HISTIDINE KINASE 4 signal transmission through membrane**

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**ABSTRACT**

Natural cytokinins are adenine-derived signalling molecules controlling plant growth and development. Cytokinin perception in *Arabidopsis* is facilitated via membrane-localized receptor histidine kinases (HKs). Although structural details of cytokinin binding to the receptor are known, the mechanism of receptor activation remains unclear. Computer simulations indicated the importance of hydrogen bond formation between the *N9* atom of the adenine ring and peptide backbone of the *Arabidopsis* histidine kinase 4 (AHK4) binding site. Possible involvement of this interaction in AHK4 active site structural rearrangement and its subsequent influence on receptor activation were investigated *in silico* and experimentally using new chemical tools, 9-deaza cytokinin analogues. Lack of ligand binding at *N9* position decreased affinity to AHK4 receptor and completely blocked its activation. New evidence is presented that cytokinin *N9* hydrogen bonding is an indispensable step in the mechanism of AHK4 activation that likely involves destabilization of the dimerization interface of the receptor.

**HIGHLIGHTS**

1. 9-deaza cytokinin analogues bind but do not activate AHK4 receptor.
2. Ligand-receptor interaction through N9 is essential for AHK4 activation.
3. Domain movement plays a role in cytokinin activation of the AHK4 signalling pathway.
4. AHK4 activation seems to involve destabilization of the dimerization interface.

**KEYWORDS**

**Cytokinin, Arabidopsis histidine kinase 4, receptor activation, 9-deaza cytokinin analogues**

## 1.1 INTRODUCTION

Hormones in general are signal molecules responsible for the coordination of growth and development of multicellular organisms. They mediate communication between individual cells as well as at an organ and tissue level (Davies, 1995). Plant hormones are small molecules produced in plant secondary metabolism that affect similar processes through non-redundant signalling pathways (Jaillais and Chory, 2010). One of the major classes of plant hormones, cytokinins (CKs), includes structurally diverse and biologically universal compounds that regulate numerous aspects of plant growth and development (Mok and Mok, 2001; Werner and Schmuelling, 2009).. For example, CKs influence cell division, promotion of seed germination, organogenesis and shoot initiation, *de novo* bud formation, phyllotaxis, apical dominance, lateral root number, root elongation, nutrient acquisition, chloroplast formation and delay of senescence, sink/source relationships and also vascular, gametophyte and photomorphogenic development (Kieber and Schaller, 2014; Osugi and Sakakibara, 2015). Chemically, CKs represent natural or synthetic structures derived from adenine or phenylurea. Natural CKs can be classified according to the arrangement of their $N^6$-side chain as isoprenoid or aromatic compounds (Mok and Mok, 2001).

CKs transduce a signal via a complex two-component system (TCS; Werner and Schmuelling, 2009). Two-component proteins occur widely in most eubacteria, but multicellular eukaryotes have representatives only in plants. The architecture of a prototypical TCS is modular according to the particular regulatory needs of the various signalling systems (West and Stock, 2001). TCS signalling pathways are usually based on transfer of a phosphate group from a particularly conserved kinase core to a receiver domain. However, instead of the TCS multi-step phosphorelay system typical for eubacteria, plants use a more complex pathway for multiplication of phosphotransfer events. It contains a hybrid histidine kinase (HK) with an additional receiver and phosphotransfer domain, which can either be linked to a HK or is completely independent (Gao and Stock, 2009).

In *Arabidopsis thaliana*, three CK sensor HKs, i.e. AHK2, AHK3 and AHK4, have been identified (Ueguchi et al., 2001a; Ueguchi et al., 2001b). Perception of CKs is realized by an N-terminally localized extracellular CHASE (cyclase/histidine kinases associated sensory extracellular) domain, which occurs between transmembrane regions in a HK (Anantharaman and Aravind, 2001; Mougel

and Zhulin, 2001). Analysis of AHK4 cocrystal structures with CKs has revealed that the active site involved in ligand-receptor interaction is a membrane-distal Per-Arnt-Sim (PAS) domain. The geometry of binding of different CKs is virtually identical. However, the mechanism of the receptor-ligand interaction as well as further signal transduction across the protein molecule remains unresolved (Hothorn et al., 2011).

Previous efforts to elucidate the mechanism of *A. thaliana* CK sensor HK function have focussed on investigations of the natural and artificially prepared mutants of the receptor. Some single mutations cause constitutive activity of AHK4 in the absence of ligand. Three single mutations, G412C (G435C), F413S (F436S) and M424T (M447T), have been characterized in the second transmembrane domain. Other constitutively active mutations, V448A (V471A), M471L and (M494L), have been described in the HisKa and HATPase_c domain (Miwa et al., 2007). Note that we use PDB numbering throughout this manuscript, Uniprot numbering is shifted by 23 amino acids (AAs), e.g. G412 is G435 in Uniprot numbering.

To date, point mutations inside the CHASE domain have been shown to cause only a decrease in, loss of or no effect on the activity and binding capacity (de Leon et al., 2004; Franco-Zorrilla et al., 2002; Heyl et al., 2007; Hothorn et al., 2011; Kuroha et al., 2006; Mahonen et al., 2006). The best known “loss of function” mutation, AHK4-T278I (T301I), named *wol-1* (Mahonen et al., 2006; AHK4-T301I, *wol*, Yamada et al., 2001), is situated in an extracellular binding domain. This mutation is directly associated with the CK suppressed phenotype. Dysfunction of the mutant gene has also been confirmed in bacterial assay (Yamada et al., 2001). Studies dealing with mutation effects on HK function have shown the importance of these particular amino acids, but the results are too fragmental (in the context of these very large proteins), precluding in-depth analysis of the protein function.

In this study, using computer modelling and simulations of the AHK4 binding site, we revealed the possible mechanism and polar contacts involved in activation of the receptor. We focused on the *N9* position of the CK adenine ring. Previously, it was hypothesized by Lomin et al. (2015) that after the CK molecule enters the binding site in the correct orientation, loop movement is initiated, leading to formation of a hydrogen bond between the main chain of a conserved Leu284 residue (Leu307) and *N9* of the hormone, thus defining a tight binding interaction. Recently, Savalieva et al. (2018) shown that the position 9 of adenine ring affects ligand binding properties of AHKs. To study the structural

features of the interaction of CKs with an AHK4 binding site, we modified the structure of the ligand instead of changing the nature of the AA residues inside the binding site. Hence, new 9-deaza analogues of two isoprenoid and two aromatic CKs (Figure 1) were synthesized and employed as chemical tools to unravel the importance of CK binding interaction.

## 2.1 RESULTS AND DISCUSSION

### 2.1. Chemo- and bioinformatics investigation of AHK4 structure reveals unique C-shaped docking platform for CK binding

Interactions between CK ligands and AAs in defined positions in the binding site as well as interactions of water molecules across the binding site of AHK4 were described in the first crystallographic study performed on a CK receptor by Hothorn et al. (2011). We used Hothorn's data and bioinformatics information about the conservation of AAs forming the binding pocket as a basis for a deeper investigation into the AHK4 structure. This information was employed for in silico modelling and designing new ligands.

The AHK4 crystal structure consists of a dimer of extracellular CHASE domains (Figure 2A, Hothorn et al., 2011). Each monomer is formed by a long N-terminal helix, which serves not only as a basis for dimerization but also as a scaffold for two Per-Arnt-Sim (PAS) domains, where the first PAS domain serves as a binding site for CKs. The binding site can be described as a beta-sheet "base" comprising mainly small aliphatic residues, which in turn is covered by flexible loop that forms a "lid". The flexible lid loop differs in length between individual AHK isoforms. All accessible crystal structures of AHK4 show that the CK is located in the binding site bound by two hydrogen bonds to "arm" helix Asp262 via $N^6$ and *N7* of the CK. An additional hydrogen bond is provided by the main chain of Leu284 (Figure 2A).

The hydrogen bonds to Asp262 via $N^6$ and *N7* and between the Leu284 backbone amide and *N9* together with water molecules bound to *N1* and *N3* form a mostly planar interconnected system that extends further into the receptor structure and connects AAs across the binding site directly or indirectly via an additional layer of water molecules (Figure 2B). The lowest half of the binding site is lined by small hydrophobic residues, which extend out from the central β-sheet. The upper half of the pocket, which is formed by two β-strands, provides additional hydrophobic contacts. Leu284 is located on a loop between the β5 and β6 sheet, which resembles a "hook" that divides the entrance towards

the binding site into two main entrances. The first entrance to the cavity is located between the “hook” and “arm” with Asp262, where C8 is positioned in the CK. The second entrance is “gated” by Tyr250, which forms a hydrogen bond to water interacting with *N3* in the CK. An additional hydrogen bond is formed between the tZ tail hydroxy group and Thr294. This interaction is responsible for specific stereorecognition of zeatin for *trans* form, the most preferred ligand for AHK4.

The overall shape of the ligand binding pocket and specific interactions of the ligands with the key AAs and water molecules explain one of key features of the receptor specificity, which distinguishes between CKs and other adenylate containing molecules. The common motifs for adenine recognition require an $N^6$ and *N1* hydrogen bond to a protein backbone carbonyl and amide (Denessiouk et al. 2001). The manner in which CK is bound to the Asp262 side chain and the lack of *N1* – backbone interaction are very different from the common modes of adenine binding and could be a possible explanation for the very limited nonspecific binding of the adenine into the receptor indicated by Romanov et al (Romanov et al., 2006).

Whereas the Asp262 interaction with CK $N^6$ and *N7* together with the CK side chain are primarily responsible for ligand stabilization, the role of the interaction between the backbone amide and *N9* is much less clear because the *N9* position of the adenine ring is not usually involved directly in the ligand-protein interaction or is “occupied” in the case of more complex molecules (e.g. ATP or ribosides). In AHK4, a hydrogen bond donor is provided by the main chain amide of Leu284 and *N9* serves as a hydrogen bond acceptor, in contrast with the ligand-free form, where N9 is a dominant hydrogen bond donor and *N7* is mainly a hydrogen bond acceptor. Together with Leu283, these two AAs form a symmetrical C-shaped docking platform, with the hydrogen bond in the centre and the plane of the CK adenine ring perpendicular (Figure 2C). This previously undescribed structure enables precise positioning of the interaction and fixes the “hook” loop to the rest of the PAS domain.

To elucidate the sequence conservation of the residues providing interactions with the ligand, including Leu283 and Leu284, we performed a bioinformatics analysis of the accessible CHASE domains obtained from the Pfam database (PF03924, http://pfam.xfam.org). Based on the conserved motif between AAs 261 and 317 (numbered according to the AHK4 sequence) of the PAS domain, 277 AAs belonging to 52 organisms were chosen for the final sequence alignment (Supplementary Figure S1). The key AAs providing ligand-receptor interactions in the PAS domain are highly conserved. Asp262 and Leu283 were found to be conserved absolutely in all 277 sequences,

confirming that these residues play a key role in the ligand binding (Supplementary Figure S1). Interestingly, the neighbouring Leu284 was also found to be conserved in about 90% of the sequences, whereas the variant AAs were predominantly other hydrophobic residues, such as Ile, Met and Phe (Figure 3, Supplementary Figure S1). These results are in good agreement with the previous study of Hothorn et al. (2011), which identified 11 invariant residues in the AHK4 binding pocket based on alignment of sequences originating from 7 different organisms. Our large alignment of 277 sequences showed that 10 of the invariant residues described previously by Hothorn et al. (2011) are indeed highly conserved, reaching similarity of 98.5% - 99.6%. Larger variability was shown for position 256, where Met exhibited lower conservation (85.4%), with variants featuring other hydrophobic residues (Val, Leu, Ile) or residues with positively charged side chains (Lys) (Figure 3, Supplementary Figure S1).

The above results indicate high sequential and functional conservation of AAs interacting with the ligand, including potentially essential leucines in positions 283 and 284 inside the binding site. Indeed, semi-quantitative analysis of AHK4 mutants' activity has shown that substitution of Leu283 and Leu284 with alanines through mutagenesis renders AHK4 inactive (Hothorn et al., 2011). Therefore, the importance of the C-shaped docking platform and functional connection of this structure to the rest of the protein were investigated further described in this study. Considering the spatial requirements of the purine ring and its tendency to form stacking interactions with hydrophobic residues, the symmetrical structure formed by Leu283 and Leu284 provides the perfect complementary shape for the interaction. Both the mutagenesis and bioinformatics results showed that not only the hydrophobic nature of the AA residue but also the presence of specific hydrophobic residues, such as leucines, at these positions is required for proper function of the receptor. The described structural features of the interaction of Leu283 and Leu284 with the ligand demonstrate the geometric constraints of fixing the ligand in a position that allows hydrogen bond formation between *N9* and the Leu284 backbone amide. Thus, to further investigate the role of this hydrogen bonding interaction without disrupting the unique structural background of the C-shaped docking platform, we applied chemical biology approach by synthetizing CK 9-deaza analogues of two isoprenoid (tZ, iP) and two aromatic (BA, 6-AP) CKs (Figure 1).

### 2.2. Synthesis of 9-deaza-CK analogues

9-deaza analogues of tZ, iP, BA and 6-AP (Figure 1) were prepared by the synthetic route shown in Figure S2. First, the pyrrolo[3,2-*d*]pyrimidine ring system was assembled as described by Furneaux and Tyler (1999) in two steps from isoxazole with an overall yield of 45%. The intermediate pyrrole ring (55% yield) was obtained from the 3-oxopropionitrile, formed from its isomer isoxazole on treatment with base and in the presence of diethyl aminomalonate. The required 3*H*,5*H*-pyrrolo[3,2-*d*]pyrimidin-4-one was obtained by condensation of the 3-amino-2-ethoxycarbonylpyrrole hydrochloride in boiling ethanol with formamidine acetate in 82% yield. In the next step, the 3*H*,5*H*-pyrrolo[3,2-*d*]pyrimidin-4-one was treated with neat phosphorous oxychloride to produce 4-chloro-5*H*-pyrrolo[3,2-*d*]pyrimidine as a yellow solid in 97% yield following the protocol of Kamath et al. (2009). Afterwards, SN2 nucleophilic substitution at the C4 position was carried out with the desired substituents as described by Sugiyama et al. (1977) to form *N*-(3-methylbut-2-en-1-yl)-5*H*-pyrrolo[3,2-*d*]pyrimidin-4-amine (9-deaza-iP), (*E*)-4-((5*H*-pyrrolo[3,2-*d*]pyrimidin-4-yl)amino)-2-methylbut-2-en-1-ol (9-deaza-tZ), *N*-phenyl-5*H*-pyrrolo[3,2-*d*]pyrimidin-4-amine (9-deaza-6-AP) and *N*-benzyl-5*H*-pyrrolo[3,2-*d*]pyrimidin-4-amine (9-deaza-BA). 9-deaza-6-AP and 9-deaza-BA were obtained in good yields (80% and 94%, respectively). In the case of 9-deaza-iP and 9-deaza-tZ, the reaction was performed in basic conditions using $K_2CO_3$ and $Et_3N$, respectively, obtaining final yields of 55% and 20%, respectively. The relatively low yield in the latter two cases was mainly due to purification issues rather than low conversion.

The activity of the 9-deaza-CK analogues was compared to their unmodified CK counterparts in classical CK bioassays and molecular-based bioassays evaluating the interaction with the CK signalling pathway, as described in the following sections.

### 2.3. Activity of 9-deaza-CK analogues in classical CK bioassays

To gain a basic insight into the effect of N-to-C substitution at position 9 of the adenine moiety the activity of the 9-deaza-CK analogues was compared to their unmodified CK counterparts in classical CK bioassays. In the Amaranthus assay, the CK activity was evaluated based on formation of betacyanin in the dark (Biddington and Thomas, 1973). In this, compared to their CK counterparts 9-deaza-tZ and 9-deaza-BA showed no activity (Figure 4A) and only weak activity was recorded at the highest concentration (10 μM) in the case of 9-deaza-iP (36%) and 9-deaza-6-AP (20%;

Supplementary Figure S2A). In the tobacco callus growth assay, CK activity was assessed based on stimulation of CK-dependent callus growth on medium with and without CK in the presence of auxin (Murashige and Skoog, 1962). In this assay, the 9-deaza-CK analogues showed a clear reduction in the CK activity compared to their CK counterparts (Figure 4B, Supplementary Figure S2B). Only 9-deaza-iP showed weak activity, reaching the level of iP at the highest concentration applied (10 μM) (Supplementary Figure S3B). In contrast, the activity of other 9-deaza CKs was reduced to 50% of that of their respective CK controls at the highest tested concentration (Figure 4B, Supplementary Figure S2B). In the senescence assay, CK-stimulated retention of chlorophyll in excised leaves of wheat in the dark was evaluated (Holub et al., 1998). Results from this assay showed completely inactive the 9-deaza-CKs (Figure 4C, Supplementary Figure S2C).

These results clearly indicate that N-to-C substitution at position 9 of the adenine moiety strongly decreases CK activity. This is in accordance with previous findings showing that substitutions at *N9* of a CK moiety through ribosylation or glucosylation decreases its biological activity in classical CK bioassays (Doležal et al., 2007; Holub et al., 1998). Recently, alkylation at N9 of BA analogues was shown to cause reduced cytokinin activity through changes in ligand binding modes (Savalieva et al., 2018). It has also been shown that rearrangements of the heteroatoms within the adenine moiety to produce CK bioisosteres with hydrogen at position 9, such as in pyrazolo[4,3-*d*]pyrimidine, also have strong effects on the activity (Hecht et al., 1971). The same is true if the *N9* of a CK or *C3* of a pyrazolo[4,3-*d*]pyrimidine iP bioisostere is substituted with a methyl group (Hecht et al., 1971). These facts indicate the high importance of the *N9* atom being a hydrogen bond acceptor provided by the main chain amide of Leu284 in the AHK4 binding site (Figure 2B; 2C) for proper CK function. Interestingly, modification of the heterocyclic purine system yielded the first CK analogues with antagonistic activity capable of a strong reduction of CK activity in bioassays (Skoog and Armstrong, 1970; Skoog et al., 1967). However, it has been shown that despite their structural similarity to natural CKs and the reversibility of their antagonistic effects by increasing the CK concentration, some strong anticytokinins, e.g. 3-methyl-7-pentylaminopyrazolo[4,3-d]pyrimidine, do not interact with CK receptors and their antagonistic activity recorded primarily in the tobacco callus assay cannot be related to interaction with a CK binding site (Spíchal et al., 2007). Thus, the first true CK antagonists, 6-(2-hydroxy-3-methylbenzylamino)purine (PI-55; Spíchal et al., 2009) and 6-(2,5-dihydroxybenzylamino)purine (LGR-991; Nisler et al., 2010), were identified not through their function

in classical CK bioassays but through their direct interaction with CK receptors. Hence, to determine whether 9-deaza-CK analogues could be used to investigate the role of hydrogen bonding with the rest of the binding site molecular-based bioassays were used, as described further.

### 2.4. Interaction of 9-deaza-CK analogues with the CK signalling pathway

Ability of the 9-deaza-CK analogues to bind and activate AHK4 receptor was studied using a live-cell hormone-binding assay (Romanov et al., 2005), the β-galactosidase activity assay (Spíchal et al., 2004) and the *ARR5::GUS* CK induction assay (Romanov et al., 2002) . The affinity of the tested compounds to the AHK4 receptor was evaluated by using the constitutively expressed receptor in *E. coli* strain KMI001 (Inoue et al., 2001; Suzuki et al., 2001; Yamada et al., 2001). In the live-cell hormone-binding assay, the tested compounds were found to effectively block binding of 3 nM 2-[$^3$H]tZ to the AHK4 receptor in the descending order of 9-deaza-tZ ~ 9-deaza-BA > 9-deaza-iP > 9-deaza-6-AP (Figure 5A). $IC_{50}$ values of the 9-deaza-CKs were in range of 1.2 μM to higher than 10 μM showing lower affinity in comparison to their aza-counterparts (Figure 5A, Table 1).

Thus, in the next assay, we tested whether the binding can be translated into activation of the CK signalling pathway. The ability of the 9-deaza analogues to trigger CK signal transduction was tested in the β-galactosidase activity assay. Here, the constitutively expressed AHK4 receptor functionally complements the bacterial signal cascade, leading to activation of a reporter gene *cps::lacZ* in the presence of CK(Suzuki et al., 2001; Yamada et al., 2001). As shown in Figures 5B and Supplementary Figure S3 compared to CKs, their 9-deaza counterparts failed to activate the CK cascade. Thus, the 9-deaza-CK analogues showed similar behaviour to the previously described competitive inhibitors of AHK4 PI-55 (Spíchal et al., 2009), LGR-991 (Nisler et al., 2010) and $N^6$-(benzyloxymethyl)adenosine (BOMA, Krivosheev et al., 2012) and have potential to be used as CK antagonists (Klimes et al., 2017).

In the above mentioned molecular bioassays, interaction of the compounds with AHK4 was tested in a bacterial system. The same *E. coli* system has been used in numerous works for characterization of the ligand specificity of various CK receptors (e.g. Kuderova et al., 2015; Spichal et al., 2004; Yonekura-Sakakibara et al., 2004) and evaluation of various chemicals as potential agonists or antagonists of CK receptors (e.g. Doležal et al., 2006; Doležal et al., 2007; Klimes et al., 2017; Krivosheev et al., 2012; Nisler et al., 2010; Spichal et al., 2009). Moreover, Lomin et al. (2015) have shown that ligand specificity profiles obtained from bacterial assay systems towards CK bases are

comparable with plant membrane assays using membranes isolated from infected tobacco leaves expressing AHK3, AHK4, and ZmHK1 receptors.

However, to further validate our results obtained from the bacterial assay, the activity of 9-deaza-CK analogues was additionally tested in the *ARR5::GUS* CK induction assay (Romanov et al., 2002) based on induction of the CK primary response gene *Arabidopsis response regulator 5* (*ARR5*; D´Agostino et al., 2000). As shown in Figure 5C, similarly to the *E. coli* assay, the 9-deaza-CK analogues did not induce the CK signalling pathway even at the highest concentration tested (10 μM).

Therefore, based on the above data showing that 9-deaza-CK analogues 1) have affinity towards AHK4 but fail to activate the receptor, and 2) show no or very weak CK activity in physiological CK bioassays, we concluded that nitrogen at the 9 position of the CK adenine ring seems to be essential for activation of the AHK4 CK receptor by ligands and its subsequent signal transduction leading to CK responses.

### 2.5. Molecular simulations

In an effort to elucidate structure/function relationships in the AHK4 PAS domain, we further applied molecular dynamics (MD) and molecular docking approaches. In the following text, we use “arm”, “hook” and “leg” terminology (Figure 2) to describe movements within the binding domain upon interaction with the 9-deaza ligand or without any ligand at all.

First, we used molecular docking into the crystal structure to replace BA in the binding site. Docking of the CKs and their analogues showed that they were all able to bind into the binding site with variable affinity. Binding into the tZ-bound structure followed the same trends as shown by the crystal structure (Table 1A), i.e. BA > 6-AP > iP ~ tZ. However, contrary to expectations from the experimental studies, the 9-deaza compounds were able to bind into AHK4 structures with even higher affinity than their aza counterparts. Since we observed a shift in the binding pose for 9-deaza analogues, we prepared several MD simulation setups to allow optimization of the protein-ligand interactions.

We have run simulations without any ligand present; with tZ in the crystal position; and with 9-deaza-tZ in a crystal-like position. The final snapshots from simulations were used for a second round of molecular docking. The ligand-free cavity was found to be closed and unable to hold any CK (Table 1B). This might explain why there is no ligand-free crystal structure available as the structure of the receptor needs to first accommodate a CK for binding. The tZ-bound structure stayed virtually the

same throughout the simulation and the docking pattern was almost the same as into the crystal structure itself (Supplementary Figure S4). However, MD simulations with 9-deaza analogues showed large shifts within the AHK4 structure (Supplementary Figures S4 and S5). Binding of the 9-deaza analogues was followed by large shifts within the structure. Hence, the proper binding affinities for those compounds were calculated against the 9-deaza-tZ-bound structure, for which all compounds bound with lower affinity than CKs in the order 9-deaza-BA > 9-deaza-tZ > 9-deaza-iP > 9-deaza-6-AP (Table 1B). This may explain why the binding patterns differed between the 9-deaza-CKs and natural CKs which could not be explained by docking into the rigid crystal structures. These results show that in accordance with the bioassay results, 9-deaza compounds bind to the AHK4 binding site weaker than their respective CKs. However, the bioassays also showed that the ability of 9-deaza analogues to activate the AHK4 receptor was severely impaired.

Interestingly, the MD simulations showed not only movements in the ligand binding site, but the movements in the binding cavity were transferred further into the AHK4 structure in both the 9-deaza-tZ occupied and ligand-free simulations. MD simulation of the AHK4 dimer and monomer with native tZ ligand showed no significant differences in the binding site structure during the 100-ns-long simulation. Therefore, we used only monomers in further studies. As stated earlier, the binding site for tZ was virtually the same as in the crystal structure (Figure 6B). When 9-deaza-tZ was used instead, the binding cavity was expanded even though the position of the ligand in comparison with the crystal pose did not move much. However, both the “arm” and the “hook” were unattached, leaving additional space for several water molecules to form a hydrogen bond network between Asp262 and $N^6$ and *N7* (Figure 6C). These movements suggest that concerted movement of the “hook” with Leu284 and “arm” with Asp262 opens up the ligand binding cavity for ligand entry. Also, as CK ribosides do not fit into the ligand binding cavity, they are not recognizable by AHK4[15,36]. However, in contrast to Lomin et al.’s[15] expectations, the ligand-free domain is not open. Instead, as it is formed mainly by hydrophobic residues, it is filled with "lid" residues, whereas the "arm" is displaced (Figure 6A). Therefore, any ligand binding requires reverse movement of the "lid" and "arm" to accommodate the ligand.

There are several models for receptor activation reported in the literature (Steklov et al., 2013) which we can compare with our modelling results. It has also been shown that the PAS domains adapt flexibly to the ligands and transmit the signal downstream (Vreede et al., 2003). Our simulations

confirmed flexible adaptation of the PAS domain to the ligand. However, how this signal is transmitted further remains to be clarified.

The first model of AHK4 activation mentions “piston-like movement” of PAS and PAS-like domains along the long stalk alpha-helix upon binding of a ligand into the PAS domain (Bartrina et al., 2017; Liu et al., 2015) transferred via motion of the second PAS-like domain towards or away from the membrane thus moving both transmembrane helices relative to each other and transmitting the binding signal into the cytosolic part of the receptor. However, no such piston movement was observed in our simulations. In fact, in all our simulated systems, the relative positions of the N- and C-terminal ends were virtually the same as in the crystal structure (see Supplementary Figure S5). The same was true for the bacterial CHASE domain mentioned in Liu et al. (2015), for which the ligand-free and ligand bound structures were aligned (see Supplementary Figure S6). Additional evidence against such movement is the presence of the disulphide bond between the stalk helix via Cys142 and the PAS-like membrane-proximal domain via Cys389. This disulphide bond effectively blocks any sliding motion that would be necessary for the piston-like mechanism of activation.

The second model of AHK4 activation considers changes in the quaternary structure of the whole HK. CHASE sensory domains are known to homodimerize (Cheung and Hendrickson, 2009) and the protein thus forms a dimeric four-helical bundle in the membrane. Both transmembrane helices are elongated in a direction towards the C-terminal end (Rost et al., 2004), forming a stalk helix elongated from the first transmembrane segment and another long helix scaffolding the kinase domain in the cytosol from the latter. Both long helices serve as a dimeric interface. In the case of AHK4 crystal structure, this interface is shortened to only the topmost part of the helix together with the following shorter helix behind PAS domain. The dimeric interface in extracellular domain is formed mainly by nonpolar AAs encompassed by positively charged ones. In the presence of CK in the binding site, both the "leg" and "arm" rotate further from the dimerization interface to support ligand binding while ligand-free or 9-deaza interface is larger and hence more stable due to movement of “leg” segment. We can expect that such movement may shrink the dimerization interface, leading to rotation or even dissociation of the monomers owing to the lack of interactions and hence receptor activation. Similar movement has been shown for other two-component proteins with the proposed inter-helix bundle movement (Gao and Stock, 2009; Hulko et al., 2006). Moreover, the constitutively active mutations are

either located in the membrane, where they lower the stability of inter-helical interaction (Supplementary Table S1), or they are located in the alpha-2 helix (rock3; T191I, E194K; Bartrina et al., 2017), where they disarm the interaction by mutation of the only negative residue Glu194 on the otherwise positively charged dimer interface. All of these constitutively active mutations destabilize the dimerization interface and change the quaternary structure of the AHK4 in a manner similar to how it is destabilized by CK binding.

On this basis, we propose that the AHK4 receptor is activated by CK binding due to destabilization of the dimerization interface. AHK4 activation depends on the hydrogen bonding with *N9* position on cytokinin, which was also confirmed *in silico* and by cytokinin assays.

### 3.1 CONCLUSIONS

Simulations showed different positions of CKs and their 9-deaza counterparts in the AHK4 binding cavity and allowed the disposition of AAs in the free or occupied binding pocket to be defined. Biological evaluation showed the influence of using 9-deaza compounds on the activity and affinity towards the AHK4 receptor. Lack of a hydrogen bond between the *N9* atom of the CK adenine ring and the peptide backbone of the AHK4 binding cavity was found to have a considerable effect on the function of the AHK4 receptor. Based on these results, we concluded that *N9* of the adenine ring is an essential group for activation of the AHK4 receptor by CKs. Moreover, simulations of the AHK4 receptor with 9-deaza-CK analogues showed similar domain movements to those of the ligand-free state but different to those with natural CKs. Our results suggest that signal transmission across the membrane in the AHK4 signalling pathway is mediated via CK activation leading to destabilization of the AHK4 receptor dimerization interface rather than by the previously hypothesized piston-like movement model of AHK4 activation.

## 4.1 EXPERIMENTAL

### 4.1. Sequence alignment

Sequences containing the CHASE domain were obtained from the Pfam database (PF03924, http://pfam.xfam.org). Alignment of 1721 domains was generated with ClustalOmega (version: 1.2.4; guidetreeout: false; dismatout: false ; dealign: false; mbed: true; mbediteration: true; iterations: 0; gtiterations: -1, hmmiterations: -1; outfmt: fa; order: aligned) by EMBL-EBI (http://www.ebi.ac.uk;). The alignment was manually modified to 277 CHASE domains originating from 52 different organisms.

### 4.2. Synthesis of 9-deaza-CKs

The general chemical methods used for synthesis and characterization of *N*-(3-methylbut-2-en-1-yl)-5*H*-pyrrolo[3,2-*d*]pyrimidin-4-amine (9-deaza-iP), (*E*)-4-[(5*H*-pyrrolo[3,2-*d*]pyrimidin-4-yl)amino]-2-methylbut-2-en-1-ol (9-deaza-tZ), *N*-benzyl-5*H*-pyrrolo[3,2-*d*]pyrimidin-4-amine (9-deaza-BA), and *N*-phenyl-5*H*-pyrrolo[3,2-*d*]pyrimidin-4-amine (9-deaza-6-AP), are described in the Supplement (see Supplementary Figure S7).

NMR spectra were recorded on a JEOL ECA-500 spectrometer in dimethyl sulfoxide-$d_6$ and were referenced to the residual peak of the solvent. Chromatographic purity was measured using the same conditions as described in Gucký et al. (2013). Melting points were determined in a Büchi Melting Point B-540 apparatus. Reagents and solvents of the highest purity available were used as purchased or purified/dried by standard procedures when necessary. Column chromatography was carried out on columns packed with silica gel (63-100 μm). 3-Amino-2-ethoxycarbonylpyrrole hydrochloride and 4-chloro-5*H*-pyrrolo[3,2-*d*]pyrimidine were prepared as described in Furneaux and Tyler (1999) and Kamath et al. (2009), respectively.

### 4.3. Synthesis of *N*-(3-methylbut-2-en-1-yl)-5*H*-pyrrolo[3,2-*d*]pyrimidin-4-amine (9-deaza-iP)

A mixture of 4-chloro-5*H*-pyrrolo[3,2-*d*]pyrimidine (100 mg, 0.52 mmol), 3-methylbut-2-en-1-amine hydrochloride (253 mg, 2.08 mmol) and $K_2CO_3$ (108 mg, 0.78 mmol) in EtOH (2.5 mL) was heated under argon at 90ºC for 16 h. The solvent was removed under vacuum and the residue was redissolved in acetone. The solid was filtered and the filtrate was purified by flash column chromatography (DCM/MeOH 1:0 to 9:1) afforded 9-deaza-iP (57 mg, 55%) as a brown solid: Mp=224-226ºC; $^1$H NMR (500 MHz, DMSO-$d_6$) δ 10.86 (bs, 1H, H-5), 8.14 (s, 1H, H-2), 7.45 (t, *J*=2.8

Hz, 1H, H-6), 6.96 (bs, 1H, NH-C4), 6.32 (1H, dd, *J*=2.9, 1.4 Hz, 1H, H-7), 5.35 (tsept, *J*=7.0, 1.4 Hz, 1H, H-2'), 4.05 (t, *J*=6.0 Hz, 2H, H-1'), 1.72 (s, 3H, H-4'), 1.71 (s, 3H, H-1''); $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ 150.19 (CH-2), 149.13 (C-4), 146.31 (C-7a), 134.60 (C-3'), 127.21 (CH-6), 121.51 (CH-2'), 113.76 (C-4a), 101.36 (CH-7), 37.68 ($CH_2$-1'), 25.44 ($CH_3$-1''), 17.82 ($CH_3$-4'); MS *m*/*z* (ESI) 204.11 $(M+H)^+$.

### 4.4. Synthesis of (*E*)-4-[(5*H*-pyrrolo[3,2-*d*]pyrimidin-4-yl)amino]-2-methylbut-2-en-1-ol (9-deaza-tZ)

A mixture of 4-chloro-5*H*-pyrrolo[3,2-*d*]pyrimidine (100 mg, 0.64 mmol), (*E*)-4-amino-2-methyl-2-buten-1-ol ethanedioate (2:1) (salt) (139 mg, 0.32 mmol) and $Et_3N$ (0.7 mL, 0.25 mmol) in a sealed tube containing EtOH (2 mL) was heated at 120ºC overnight. The solvent was removed, the residue was diluted with $CH_3CN$ and filtered. The resulting filtrate was concentrated in vacuum and then purified by flash chromatography (DCM/MeOH 1:0 to 8:2) to afford 9-deaza-tZ (28 mg, 20%) as an orange oil. Rf (EtOAc) = 0.13; $^1$H NMR (500 MHz, DMSO-$d_6$) δ 11.48 (bs, 1H, H-5'), 8.39 (s, 1H, H-2'), 8.08 (bs, 1H, NH-C4'), 7.62 (s, 1H, H-6'), 6.42 (d, *J*=2.4 Hz, 1H, H-7'), 5.57 (tsept, *J*=7.0, 1.3 Hz, 1H, H-3), 4.87 (t, *J*=5.5 Hz, 1H, OH), 4.19 (t, *J*=5.8 Hz, 2H, H-4), 3.83 (d, *J*=4.6 Hz, 2H, H-1), 1.67 (s, 3H, $CH_3$); $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ 150.43 (CH-2'), 145.43 (C4'), 139.65 (C-7a'), 134.04 (C-2), 130.65 (CH-6'), 117.97(CH-3), 113.10 (C-4a'), 96.94 (CH-7'), 65.48 ($CH_2$-1), 38.06 ($CH_2$-4), 13.74 (Me-$C_2$); MS *m*/*z* (ESI) 219.02 $(M+H)^+$.

### 4.5. Synthesis of *N*-benzyl-5*H*-pyrrolo[3,2-*d*]pyrimidin-4-amine (9-deaza-BA)

A mixture of 4-chloro-5*H*-pyrrolo[3,2-*d*]pyrimidine (100 mg, 0.651 mmol) and benzylamine (288 µL, 2.64 mmol) in a sealed tube containing EtOH (1 mL) was heated at 100ºC overnight. The solvent was removed, then the residue was diluted with $CH_3CN$ and filtered. The resulting filtrate was concentrated in vacuum and then purified by flash chromatography (DCM/MeOH 1:0 to 9:1) to afford 9-deaza-BA (137 mg, 94%) as a pale yellow solid. Rf (DCM/MeOH 9:1) = 0.17; Mp=213-215ºC; $^1$H NMR (500 MHz, DMSO-$d_6$) δ 10.90 (s, 1H, H-5), 8.16 (s, 1H, H-2), 7.48 (t, *J*=2.6 Hz, 1H, H-6), 7.44 (t, *J*=4.9 Hz, 1H, NH-C4), 7.38 (d, *J*=7.3 Hz, 2H, $H_o$-Ph), 7.34 (t, *J*=7.5 Hz, 2H, $H_m$-Ph), 7.26 (t, *J*=7.2 Hz, 1H, $H_p$-Ph), 6.36 (s, 1H, H-7), 4.71 (d, *J*=5.2 Hz, 2H, $CH_2$-Ph); $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ 150.13 (CH-2), 149.22 (C-4), 146.57 (C-7a), 139.69 (C-Ph), 128.43 ($CH_o$-Ph), 127.61 ($CH_m$-Ph), 127.52 (CH-6), 126.97 ($CH_p$-Ph), 113.70 (C-4a), 101.39 (CH-7), 43.21 ($CH_2$-Ph); MS *m*/*z* (ESI) 225.08 $(M+H)^+$.

### 4.6. Synthesis of *N*-phenyl-5*H*-pyrrolo[3,2-*d*]pyrimidin-4-amine (9-deaza-6-AP)

A mixture of 4-chloro-5*H*-pyrrolo[3,2-*d*]pyrimidine (100 mg, 0.651 mmol) and aniline (236 µL, 2.60 mmol) in a sealed tube containing EtOH (2 mL) was heated at 100ºC overnight. Afterwards, the solvent was removed and the residue was purified by flash column chromatography (DCM/MeOH 1:0 to 9:1) to afford 9-deaza-6-AP (110 mg, 80%) as a brown solid. Rf (DCM/MeOH 9:1) = 0.40; Mp=248-250ºC; $^{1}$H NMR (500 MHz, DMSO-$d_6$) δ 11.18 (s, 1H, H-5), 9.22 (s, 1H, NH-C4), 8.33 (s, 1H, H-2), 7.86 (d, *J*=8.4 Hz, 2H, H$_o$-Ph), 7.65 (s, 1H, H-6), 7.35 (t, *J*=7.5 Hz, 2H, H$_m$-Ph), 7.01 (t, *J*=7.3 Hz, 1H, H$_p$-Ph), 6.46 (s, 1H, H-7); $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ 148.51 (C-4), 145.09 (CH-2), 137.69 (C-7a), 134.95 (C-Ph), 132.27 (CH-6), 128.95 (CH$_m$-Ph), 124.93 (CH$_p$-Ph), 121.62 (CH$_o$-Ph), 113.87 (C-4a), 97.07 (CH-7); MS *m*/*z* (ESI) 211.05 (M+H)$^+$.

### 4.7. Molecular dynamics simulations

The crystal structure of *Arabidopsis thaliana* histidine kinase 4 (AHK4) with *N*$^6$-benzyladenine (PDB ID: 3T4K) at 1.77 Å resolution was used for MD simulations (Hothorn et al., 2011). The crystal structure contains a dimer of the AHK4 sensor domain structure (AAs 149-416 of UniprotID: Q9C5U0). The protein was immersed into a water box as either a dimer (30 000 SPC/E molecules) or monomer (18 000 SPC/E molecules) and Na+ and Cl- ions were added to mimic physiological concentrations and to neutralize the system prepared for simulation. Topologies of both tZ and 9-deaza-tZ ligands were prepared in Ambertools (Case et al., 2017) with gaff nonbonded parameters and RESP (B3LYP/cc-pVDZ) charges obtained from the Gaussian 09 program suite (Frisch et al., 2016). MD simulations were executed in the Gromacs 4.5.4 program suite (Pronk et al., 2013) with the Amber03 force field for the protein (Duan et al., 2003). Simulations of the dimer and monomer with natural ligand tZ in the active site in the crystal position were followed by simulations with 9-deaza-tZ in the same position and with ligand-free monomer. After energy minimization using the steepest descent algorithm, 10-ns-long simulations were used to equilibrate the systems using a 2 fs timestep together with the V-rescale thermostat to set the temperature to 310 K and the isotropic Berendsen barostat to set the pressure to 1 bar. The cutoff for noncovalent interactions was set to 1.0 nm. Finally, 100-ns-

long production simulations were carried out (310 K and 1 atm.) with Parrinello-Rahman pressure coupling to collect structures of AHK4 with and without individual ligands.

### 4.8. Molecular docking

The geometry of CKs and their 9-deaza analogues was modelled with Marvin (http://www.chemaxon.com), and then the compounds were prepared for docking in the AutoDockTools program suite[41]. The Autodock Vina program[42] was used for docking four CKs and their 9-deaza ligands into the set of AHK4 structures obtained from MD, i.e. CK-bound, 9-deaza-bound and ligand-free structures, and into the crystal structure. A 15 Å box centred at the CK-binding position was used. The exhaustiveness parameter was set to 20. Only crystal-like poses were selected for the analysis.

### 4.9. Cytokinin bioassays

Amaranthus assay, Tobacco callus growth bioassay and Wheat senescence assay were performed according to Holub et al. (1998) with slight modifications. In Tobacco callus growth bioassay, six-well plates were used for growing of calli. Each well contains 3 ml of MS medium, were 0.1 g of callus was placed. Tested cytokinin and their 9-deaza derivatives were dissolved in dimethylsulfoxide (DMSO) and diluted with distilled water to $5 \times 10^{-2}$ M solutions. This stock solution was further diluted in the media appropriate to each biotest to concentrations from $10^{-9}$ to $10^{-4}$ M. The final concentration of DMSO in the media did not exceed 0.2% and thus did not affect the biological activity of the substance tested in the assays. Five replicates were prepared for each compound concentration and the entire tests were repeated at least twice.

### 4.10. Bacterial strain and CK standards

CK receptor AHK4 was expressed by *E. coli* strain KMI001 carrying the pINIIIΔEH vector (Suzuki *et al.*, 2001; Takeda et al., 2001; Yamada *et al.*, 2001). Non-labelled phytohormones used in the β-galactosidase and CK binding assays were obtained from OlChemIm Ltd. (Olomouc, Czech Republic). Radiolabelled *trans*-zeatin (2-[$^3$H]tZ, 592 GBq/mmol) was obtained from the Isotope Laboratory, Institute of Experimental Botany, *The Czech Academy of Science*, Prague, Czech Republic.

### 4.11. β-galactosidase assay

*E. coli* bacterial precultures were grown overnight at 25°C with shaking to $OD_{600}$~1. The cultivation was carried out in M9 medium containing 0.1% (w/v) casAAs and appropriate antibiotic at 100 µg/mL. The next day, the preculture was diluted to a ratio of 1:600 using fresh M9 medium with casAAs and antibiotic and a final volume of 20 mL was applied to one 96-well microtiter plate (200 µL/each well). CK stock solution or the tested compound was added to the 96-well microtiter plate filled with diluted bacterial culture to obtain a final concentration of 50 µM. Subsequently, compounds in the cell culture were diluted to 0.01 µM. The microtiter plate was incubated at 25°C for 17 h under 450 rpm shaking in a thermomixer. After measuring the optical density at 600 nm, 50 µL of each sample of the bacterial culture with compound was incubated with 2 µL 25 mM 4-methylumbelliferyl galactoside substrate at 37°C for 30 min. The reaction was stopped by adding glycine carbonate buffer at pH 10.7. Quantification was performed based on the fluorescence signal using an excitation peak of 365 nm and emission wavelength of 460 nm (Synergy Multi-Mode Microplate Reader).

### 4.12. CK binding assay

CK binding assay was performed according to the method described by Romanov et al. (2005, 2006) with slight modifications. Cell cultures were grown at 25°C overnight under shaking to $OD_{600}$~0.7–0.8 in M9 liquid medium supplemented with 0.1% (w/v) casAAs and 100 µg/mL ampicillin. The binding assay was performed according to the method described by Romanov et al. (2005, 2006) with slight modifications.

For assays, each sample contained 1 mL of the cell suspension, 3 pmol of 2-[$^3$H]tZ and various concentrations of unlabelled tZ or other tested competitors. As a control, 0.1% (v/v) dimethyl sulfoxide (DMSO; solvent) was added instead of the unlabelled compound. After 30 min incubation at 4°C, the sample was centrifuged (6000 *g*, 6 min, 4°C). The supernatant was removed and the bacterial pellet was resuspended in 20 µL $dH_2O$ and then 1 mL of scintillation cocktail (Beckman, Ramsey, MN, USA). Radioactivity was measured by a Hidex 300 SL scintillation counter (Hidex, Finland). To discriminate between specific and nonspecific binding, a high excess of unlabelled tZ (at least 3000-fold) was used for competition, as described by Romanov et al. (2006). $IC_{50}$ values were determined as average values from three independent measurements. GraphPad Prism 5.1 was used for calculation of $IC_{50}$ and preparing graphs (http://www.graphpad.com/scientific-software/prism/).

### 4.13. *ARR5::GUS* CK induction assay

*ARR5::GUS* cytokinin induction assay was done according to Romanov et al. (2002). Transgenic Arabidopsis *ARR5::GUS* seeds were sterilized, placed in the wells of 6-well plates containing 1/2 MS medium and cultivated in darkness (4 °C) for 4 days. Then seeds were transferred to an orbital shaker (16 h light/8 h dark) for 3 days in a growth chamber (22 °C). Transgenic *ARR5::GUS* Arabidopsis seedlings were treated with cytokinins (tZ, iP, BA, 6-AP) and their 9-deaza analogues. DMSO was used as a control (final concentration 0,1%). Seedlings were cultivated for another 17 h. GUS activity measurements were performed in accordance with previously published procedure (Romanov et al., 2002). Fluorescence was measured using a Fluoroscan Ascent microplate reader (Labsystems, Helsinki, Finland) at excitation and emission wavelengths of 365/450 nm.

**ACKNOWLEDGMENTS**

This work was supported by the Czech Science Foundation (15-19266S), the Ministry of Education Youth and Sports, Czech Republic (grants LO1204 and LO1305 from the National Program of Sustainability I), ERDF project "Development of pre-applied research in nanotechnogy and biotechnology" (No. CZ.02.1.01/0.0/0.0/17_048/0007323) and by the European Social Fund and the state budget of the Czech Republic (project CZ.1.07/2.3.00/30.00041). L.H. was supported by grant IGA_PrF_2018_023, V.B. was supported by grant IGA_PrF_2018_032. K.B. was supported by Czech Science Foundation (17-21122S). This work was supported by ELIXIR CZ and MetaCentrum research infrastructure projects (MEYS Grant No: LM2015047 and CESNET LM2015042) including access to computing and storage facilities. The authors declare no conflict of interest.

**Appendix A.** The Supplementary Material file contains Supplementary Figures S1 - S7, Supplementary Table S1 and their captions.

## FIGURES, TABLES AND LEGENDS

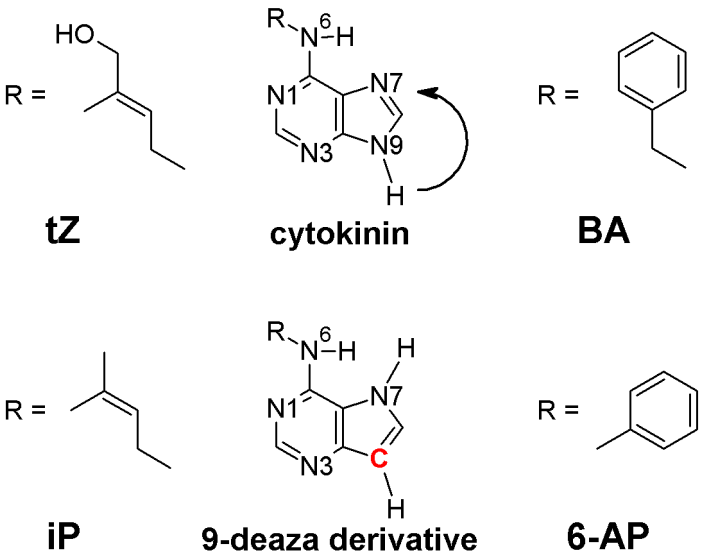


**Figure 1.** Structures of compounds used in this study to investigate binding into the AHK4 receptor. Structures of classical CKs in a non-bound state and their corresponding 9-deaza-derivatives. tZ – *trans*-zeatin; iP – isopentenyladenine; BA – $N^6$-benzyladenine; 6-AP – 6-anilinopurine. Shift of a hydrogen atom during change from non-bound to bound state is depicted by black arrow.

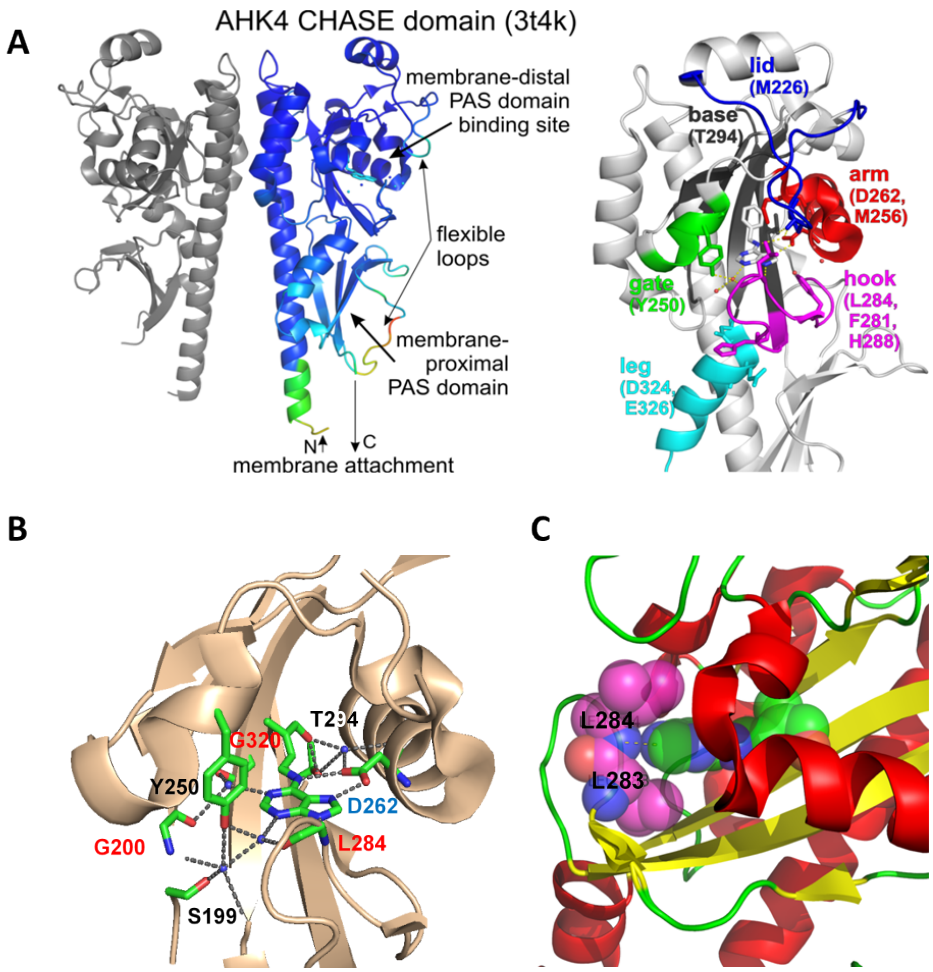


**Figure 2**. Structure of the AHK4 receptor. A) Visualization of the AHK4 CHASE domain crystal structure (3T4K) and its CK binding site. The left image shows the overall structure of the extracellular CHASE domain dimer with colour coding for flexibility from b-factors. The right image shows the annotated CK binding site with highlighted AAs. B) Interconnected system of hydrogen bonds stabilizing tZ inside the binding cavity of AHK4 (3T4L). Blue balls – water; dashed lines – H-bonds; red residues - main chain H-bond forming; black residues - side chain H-bond forming; blue residues - both side chain and main chain H-bond forming. C) The structure of the "hook" loop. Leu284 and Leu283 (magenta spheres) form a symmetrical C-shaped docking platform stabilizing ligand tZ (green spheres) in a position that enables hydrogen bond formation between tZ *N9* and the Leu284 backbone amide (visualized by the yellow dashes).


**Figure 3.** Selection of sequence alignment of CHASE domains with a conserved motif between AA positions 261 and 317 in PAS domains. Comparison of two conserved sequences (AHK4 and AHK2 in

grey) and 35 other sequences which differ by at least one of the discussed AAs at the following positions: A) 256, B) 262, C) 281, D) 283, and E) 284 (numbered according to the AHK4 sequence).

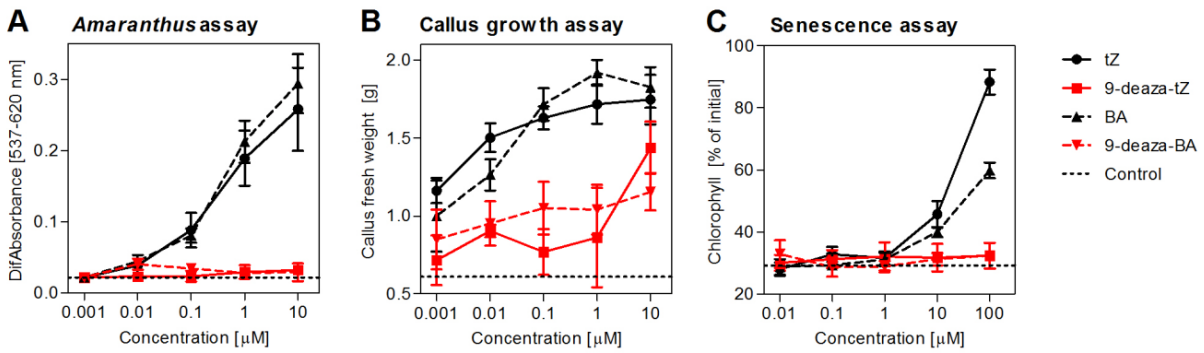


**Figure 4.** Activity of 9-deaza-CK analogues in classical CK bioassays. A) Betacyanin formation under dark induction in *Amaranthus* cotyledon-hypocotyl explants (± SD of the mean for 5 biological replicates). Control incubation without any compounds is marked by the dashed line (0.022 Dif/Absorbance [537/620 nm]). B) Effect of tested compounds on tobacco callus growth (± SD of the mean for 6 replicates for each concentration of compound). Biological activity was determined by the increase of callus fresh weight after four weeks of cultivation. The weight of control calli, growing without any of the tested compounds, is marked by the dashed line (0.679 g). C) Retention of chlorophyll in excised wheat leaves (± SD of the mean for 5 replicates). Control incubation without any compounds is marked by the dashed line (29.3 % of initial chlorophyll).

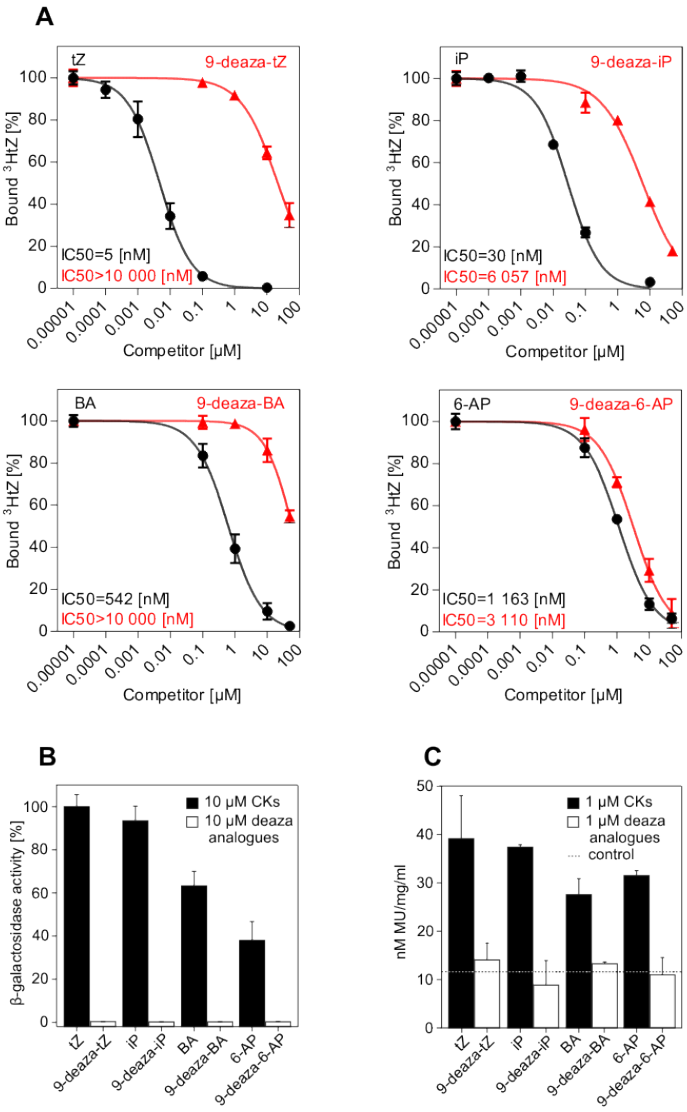


**Figure 5.** Interaction of 9-deaza-CK analogues with the CK signalling pathway. A) Affinity of 9-deaza and aza-compounds towards the AHK4 CK receptor expressed in *E. coli* cells. $IC_{50}$ values were determined based on competition of the tested substances with dose-dependent binding of 2-[$^3$H]tZ and in a live-cell binding assay. $IC_{50}$ values were evaluated from at least 3 biological replicates, each consisting of 3 samples (±SD). B) Activation of the AHK4 CK receptor by CKs and their deaza-derivatives in a bacterial system. β-galactosidase activity was measured in a transformed *E. coli* strain expressing the AHK4 receptor and gene *cps::lacZ* activated in the presence of CK. Data were obtained from 2 independent experiments, where each sample was tested in 3 repetitions and standard deviation was calculated from the mean (see also Supplementary Figure S3). C) Induction of *ARR5::GUS* in transgenic *Arabidopsis* plants. The dashed line represents GUS activity in non-induced plants. Standard deviation was calculated from 2 repetitions.

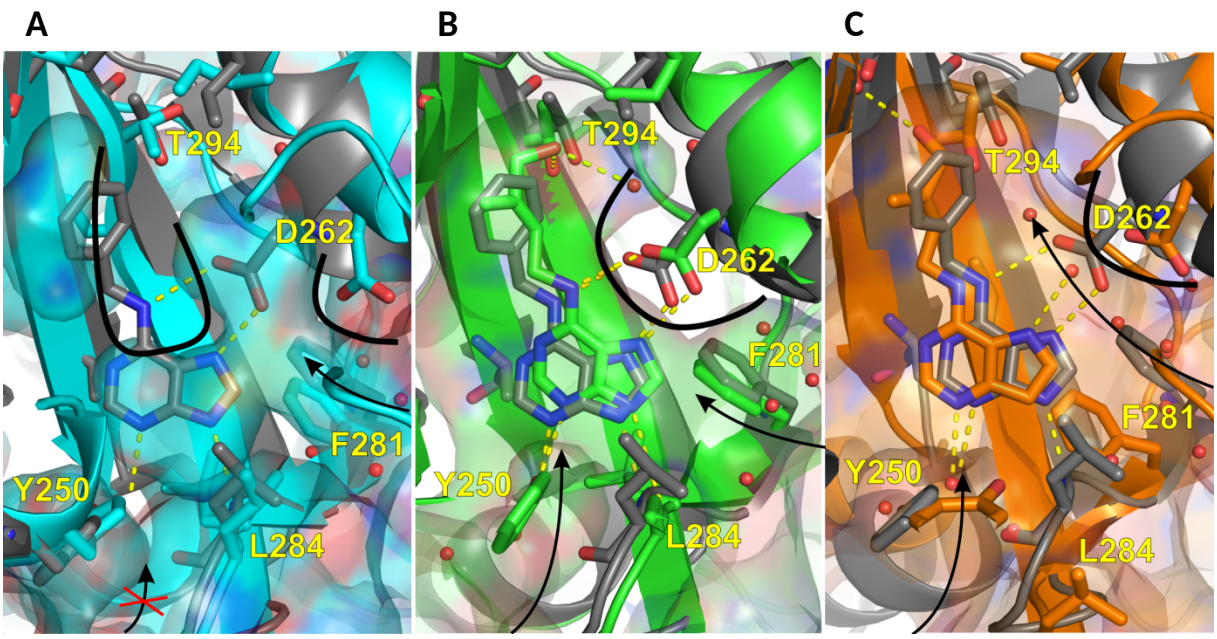


**Figure 6**. Binding site in the ligand-free (A), tZ (B) and 9-deaza-tZ (C) occupied state from MD simulation in comparison with the crystal structure with BA (in grey; PDB ID: 3T4K). Arrows show open water paths into the cavity; Black contours show changes in the cavity space.

A

| Receptors/ligands | Crystal 3T4K [kcal/mol] | $IC_{50}$ [nM] |
|---|---|---|
| **tZ** | **-7.6** | **5** |
| **9-deaza-tZ** | -8.5 | >10 000 |
| **iP** | **-7.7** | **30** |
| **9-deaza-iP** | -7.7 | 6 057 |
| **BA** | **-8.6** | **542** |
| **9-deaza-BA** | -8.9 | >10 000 |
| **6-AP** | **-7.8** | **1 163** |
| **9-deaza-6-AP** | -8.9 | 3 110 |

B

| Receptors/ligands | Ligand-free | AHK4-deaza [kcal/mol] | AHK4-normal [kcal/mol] |
|---|---|---|---|
| **tZ** | NA | -6.4 | **-7.1** |
| **9-deaza-tZ** | NA | **-7.1** | -7.7 |
| **iP** | NA | -6.4 | **-7.2** |
| **9-deaza-iP** | NA | **-6.5** | -7.4 |
| **BA** | NA | -7 | **-8.2** |
| **9-deaza-BA** | NA | **-7.3** | -8.2 |
| **6-AP** | NA | -6.3 | **-8.0** |
| **9-deaza-6-AP** | NA | **-6.3** | -8.2 |

**Table 1.** Binding affinities of CKs and their 9-deaza analogues in AHK4. (A) Comparison of binding affinities based on the crystal structure (PDB ID: 3T4K) of AHK4 [kcal/mol] and measured in the *E. coli* live cell assay [nM]. (B) Binding affinities of individual compounds docking into AHK4 for the ligand-free cavity and tZ-bound structure with ligand [kcal/mol].

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frontiers in Plant Science

METHODS
published: 08 June 2017
doi: 10.3389/fpls.2017.00947

# High Throughput Screening Method for Identifying Potential Agonists and Antagonists of *Arabidopsis thaliana* Cytokinin Receptor CRE1/AHK4

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The CRE1/AHK4 cytokinin receptor is an important component of plants' hormone signaling systems, and compounds that can alter its activity have potential utility for studying the receptor's functions and/or developing new plant growth regulators. A high throughput method was developed for screening compounds with agonist or antagonist properties toward the CRE1/AHK4 cytokinin receptor in a single experiment using the Nanodrop II liquid handling system and 384-well plates. Potential ligands are screened directly, using a reporter system in which receptor signaling activity triggers expression of β-galactosidase in *Escherichia coli*. This enzyme generates a fluorescent product from a non-fluorescent substrate, allowing the agonistic/antagonistic behavior of tested compounds to be assayed in relation to that of an internal standard (here the natural ligand, *trans*-zeatin). The method includes a robust control procedure to determine false positive or false negative effects of the tested compounds arising from their fluorescent or fluorescent-quenching properties. The presented method enables robust, automated screening of large libraries of compounds for ability to activate or inhibit the *Arabidopsis thaliana* cytokinin receptor CRE1/AHK4.

**Keywords: high throughput screening, plant growth regulators, cytokinin, CRE1/AHK4 receptor, laboratory automation**

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***Edited by:***
*Roger Deal,*
*Emory University, United States*

***Reviewed by:***
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***Specialty section:***
*This article was submitted to Technical Advances in Plant Science, a section of the journal Frontiers in Plant Science*

***Received:*** *13 February 2017*
***Accepted:*** *22 May 2017*
***Published:*** *08 June 2017*

***Citation:***
*Klimeš P, Turek D, Mazura P, Gallová L, Spíchal L and Brzobohatý B (2017) High Throughput Screening Method for Identifying Potential Agonists and Antagonists of Arabidopsis thaliana Cytokinin Receptor CRE1/AHK4. Front. Plant Sci. 8:947. doi: 10.3389/fpls.2017.00947*

## INTRODUCTION

Plant growth regulators are diverse groups of chemical messengers, including cytokinins, that affect various processes in plant growth and development. Compounds that specifically interact with plant hormone pathways have high utility in research and potential agronomic applications for improving crop yields or other aspects of crops' performance (Basra and Lovatt, 2016; Koprna et al., 2016).

Currently there are several methods for testing substances' effects on plants. The oldest and most straightforward is to apply them to whole plants in different developmental stages, parts of plants or plant cell cultures, then monitor the physiological, morphological, anatomic, and/or biochemical responses. However, these classical methods are not usually suitable for high throughput screening (HTS) due to the laboriousness and costs of both preparing the test materials (e.g., whole plants) and evaluating phenotypic effects of the treatments. Furthermore, although such techniques

may identify substances with general effects on plant growth and development (for example, see Rodriguez-Furlán et al., 2016), they provide little or no information on the molecular level mechanisms involved.

A potentially more convenient and informative approach is to use an assay based directly on interactions between the tested compounds and proteins of interest (e.g., a cytokinin receptor, or reporter acting downstream of a targeted receptor) in a less complex system. Notably, heterologous systems have been used to express cytokinin receptors in a manner that functionally complements a two-component signaling pathway in a genetically modified yeast (Suzuki et al., 2001; Ueguchi et al., 2001) or *Escherichia coli* (Yamada et al., 2001). These systems can provide precise information about receptors' signal transduction activities and ligand specificities. Moreover, transformed yeast expressing the CRE1/AHK4 receptor has been successfully used to screen for compounds with antagonistic activity (Arata et al., 2010), based on differences in the yeast's growth (measured as changes in optical density at 600 nm, $OD_{600}$) in 96-well plates. However, the method has several disadvantages for use in HTS applications, including complications associated with the yeast's growth requirements and monitoring changes in the optical density.

The methodology reported in this work overcomes these disadvantages by using a strain of *E. coli* expressing a CRE1/AHK4 cytokinin receptor (Spíchal et al., 2004). CRE1/AHK4 signaling triggers expression of a β-galactosidase reporter gene, which can be detected by highly sensitive fluorescence measurements suitable for HTS. The described method provides a novel approach for screening cytokinin receptor agonists and antagonists in a single experiment, thereby identifying interesting compounds for further research and potential agronomical applications.

## MATERIALS AND METHODS

### *E. coli* Strain and Plasmid

*Escherichia coli* strain KMI001 (Δ*rcsC, cps::lacZ*, Takeda et al., 2001) harboring the plasmid pIN-III – AHK4 was used in all the experiments reported here. The Arabidopsis cDNA that encodes the entire amino acid sequence of CRE1/AHK4 (UNIPROT ID: Q9C5U0) is cloned downstream of the lpp-lac promoter, followed by a prokaryotic ribosome-binding site, on pIN-III (between the unique *Bam*HI and *Sal*I sites) as described by Suzuki et al. (2001). Entire size of the construct is 10.7 kb.

### Materials and Reagents

Sodium carbonate, citric acid, disodium phosphate, magnesium sulfate, and dimethyl sulfoxide were purchased from PENTA s.r.o. (CZ). 4-methylumbelliferyl-β-D-galactopyranoside (4-MUGal), 4-methylumbelliferon (4-MU), 7-amino-4-methylcoumarin (7-AMC) and rifampicin were purchased from Sigma-Aldrich. A library of 93 newly synthesized compounds and ZOGA-090 was obtained from Palacký University Olomouc (CZ) for testing the screening setup. Chemical structure of ZOGA-090 is shown in **Supplementary Figure S1**. Chemical structures of the ligands were not disclosed due to intellectual property protection. Bacto$^{TM}$ Casamino Acids were purchased from Becton, Dickinson and Company (United States). *Trans*-zeatin (tZ) was purchased from OlChemIm (CZ), and lysogeny broth (LB) medium from Duchefa Biochemie (NL).

Minimal (M9-505) medium, consisting of ZYM-505 medium (Studier, 2005) without N-Z-amine AS and yeast extract, was prepared and enriched with 0.1% v/v Casamino acids. The final concentration of ampicillin in this medium was 50 μg/ml and final concentration of the solvent (DMSO) was ∼1% (v/v). Concentration of DMSO up to 3% did not significantly change response of the detection culture. Radioactive-labeled [$^3$H]tZ (1,3 TBq/mmol) was obtained from the Isotope Laboratory, Institute of Experimental Botany, AS CR, Prague (CZ) scintillation cocktail was purchased from Perkin Elmer (United States).

### Live-Cell Cytokinin-Binding Assay

Intact *E. coli* cultures (strain KMI001), expressing CRE1/AHK4 cytokinin receptor (Suzuki et al., 2001; Yamada et al., 2001), were grown at 25°C overnight. M9 liquid medium, supplemented with casamino acids [0.1% (w/v)] and ampicillin (100 μg/ml), were used to reach $OD_{600}$ ∼1–1,4. The assay described by Romanov et al. (2005) was performed with slight modifications.

Each sample contained 1 ml of the overnight cell culture, 3 pmol of [$^3$H]tZ and various concentrations of unlabeled tZ/other tested compound (0.1 nM–50 μM). Negative control contained 3 pmol of [$^3$H]tZ and 0.1% (v/v) dimethylsulfoxide (DMSO; solvent), instead of the unlabeled compound. After 30 min incubation at 4°C, the sample was centrifuged (8,000 rpm, 4 min, 4°C) and supernatant was removed. Bacterial pellet was resuspended in 50 μl $dH_2O$. Subsequently, 1 ml of scintillation cocktail was added. Radioactivity was measured by a Hidex 300 SL scintillation counter Hidex (FL). High excess of unlabeled tZ (at least 3000-fold) was used for competition, to discriminate between specific and non-specific binding.

### HTS Equipment

A Nanodrop II liquid handling system (BioNex Solutions, San Jose, CA, United States), was used for all pipetting steps. BioNex Nanodrop II accessories can be mounted on two nests, mostly used for microtitration plates. There are also two positions for trays (containing in this case *E. coli* suspension and decontaminating bleach solution) or PCR tube holders. *E. coli* was cultivated using a microplate shaker with a controlled heating platform (ThermoMixer C, Eppendorf) and heated lid (ThermoTop, Eppendorf). For screening, sterile transparent 384-well plates (Corning, United States) were used. Optical densities ($OD_{600}$) and fluorescence intensities of the β-galactosidase-catalyzed reaction product (excitation and emission maxima: 365 and 448 nm, respectively) were measured using an Infinite M1000Pro plate reader (Tecan, CH). In case the HTS automation is not available the method could be downscaled and adapted for manual pipetting similarly as described by Spíchal (2011).

### Statistical Analysis

For multiple comparison analysis of the acquired data sets *t*-tests were used. Mean responses to test compounds, relative to DMSO

controls, were assessed by calculating *P*-values. These were deemed significant if lower than $\alpha_{ADJ}$, the nominal significance level following Šidák correction, derived from $\alpha_{ADJ} = (1-(1-\alpha)\hat{}1/m) = 0.00054$, where $\alpha = 0.05$ and $m = 95$ (Šidák, 1967). To describe the separation between responses to an internal standard (tZ at 50 nM) and both a positive control and a negative control (50 μM tZ and ZOGA-090, respectively), the Z′-factor described by Zhang et al. (1999) was used. All calculations were performed in MS Excel 2013.

# RESULTS

## Preparation and Optimization of Use of the *E. coli* Detection Culture

### General Description of the *E. coli* Detection Culture

As described by Spíchal et al. (2004), *E. coli* strain KMI001 expressing the CRE1/AHK4 cytokinin receptor has been used to develop a system for studying the receptor's interaction with potential agonists/antagonists. In this system, the CRE1/AHK4 receptor (a kinase) generates signal after interacting with an activating ligand presented in the growth medium. Further signal transduction triggers an engineered operon leading to expression of the reporter enzyme β-D-galactosidase (Suzuki et al., 2001), at a level related to the ligand's concentration, activating properties and duration of interaction with the receptor (Spíchal et al., 2004), up to a saturation level, beyond which increases in ligand concentration only result in marginal increases in signaling intensity (**Figure 1**). The assay results are expressed in terms of optical density of the bacterial culture and fluorescence intensity of the β-galactosidase-catalyzed reaction product, and the strength of the ligand-receptor interaction is described as the ratio between fluorescence intensity and optical density. In the study reported here, limitations of the detection system and optimal conditions were experimentally investigated.

### Selection of Growth Medium

The composition of the culture medium profoundly affects the *E. coli* strain's performance. Here, its performance was compared in tests with tZ and two types of media – Lysogeny broth (LB) medium and M9-505 minimal medium (modified ZYM-505 medium, Studier, 2005). LB medium is commonly used because it is simple to prepare and allows stable growth of bacteria, while minimal medium is used in situations where the medium's composition must be fully defined. As expected, *E. coli* grew faster in LB medium than in M9-505 medium, but although the cultures reached higher optical densities in LB medium, the measured fluorescence was higher in M9-505 medium, as shown by the final relative fluorescence unit (RFU)/$OD_{600}$ ratios (**Figure 1**). Thus, the M9-505 medium affords higher sensitivity. Moreover, further tests showed that the LB medium quenches the fluorescence signal by ∼20% (**Figure 2**). Thus, M9-505 medium was used in all further experiments.

### Effects of the Pre-cultivation Procedure on the Detection Culture's Sensitivity

Fresh KMI001-AHK4 detection culture must be prepared for each experiment. In the standard previously reported procedure a colony picked from a Petri dish or a portion of frozen stock of *E. coli* strain KMI001-AHK4 is used to start a 1 ml overnight culture, which is inoculated the following day into fresh medium to prepare the detection culture used to test compounds' interaction with the receptor (Mizuno and Yamashino, 2010; Spíchal, 2011). However, this straightforward procedure provides inconsistent responses to a standardized concentration of tZ due to variations in duration of cultivation of the *E. coli* (from the same frozen stock), and both the volume and dilution of the overnight culture (**Supplementary Figure S2**).

To avoid this variability, we developed the following procedure. First, a larger volume (50 ml) of overnight culture is prepared from a stock of frozen culture with verified activity

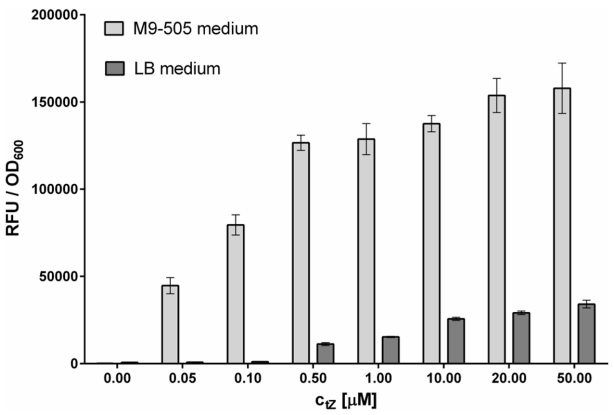


**FIGURE 1 |** Comparison of sensitivity of *Escherichia coli* reporter cultures grown on LB and M9-505 media. Experiments were performed in 96-well format involving incubation with tZ for 5 h at 25°C and 800 rpm shaking, followed by 30 min incubation with 4-MUGal substrate at 37°C and 800 rpm shaking, and finally measurement of fluorescence from mixtures of 50 μl of *E. coli* suspension and 100 μl of 0.2 M sodium carbonate. Means ± SD ($n = 4$) of ratios of final RFU to $OD_{600}$ shown. Cultures grown on M9-505 medium clearly provide much higher sensitivity than cultures grown on LB medium.

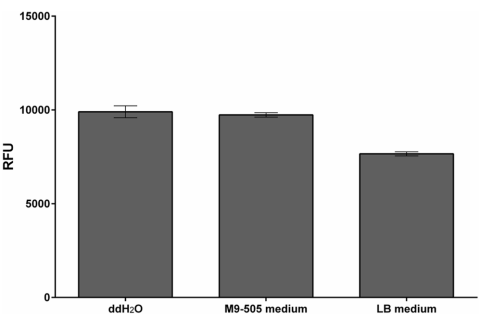


**FIGURE 2 |** Quenching properties of M9-505 and LB media. Comparison of quenching of fluorescence of the enzymatic reaction product (4-MU) by M9-505 and LB media. Means ± SD ($n = 3$) obtained in tests with solutions consisting of 30 μl of the media (or water), 9 μl of 1.2 M sodium carbonate and 1 μl of 2 mM 4-MU contained in wells of a 384-well plate. LB medium clearly quenched the fluorescence substantially more than M9-505 medium.

in tests with tZ. The next day the overnight culture is harvested, then prepared portions are frozen in liquid nitrogen, and stored at −80°C. These portions are directly diluted in medium and ready for use in screening. The procedure has several important advantages. The step that generates most variability in properties of the detection culture (cultivation of an overnight culture), is omitted. Frozen stocks are immediately available for HTS screening, experiments can be started at any time, and they provide highly stable responses to tZ. These responses are not linear but provide (in our experimental setup) a measurement window between 50 nM and 50 μM, which sacrifices resolution at the upper end of the concentration scale, but enables reliable detection of low concentrations of tZ, which is crucial for detecting antagonists by the HTS method (**Figure 3**).

### Effect of Detection Culture Cultivation Temperature on CRE1/AHK4 Signaling

The first phase of the assay involves incubating portions of the *E. coli* KMI001-AHK4 detection culture with test compounds for several hours at 25°C (Mizuno and Yamashino, 2010; Spíchal, 2011). Growth rates of *E. coli* are influenced by available oxygen levels in wells of the 384-well plates used (Duetz et al., 2000). Thus, to ensure sufficient aeration of 30 μl suspensions of bacteria growing in the M9-505 medium in the plates a high-speed microplate shaker (MixMate, Eppendorf, Germany) was initially used. However, results of experiments with this shaker had poor reproducibility, possibly due to transfer of excess heat from the shaker into the 384-well plate, which increased the cultivation temperature up to 29°C and thus apparently affected the signaling pathway. To confirm this hypothesis, further experiments were performed with incubation at both 25 and 29°C, using plates shaken in microplate shakers with a controlled heating platform (ThermoMixer C, Eppendorf) and heated lid (ThermoTop, Eppendorf). The results show that cultivation at 29°C reduces CRE1/AHK4 signaling activity in the *E. coli* KMI001-AHK4 detection system by 95% relative to the activity at 25°C (**Figure 4A**), although the bacteria grew faster (**Figure 4B**). Thus, the better growth conditions clearly suppress the signaling pathway or expression of the reporter enzyme, even in the presence of the strong agonist tZ (1 μM). Moreover, during the (5-h) incubation phase water evaporated from medium condensed on the inner side of the plate cover. However, using the heated lid in experiments stops water condensation and ensures thermal homogeneity across the plate. This setup also avoids the need for methods such as placing a wet paper towel under the plate in the plate holder to compensate for evaporation (Zhao, 2003).

The results as well as published data (Mizuno and Yamashino, 2010; Spíchal, 2011) clearly show that the receptor signaling pathway is suppressed by the higher temperature during the incubation of compounds with detection culture, thus it is highly important to keep the temperature constant (preferably at 25°C, unless further analyses indicate that another temperature is closer to optimal). They also show that the temperature can be robustly controlled using a microplate shaker that can control the temperature of both the shaking platform and lid.

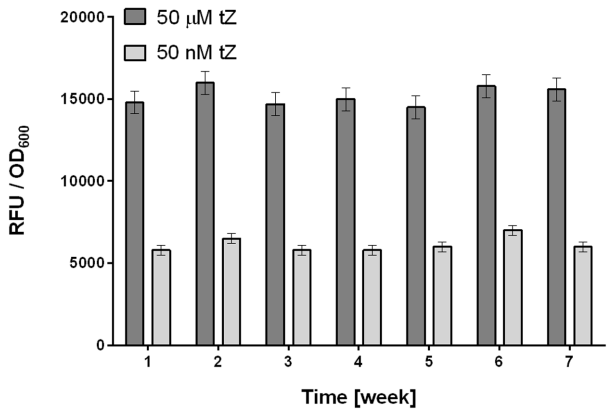


**FIGURE 3 |** Stability of responses to tZ of the optimally prepared *E. coli* KMI001-AHK4 detection culture. Frozen stocks of detection culture were tested weekly for 7 weeks, and provided stable responses to tZ at both low (50 nM) and high (50 μM) concentrations. Stocks of detection culture were prepared as follows. 50 ml of M9-505 medium in a 250 ml Erlenmeyer flask was inoculated with a stock KMI001-AHK4 culture then incubated at 25°C, with 250 rpm shaking for 20 h. The *E. coli* was then harvested by centrifugation (10,000 *g* for 10 min) and resuspended in 1 ml of 50% glycerol and M9-505 medium (1:1). Portions (50 μl) of this mixture were frozen in liquid nitrogen and stored at –80°C, then resuspended in 40 ml of M9-505 medium immediately before use as a detection culture in tZ response tests involving fluorescence measurement as in the standard high throughput screening (HTS) protocol (see below). Bars in the graph represent means ± SD ($n$ = 4).

## Properties of Compounds that Interfere with the HTS

### Interference by Compounds' Optical Properties with the Optical Density Measurements

After mixing *E. coli* detection culture with test compounds, optical densities were measured at 600 nm. Clearly the $OD_{600}$ will be increased, at least initially, by any compound that absorbs light at 600 nm, and such changes in $OD_{600}$ must be considered at the end of the experiment. This is not straightforward because compounds may be metabolized or degraded during the incubation. However, significant differences in $OD_{600}$ of detection cultures incubated with test compounds, relative to those of controls incubated with DMSO, that persist throughout the incubation must be accounted for when evaluating the data and identifying potential agonists/antagonists.

### Interference of Fluorescent Compounds, Fluorescence Quenchers, or Their Precursors, with Fluorescence Measurements

When large libraries of compounds are screened in HTS programs some could have similar fluorescent properties to 4-MU (measured at the end of experiment), or quench fluorescence from the formed 4-MU, thereby interfering with its determination. In addition, degradation or metabolization of compounds during their incubation with the *E. coli* detection culture could affect their fluorescent properties. To assess such potential effects (which clearly must be distinguished

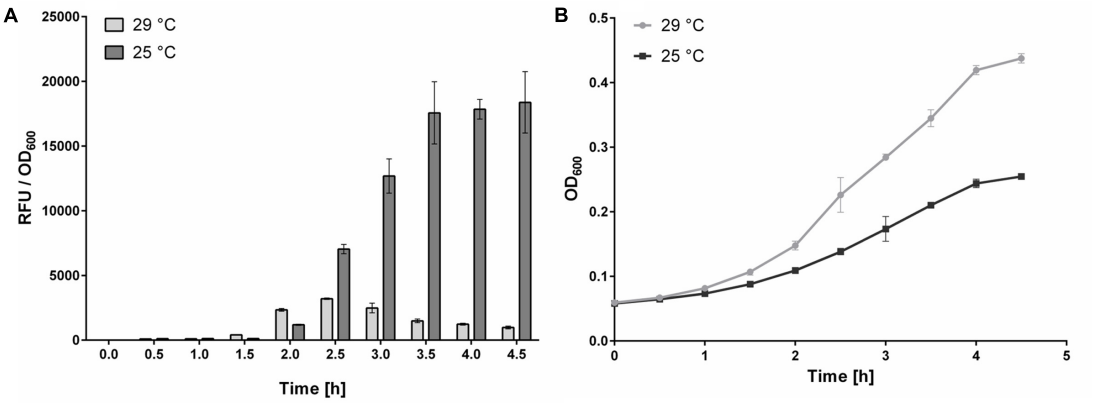


**FIGURE 4 |** Effect of temperature on AHK4 signaling. Signal to bacterial density (RFU to $OD_{600}$) ratios, and bacterial densities, obtained following incubation (for indicated times) of *E. coli* KMI001-AHK4 at 25 and 29°C in M9-505 medium supplemented with 1 μM tZ. 200 μl portions of KMI001-AHK4 detection culture were dispensed into two 96-well plates with tZ. One was cultivated at 25°C, and the other at 29°C, for 5 h with shaking at 800 rpm in a ThermoMixer C (Eppendorf). Every 30 min. three 30 μl samples were taken from both sets of *E. coli* suspensions, their optical density was measured then 0.3 μl of 50 mM 4-MUGal was added and incubation continued in a 384-well plate at 37°C with shaking at 1,400 rpm for 30 min. Then the reaction was stopped by adding 9 μl of 1.2 M sodium carbonate and fluorescence was measured. **(A)** The RFU/$OD_{600}$ ratios clearly show that detection culture cultivated at 29°C was much less sensitive than otherwise identically cultivated culture at 25°C. **(B)** Growth curves of detection cultures follow the classical trend, but in contrast to expectations the higher densities of *E. coli* cultivated at 29°C are associated with substantially lower RFU/$OD_{600}$ ratios. Thus, the KMI001-AHK4 detection culture must be incubated with test compounds at a constant temperature to obtain reproducible responses. In both **(A,B)**, data shown are means ± SD ($n \geq 3$).

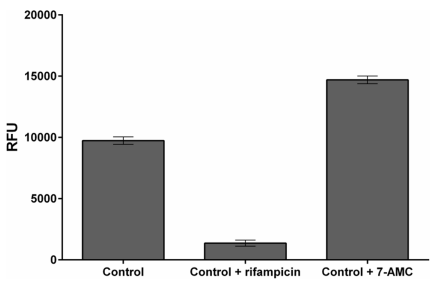


**FIGURE 5 |** Example of fluorescent analog and fluorescence-quencher. Effects of rifampicin and 7-AMC in M9-505 medium on fluorescence from 4-MU (Control), in wells of a 384-well plate. Final control solutions contained 30 μl medium (M9-505), 9 μl of 1.2 M sodium carbonate, and 1 μl of 0.2 mM 4-MU. 7-AMC and rifampicin were added to solutions (at a final concentration of 50 μM), as a fluorescent analog of 4-MU and a fluorescence-quencher, respectively, under the same conditions as test compounds.

and accounted for) we investigated the interference caused by the model fluorescent compound 7-amino-4-methylcoumarin (7-AMC) and fluorescence-quenching compound rifampicin (Richter et al., 2015; Żamojć et al., 2015). As anticipated, these compounds respectively increased and decreased fluorescence recorded in the assay (**Figure 5**).

The results show that fluorescent or fluorescence-quenching compounds may substantially affect results of the screening procedure, so we recommend inclusion of a reference plate to identify such compounds in the compound library.

### Effects of Compounds on Growth of *E. coli*

After incubating test compounds with *E. coli* detection culture for 5 h the optical densities of all samples in the 384-well plate are measured. In the presence of a strong agonist activation of the signaling pathway reduces growth of the *E. coli* due to intense expression of the reporter enzyme, as shown by differences (of about 20%) in the optical densities of negative and positive controls (containing DMSO and tZ, respectively). This significant decrease in optical density can be used as a secondary marker (in addition to high fluorescence) for identifying agonists. False positive agonists usually lack this property, as inhibitors of the signaling pathway generally do not perturb growth of the *E. coli*, although the theoretical possibility cannot be excluded. Some screened compounds may also potentially inhibit or promote *E. coli* growth. The possibility that a few test compounds may have complex effects that complicate detection of effects on the CRE1/AHK4 receptor *per se* is a limitation of the described HTS method.

## HTS Method

### Internal Standard

In order to distinguish between agonists, antagonists, and compounds that do not interact with the receptor in a single experiment, we introduced an internal standard procedure by adding the natural ligand tZ to the growth medium (to a final concentration of 50 nM) at the start of the experiment. At this concentration, tZ triggers a predetermined weak response of the receptor signaling pathway. In this setup agonists are able to trigger signaling pathway even to higher level leading into increase of the fluorescence intensity at the end of the experiment. Competitive antagonists compete with tZ for the same binding site of the receptor and thereby decrease fluorescence output. In the case of uncompetitive antagonism the decrease in the fluorescence output is also recorded. Compounds

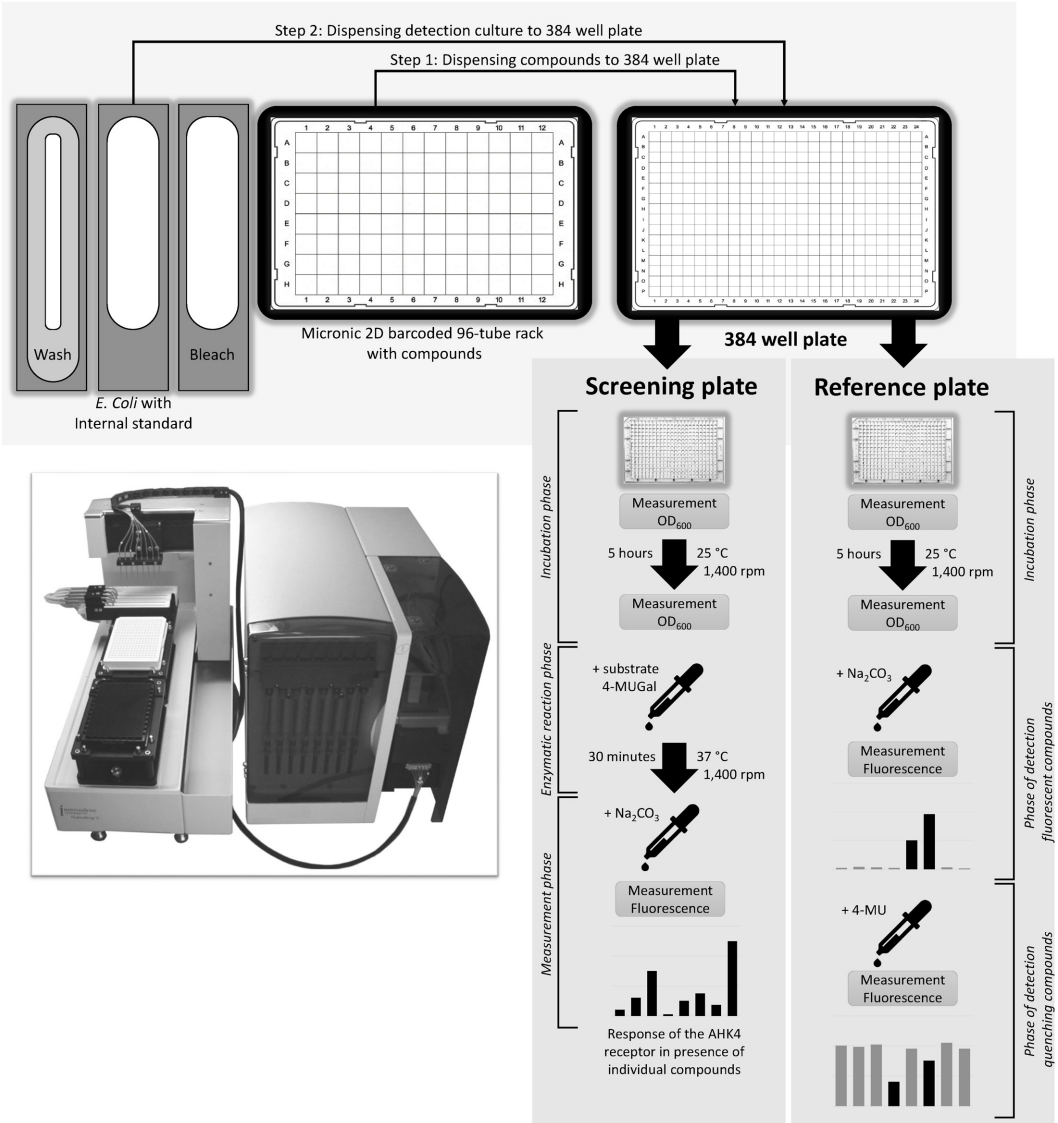


**FIGURE 6 |** High throughput screening scheme. A liquid handling system is used for pipetting compounds, *E. coli* detection culture, 4-MUG substrate, stop solution ($Na_2CO_3$) and fluorescence standard (4-MU). The screening plate is used for direct investigation of test compounds' effects on the AHK4 receptor (agonistic, antagonistic, or neutral). The reference plate is used for determining properties of test compounds that could interfere with the fluorescence measurements (by fluorescing like 4-MU or quenching fluorescence).

that do not interact with the receptor have no effect on fluorescence.

## Reference and Screening Plate Setup

As compounds tested in a HTS may have properties that interfere with fluorescence from 4-MU (**Figure 5**), two 384-well plates (screening and reference) are used in parallel to investigate the screened compounds' fluorescence properties and preclude artifacts.

Wells of the screening plate are filled with test compound solutions (concentration during test 50 μM), mixtures containing the *E. coli* KMI001-AHK4 detection culture and 50 nM of tZ then incubated for 5 h with shaking at 1,400 rpm. After this the substrate (4-MUGal) is added and the resulting mixtures are incubated for another 30 min at 37°C. Finally, the enzymatic reaction is stopped by adding 1.2 M sodium carbonate solution and fluorescence of the formed 4-MU is measured.

The reference 384-well plate is treated identically, except that addition of the 4-MUGal substrate and incubation at 37°C are omitted, and the fluorescence measurements (after addition of sodium carbonate) identify compounds that may fluoresce like 4-MU, by yielding stronger fluorescence than controls containing DMSO. A second fluorescence measurement is taken after adding 300 nl of 6 mM 4-MU to each well, to identify compounds that significantly reduce fluorescence relative to DMSO controls and thus are fluorescence quenchers. At this point the fluorescence

resulting from incubations with some test compounds may also exceed control levels, indicating non-specific additive interaction between 4-MU and those compounds. Thus, in all cases fluorescence properties of the hits must be evaluated by comparing results obtained from the screening and reference plates, as schematically illustrated in (**Figure 6**).

## Statistical Evaluation of the Screening Results and Empirical Rules for Hit Sorting

In the screening reported here each test rack contained 93 compounds and three controls—a positive control (the natural ligand tZ), a negative control (the antagonist ZOGA-090; Gallová et al., 2016) and a neutral control (DMSO, for quantifying the response solely to the internal standard)—in four technical replicates. The fluorescence to optical density ratio obtained in each case was calculated to normalize the results with respect to growth of the detection culture. The screening hits were defined as ligands that interact with the CRE1/AHK4 receptor and significantly increase or decrease the response induced by the internal standard. The means of the measured responses to tested compounds and the DMSO control were compared using Student's *t*-test. Due to the high number (95) of independent tests, the α (nominal significance) level were subjected to Šidák adjustment ($\alpha_{ADJ} = 0.00054$) to avoid type I errors (statistically significant but false positive test results; Abdi, 2007). Thus, statistically significant results (hits) must be checked to exclude possible false positive or negative effects caused by the compounds' non-specific interference with either fluorescence/absorbance measurements or effects on *E. coli* growth. Due to a high discovery rate of hits, to facilitate distinction of those warranting the closest further attention we applied empirical rules to sort identified hits into five groups: strong agonists, agonists, neutral agents (which do not induce a significantly different response from DMSO controls), antagonists and strong antagonists. Strong agonists were defined as compounds that induced responses at least mid-way between those of tZ at the test and internal standard levels (50 μM and 50 nM, respectively). The strong antagonists were defined as compounds inducing responses weaker than midway between responses to the internal standard (plus DMSO) and ZOGA-090. The agonists and antagonists were defined as compounds that induced responses that were weaker than these hits defining level, but significantly different from those induced by the internal standard.

## Hit Validation

The identification of HTS hits is based on the activity of the *lacZ* reporter down-stream of the heterologous signaling cascade, thus, it can't be excluded that the hit molecule influences the reporter activity by mechanism different from the interaction with the receptor active site, e.g., through interaction with other components of the cascade. For this reason a validation step is included into the screening procedure. The validation is based on the analysis of the affinity of the hit to the receptor ligand-binding site. Compounds selected by HTS screen as potential agonists and/or antagonists of CRE1/AHK4 were tested for their

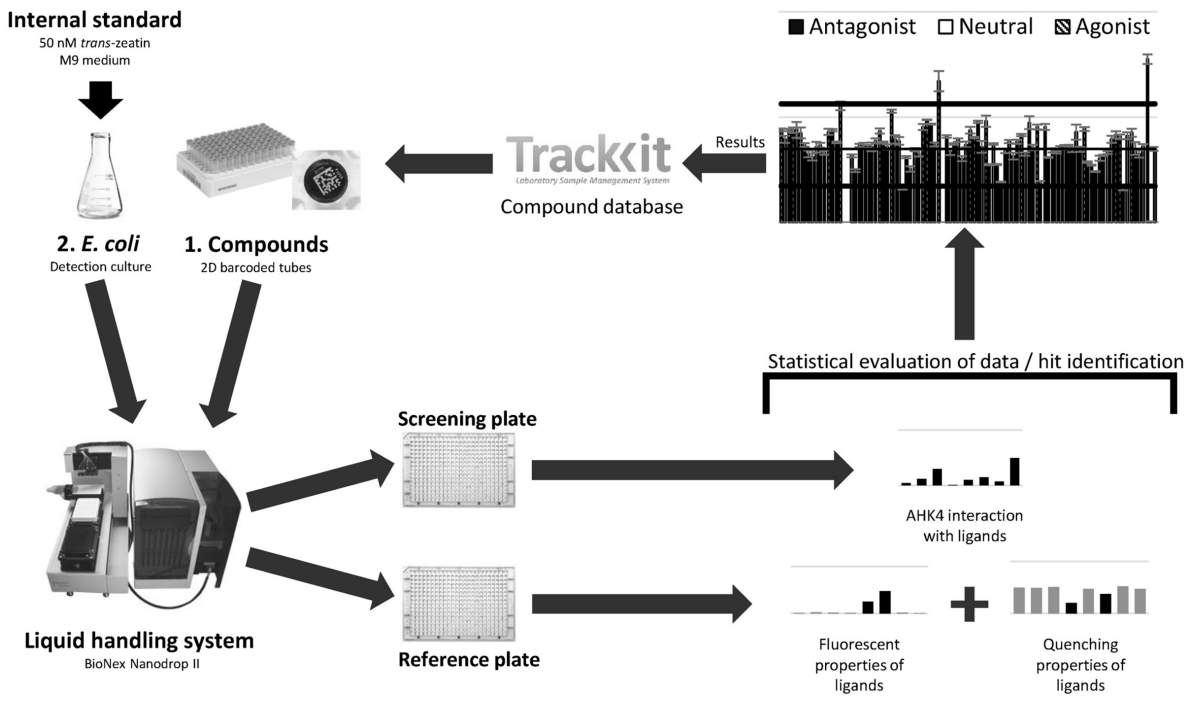


**FIGURE 7 |** High throughput screening workflow scheme. This is a complex procedure that involves selecting compounds for screening from the database, preparation of samples in 2D barcoded tubes, the screening procedure using the liquid handling system, statistical evaluation of obtained data and finally uploading of the results into the database.

ability to compete with tritium labeled natural ligand [$^3$H]tZ for binding into the receptor active site using the live-cell cytokinin-binding assay published by Romanov et al. (2005). The validation step shows whether the tested compound directly interacts with the receptor active site and unambitiously confirms the mode of action of the HTS selected hit. In positive case the hit is classified as a confirmed specific agonist or antagonist of the receptor CRE1/AHK4.

## HTS Workflow

The starting point of a HTS program is selection of compounds from a suitable database (here in TrackIt software). Selected compounds are prepared in the standard format, with 93 new compounds (one full rack with three positions for controls) diluted in DMSO at a stock concentration of 5 mM. Sample tubes are barcoded to enable tracking of compounds in the screening and selection of tubes for further analysis of hits. The results obtained for the screened compounds are evaluated and stored, together with procedure details, in the database for later processing, thereby closing a cycle (**Figure 7**).

## Example of HTS Performance with a Full Set of 93 Compounds

### Preparation of Bacterial Detection Culture

Fifty μl of frozen (−80°C) *E. coli* KMI001-AHK4 bacterial detection culture was thawed on ice (10 min) and then diluted with 40 ml of M9-505 medium. The resulting suspension was supplemented with the internal standard (2 μl of 1 mM tZ; final concentration, 50 nM).

### Automatic Liquid Handling Procedure

All pipetting steps were performed by a Nanodrop II liquid handling system (Bionex, NL). Each test compound and each of the control (0.3 μl) was dispensed from a tube rack (Micronic, NL) into 4 wells of a 384-well transparent microtitration screening plate (Corning, United States), then 30 μl of detection culture was dispensed into each well of the plate (Supplementary Data Sheet 1). The procedure is captured on video[1].

### Incubation and Detection Phases

The screening plate was incubated at 25°C with shaking at 1,400 rpm for 5 h (ThermoMixer C, Eppendorf). The optical density ($OD_{600}$) of the bacterial culture was then measured using an Infinite M1000Pro plate reader (Tecan, CH). 0.5 μl of 50 mM of the β-galactosidase substrate (4-MUGal in DMSO, final concentration 0.80 mM) was added to all wells and the plate was incubated at 37°C with shaking at 1,400 rpm for 30 min in a ThermoMixer C instrument (Eppendorf). The 4-MUGal hydrolysis catalyzed by the β-galactosidase reporter enzyme was stopped by adding 9 μl of sodium carbonate (1.2 M). The fluorescence resulting from incubation with each test compound was measured using an Infinite M1000Pro plate reader (Tecan), with excitation at 365 nm and emission at 448 nm, then expressed in terms of a RFU/$OD_{600}$ ratio.

A reference plate with wells filled with compounds and *E. coli* was also incubated for 5 h, as above. Then 9 μl of the sodium carbonate solution was dispensed to each well, fluorescence was measured and fluorescent compounds were

[1]https://youtu.be/EqW_bHA8Gd8

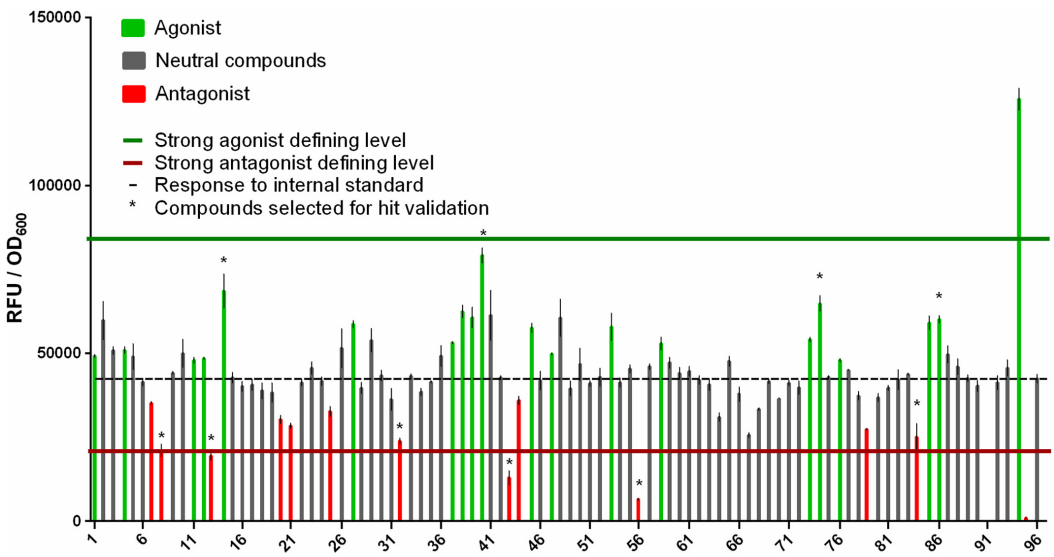


**FIGURE 8 |** Results of the test screen of 93 unknown compounds for ligands that interact agonistically or antagonistically with the CRE1/AHK4 receptor. Comparison of responses (RFU/$OD_{600}$ ratios) induced by each compound and the internal standard (no. 96, dashed line). The upper horizontal (green) line indicates the hit defining level for strong agonists (inducing stronger responses than the midpoint between responses induced by the internal standard and 50 μM tZ [no. 94]), while the lower horizontal (red) line indicates the hit defining level for strong antagonists (inducing weaker responses than the midpoint between responses induced by the internal standard and 50 μM ZOGA-090 [no. 95]). Ligand 91 suppresses growth of the detection culture, and thus yields no fluorescence. *Compounds selected for hit validation.

detected. Finally, 300 nl of 6 mM 4-MU was dispensed to each well and fluorescence was measured again to detect compounds that quench the fluorescence.

### Results of Test Run with a Full Set of 93 Compounds

In our setup a test set of 93 compounds is optimal because it completely fills a 96-well source plate (together with three controls). Thus, if large numbers of compounds are to be screened they should ideally be divided into groups of 93. Therefore, to assess the screening method's performance we subjected 93 compounds for which no information regarding activity was available, and subjected them to the screening procedure described above.

The wells in the 384-well screening plate were occupied by quadruplicate mixtures containing the 93 unknown compounds, at 372 positions in total, and three controls (tZ as a natural agonist of the receptor, ZOGA-090 as a known antagonist and DMSO to quantify the response to the internal standard) at 12 positions in total.

The responses induced by the internal standard, natural agonist tZ and antagonist ZOGA-090, expressed as RFU/$OD_{600}$ ratios, were 42,427 ± 1,292, 125,728 ± 3,235, and 898 ± 143, respectively. In addition, the separations between the response to the internal standard and both the positive control (tZ) and negative control (ZOGA-090) were very good, with Z′-factors of 0.84 and 0.90, respectively. Moreover 19 agonists and 12 antagonists (three defined as strong antagonists) were identified among the 93 unknown compounds. One compound suppressed growth of the detection culture, so its effect on the CRE1/AHK4 receptor could not be determined using our reporter system (**Figure 8**). None of the compounds significantly influenced the fluorescence measurement (**Supplementary Figures S3**, **S4**).

Ten compounds selected by HTS screen as potential agonists and/or antagonists of CRE1/AHK4 were tested for their ability to compete with tritium labeled natural ligand [$^3$H]tZ for binding into the CRE1/AHK4 active site. The excess of the tested compounds (concentration > 6,500-times higher than [$^3$H]tZ) was used to select the compounds capable of the effective ligand displacement. Cytokinin tZ (10 μM) and cytokinin antagonist ZOGA-090 (20 μM) were used as the agonist and antagonist positive controls, respectively. From the 10 HTS-selected hits one potential agonist (compound 14) showed effective displacement of the natural ligand from CRE1/AHK4 active site (**Figure 9A**). The detailed analyses confirmed that compound 14 competes for binding into the CRE1/AHK4 cytokinin binding site in the dose dependent manner (**Figure 9B**).

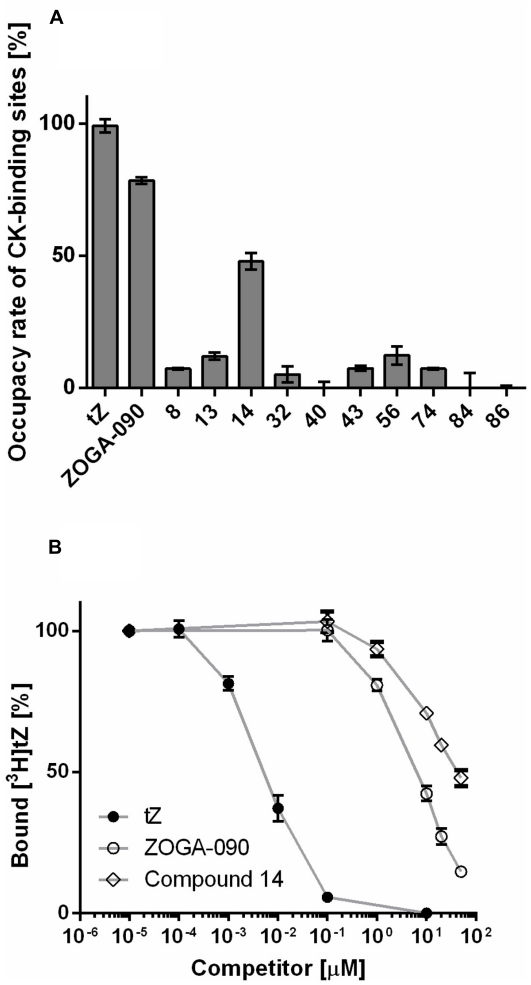


**FIGURE 9 |** Hit validation. **(A)** Live-cell cytokinin-binding assay with CRE1/AHK4 cytokinin receptor. Tested compounds (in 20 μM concentration) competed with radioactive-labeled [$^3$H]tZ at the receptor active site. Maximum binding affinity [100%] is related to 10 μM tZ. **(B)** The detailed analyses confirmed that compound 14 competes for binding into the CRE1/AHK4 cytokinin binding site in the dose dependent manner although the binding is weaker than for ZOGA-090.

## DISCUSSION

The optimization of the screening method described here shows that M9-505 medium rather than rich LB medium should be used to cultivate the detection culture as it provides high fluorescence intensity at the end of the experiment (and thus higher sensitivity). In addition, the test compounds should be incubated with the detection culture at a carefully controlled temperature at 25°C because the higher temperature suppresses activation of the signaling pathway. Use of a Thermomixer C instrument enables the required control via its platform and lid heating systems. The biological variability in *E. coli* cultures cultivated overnight could cause significant problems, but they can be avoided by preparing frozen stocks of *E. coli* KMI001-AHK4 culture with verified activity for use in the screening procedure.

The screening setup was designed to distinguish both agonists and antagonists in a single run by including a low concentration of the natural agonist tZ in each assay mixture. Thus, agonists and antagonists should respectively increase and decrease the response of the CRE1/AHK4 receptor to the natural agonist, provided false hits caused by compounds with fluorescent or fluorescence-quenching properties are distinguished using a reference plate.

Comparison of screening and reference plates enables identification of compounds with properties that interfere with fluorescence signals from 4-MU, thereby complicating evaluation of their effects on CRE1/AHK4 signaling. Thus, incorporation of this step eliminates false agonist/antagonist hits during the screening procedure and saves the work and time needed for reevaluation of false hits.

A suitable system for storing and tracking samples is vital for HTS. Thus, the potential ligands library was stored in Micronic 2D barcoded tube racks, thereby assigning a unique number and rack position to every compound. In addition, rack positions and screening results were uploaded into an online (TrackIt) database, thus facilitating searches for specific compound and maintaining direct links between the screening information and individual compounds.

The procedure for identifying hits in HTS depends on two sets of factors. One set consists of inherent features of the screening assay that influence the system's technological and biological variability, and thus the ability to separate signal and noise. The other set consists of factors associated with hits' significance, such as the screening objectives, planned follow-up analyses, and budgetary constraints.

The assay presented here exploits a signaling cascade in living *E. coli* cells, so inevitably there is a significant amount of inherent biological variability in the system. Nevertheless, the optimized assay behaves highly consistently, enabling easy discrimination of small differences between responses induced by test compounds. A major screening objective was to identify the best agonists/antagonists of the CRE1/AHK4 receptor, so we decided to define strong agonists and strong antagonists by comparing compounds' responses with those of the well-defined agonist tZ and antagonist ZOGA-090 using robust statistical criteria. Use of such criteria for detecting important hits from a test library of compounds could be extended to hit selection in HTS more generally. HTS should select the potential agonists and/or antagonists of the receptor CRE1/AHK4, i.e., compounds with specific affinity to the receptor active site. However, the selection can contain also false positive hits influencing the assay read-out message through interaction with other members of the down-stream signaling cascade and/or the reporter activity. A validation step is thus needed to discriminate the compounds interacting with the receptor out of its active site, e.g., with kinase out-put domain, non-specific inhibitors of phosphotransfer, or inhibitors of β-galactosidase. In the presented method the validation step is based on the direct confirmation of the hit interaction with the ligand-binding site. However, whatever cytokinin-specific bioassay can be used for the further functional validation of a cytokinin receptor agonist or antagonist as described, e.g., by Doležal et al. (2006) and Spíchal et al. (2007), respectively.

The developed method can be used for screening libraries of compounds to find potential plant growth regulators that specifically target cytokinin perception. Moreover, its automation, miniaturization and ability to identify agonistic or antagonistic compounds in a single step enable highly efficient screening, and the extension of the method enables detection of false hits due to test compounds having either fluorescence or fluorescence-quenching properties. The method is capable to uncover interfering properties of screened ligands even when these properties are developed just during the screening procedure.

## AUTHOR CONTRIBUTIONS

PK programmed the robot, DT optimized cultivation of *E. coli* cultures, PK and DT performed the experiments, and analyzed data, PM invented the method, designed the research and analyzed data, LG created compound library, designed and performed the validation step, LS designed the research and interpreted data, BB supervised all facets of the project. All authors contributed to write the manuscript.

## FUNDING

This research has been financially supported by the Czech Science Foundation under project GA15-19266S, the Ministry of Education, Youth and Sports of the Czech Republic under project CEITEC 2020 (LQ1601) and grant LO1204 from the National Program of Sustainability I, and the Internal Grant Agency of Palacký University (IGA_PrF_2017_010).

## ACKNOWLEDGMENTS

We thank the SCALED BIOSYSTEMS team for help and useful ideas and suggestions. Access to the MetaCentrum computing facilities provided under the program "Projects of Large Infrastructure for Research, Development, and Innovations" LM2010005, funded by the Ministry of Education, Youth, and Sports of the Czech Republic, is highly appreciated.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00947/full#supplementary-material

**FIGURE S1 |** ZOGA-090 (*N*-(3-methylbut-2-en-1-yl)-5*H*-pyrrolo[3,2-*d*]pyrimidin-4-amine) is a 9-deaza derivative of a natural cytokinin isopentenyladenine (iP). The lack of nitrogen in position 9 decreases affinity to the cytokinin receptors and mainly causes complete loss the receptor activation capacity.

**FIGURE S2 |** A detection culture that provides a stable response is required for the HTS procedure. Responses to 100 nM tZ of detection cultures prepared from cultures grown overnight (from frozen stocks) for times varying from 15 h to 23 h cover a 2-fold range (S12 A). Volumes of detection cultures ranging from 1 to 4 ml prepared from overnight cultures (started at the same time) provide (at least in the illustrated case) linear responses to 100 nM tZ (S12 B). However, the dilution also affects properties of the detection culture, as illustrated by results obtained with 20-, 50- and 100-fold dilutions of overnight culture (S12 C). Thus, even after optimizing the duration of cultivation, cultivation volume and dilution of overnight culture, our efforts to prepare stable detection cultures from overnight cultures for every experiment were not entirely successful.

**FIGURE S3 |** Fluorescence of compounds measured in a 384-well reference plate after 5 hours incubation with *E. coli* strain KMI001-AHK4 and addition of sodium carbonate solution. There were significant differences between fluorescence induced by several compounds and DMSO (in wells O23, O24, P23, P24), but these measured values did not affect the evaluation of the data set (the response induced by the internal standard was ∼6000 RFU).

**FIGURE S4 |** Quenching of fluorescence of compounds measured in a 384-well reference plate after 5 hours incubation with *E. coli* strain KMI001-AHK4 and addition of sodium carbonate solution and 4-MU. The measured fluorescence intensities were not significantly different from those induced by control (DMSO) (No. 96) at the significance level $\alpha_{ADJ} = 0.00054$ according to Šidák correction.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**PALACKÝ UNIVERSITY IN OLOMOUC**

FACULTY OF SCIENCE

DEPARTMENT OF BOTANY

&

DEPARTMENT OF CHEMICAL BIOLOGY AND GENETICS

# Study of the Ligand Specificity of Cytokinin Receptors

Ph.D. THESIS

BY

Msc. LUCIA HLUSKOVÁ

IN PROGRAMME BIOLOGY-BOTANY

P1527

SUPERVISED BY

Msc. Lukáš Spíchal, Ph.D.

2019

This thesis has been completed due to work of the Ph.D. study program P1527 Biology-Botany (1507V004), guaranteed by the Department of Botany, Faculty of Science, Palacký University in Olomouc in the period 2011-2019.


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# SÚHR

Cytokiníny patria medzi dôležité rastlinné hormóny, ktoré sú prijímané hybridnými histidinovými kinázami. Receptory cytokinínov už boli identifikované a charakterizované medzi mnohými modelovými rastlinami. Identifikovali sme 5-člennú rodinu cytokinínových receptorov u agronomicky dôležitej plodiny repky olejnej (*Brassica napus*). Naklonovali sme 3 receptory (BnCHK1, 3, 5) a určili ich väzbovú špecifitu.

Štruktúra cytokinín-vnímajúcej CHASE domény už bola vopred objasnená, ale ostáva nejasný mechanizmus aktivácie receptora a prenos signálu cez membránu. Dva leucíny tvoria platformu v tvare písmena C, ktorá interaguje s N9 atómom na molekule cytokinínu. Na potvrdenie dôležitosti tejto väzby sme nasyntetizovali 9-deaza analógy cytokinínov a testovali sme ich vplyv na afinitu a aktiváciu CRE1/AHK4.

Vnesením bodových mutácií do vnútra CHASE domény CRE1/AHK4 sme analyzovali dôležitost konzervovaných zvyškov vo väzbovom mieste. Využili sme miestne riadenú mutagenézu za účelom zmeny preferencie ligandov špecifickejšie k vlastnostiam AHK3.

# SUMMARY

The important plant hormones cytokinins are perceived by hybrid histidine kinases. The cytokinin receptors have been identified and characterised already in many model plants. Here, we have identified 5 member-family of cytokinin receptors in agronomically important plant *Brassica napus*. We have cloned three receptors (BnCHK1, 3, 5) and determined their ligand specificity.

The structure of the cytokinin-sensing CHASE domain has been elucidated previously, but the mechanism of activation of the receptor and of the transfer of the signal accross the membrain is still unclear. Two leucines form a C-shaped docking platform interacting with the N9-atom of bound cytokinin. To confirm importance of this bond, we have synthesised 9-deaza-analogues of cytokinins and tested its influence on affinity and activation of CRE1/AHK4.

To analyse the importance of conserved residues in the binding pocket, point mutations within the CHASE domain of CRE1/AHK4 were introduced. Side-directed mutagenesis was used to change the ligand preference to obtain an AHK3-like profile.

# LIST OF CONTENTS

# AIMS OF THE WORK

1) Characterisation of ligand specifity of the *Brassica napus* cytokinin receptors
2) Testing of specifity of interactions of selected ligands and mutant forms of cytokinin receptors for better understanding of the plant signalling system using molecular biology and chemical biology approaches
    a. Studies of cytokinin receptor affinity and activation using a chemical biology approach.
    b. Analysis of the importance of the conserved residues in the binding pocket of AHK3 and AHK4

The interaction between individual cells at the organ and tissue levels is mediated by extracellular signal molecules that regulate growth and development of multicellular organisms (Davies, 1995). Cytokinins influence virtually all physiological processes: among the most important ones belong stimulation of cell division, branching, vascular bundle differentiation, chloroplast differentiation and delay of chlorophyll degradation and hence of plant senescence (Schmülling, 2004). Different processes of cytokinin metabolism are subcellularly compartmentalised, probably contributing to the regulation of the amount of active hormones (Kiran et al., 2012).

## Cytokinin Signaling and its Origins

Stock et al. (1990) defined two families of homologous proteins transferring signals among 11 prokaryote species. They included sensory histidine kinases (HKs) and response regulators (RRs). The level of RR phosphorylation is further regulated by the activity of phosphatases. His-Asp phosphotransfer system is present mainly in bacteria, but it is rather rare in eukaryotes, which rely mostly on Ser, Thr and Tyr phosphorylations.

The response regulators control a wide range of processes in the cell by transferring the signal from receptors to elements responsible for regulation of movement or gene expression (Stock et al., 1990).

### A. Molecular Mechanisms of the His-Asp Phosphorylation System

The cytokinin signal transduction pathway in the model plant *Arabidopsis thaliana* is based also on the TCS system. It is composed of four principal steps (Fig. 1): first, sensing and signalling by histidine protein kinase, next translocation of a phosphotransfer protein, Arabidopsis Response Regulator-dependent trancriptional activation and a negative feedback loop *via* cytokinin-induced expression of some *ARR* genes.

The cytokinin signal is perceived by a membrane-bound CHASE domain. Cytoplasmic-to-nuclear transfer of the phosphate residues is mediated by *Arabidopsis* phosphotransfer (HPt) proteins (AHP1-5), that phosphorylate *Arabidopsis* type-B RRs (ARR-B 1,2,10-14,18-21) in the nucleus (Hwang et al., 2012; Hwang and Sheen, 2001). ARRs are clasified into three types: type-A, type-B and type-C. ARR-As have a function as negative-feedback regulators of the cytokinin signalling pathway (To et al., 2004). The last group of ARRs are type-C (ARR22 and ARR24), whose transcription level is not activated by cytokinins (Kiba et al., 2004). Consequently the role of the ARR-Cs in the TCS is currently not clear. Cytokinin response factors are also involved in regulating the transcriptional response to cytokinin. Their interaction with members of the TCS has been shown (Cutcliffe et al., 2011).

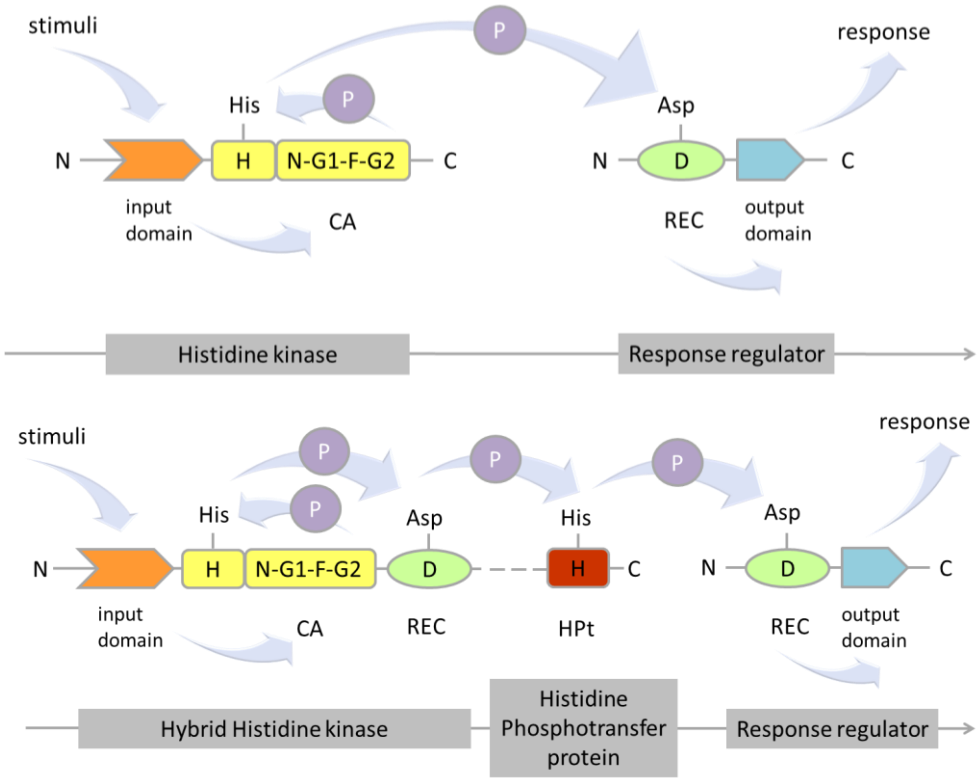


Figure 1: Scheme of two-component systems (TCS).
a) The prototype of the TCS signalling pathway has features as a conserved phosphoryl transfer to link various input stimuli and output responses. A phosphate residue is transferred from the highly conserved kinase core (CA – catalytic domain) to receiver (REC) domain.
b) The multi-step phosphorelay system uses a more complex pathway for multiplication of phosphotransfer events in comparison with TCS (figures modified from Gao and Stock, 2009).

## B. Histidine Kinases in *Arabidopsis thaliana*

*Arabidopsis* contains 6 histidine kinase receptors that do not belong to ethylene or phytochrome receptors. These are: AHK1 (AtHK1) – an osmosensor (Urao et al., 1999; Wohlbach et al., 2008); three cytokinin receptors AHK2, AHK3 and CRE1/AHK4 (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001b; Yamada et al., 2001); AHK5 (CKI2; Desikan et al., 2008; Iwama et al., 2007; Mira-Rodado et al., 2012) and CKI1 (Hwang et al., 2012; Kakimoto, 1996).

The first cytokinin receptor was unambiguously identified only at the beginning of the 21st century (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001b; Yamada et al., 2001). CRE1 successfully complemented a dysfunctional histidine kinase receptor in a yeast system in a cytokinin-dependent manner (Inoue et al., 2001). Further, it was confirmed in the same year, that CRE1 (also called Arabidopsis HISTIDINE KINASE4 (AHK4) or WOODEN LEG (WOL)) binds cytokinins specifically in the extracellular domain and transfers the signal through the membrane (Suzuki et al., 2001; Ueguchi et al., 2001b; Yamada et al., 2001). However, if cytokinin

is absent, CRE1/AHK4 acts as a phosphatase and removes phosphate from AHP proteins to decrease phosphate load in the system (Mähönen et al., 2006).

Further, additional genes *AHK2* and *AHK3* were identified as cytokinin receptors in *Arabidopsis* (Higuchi et al., 2004; Hwang and Sheen, 2001; Nishimura et al., 2004). The key property which distinguishes the cytokinin receptors from other histidine kinases is the presence of CHASE (Cyclases/Histidine Kinases Associated Sensory Extracellular) domain (Mougel and Zhulin, 2001). There are 2 (CRE1/AHK4) or 3 (AHK2 and AHK3) transmembrane domains at the N-terminus of the receptors.

The first model of *Arabidopsis* cytokinin receptor localisation was based on bioinformatic predictions and on homology with bacterial and yeast histidine kinases (Inoue et al., 2001; Ueguchi et al., 2001a). The first experimental evidence came from *Arabidopsis* protoplasts in which a fusion protein of AHK3 with green fluorescent protein (AHK3-GFP) was observed in the plasma membrane (Kim et al., 2006). The optimal condition for AHK3 to bind *t*Z is a basic environment; for CRE1/AHK4, it is a basic to neutral environment (Romanov et al., 2006). These conditions are typical for the cytoplasm, nucleus, endoplasmic reticulum (ER) or mitochondria, while the apoplast is characterised by an acidic environment. Thus, Romanov et al. (2006) postulated location of the CHASE domain inside of the cell. Further experiments showed sensitivity of AHK3 to endoglycosidase H. Thus, AHK3 contains mannose structures, which are sensitive to endoglycosidase H, characteristic for glycoproteins in the ER (Caesar et al., 2011).

It is assumed that the CHASE domain is in the lumen of the ER and the C-terminal kinase domain is localised in the cytoplasm (Caesar et al., 2011). Based on results from membrane fractionation and subsequent cytokinin-binding assays, the localization of AHK2 and AHK3 receptors was confirmed mostly in the internal membrane system. In contrast, the CRE1/AHK4 receptor was detected also in the plasma membrane. Zürcher et al. (2016) characterised PUP14 (purine permease 14) cytokinin transporter with plasma membrane localization, which import bioactive cytokinins into the cytosol. By taking up ligands from the apoplast, they suppressed the response to cytokinins (Zürcher et al., 2016). Thus, the localization of cytokinin receptors remains unclear.

## C. Characterisation of the CHASE Domain of CRE1/AHK4

By comparing the CRE1/AHK4 amino acid sequence with sequences from different bacterial membrane proteins, a conserved region called the CHASE domain was characterized. A large number of CHASE domain-containing proteins were found in bacterial genomes. Some are histidine kinases, hybrid forms of histidine kinases or various types of receptors with other catalytic function, especially adenylate or guanylate cyclases (Mougel and Zhulin, 2001).

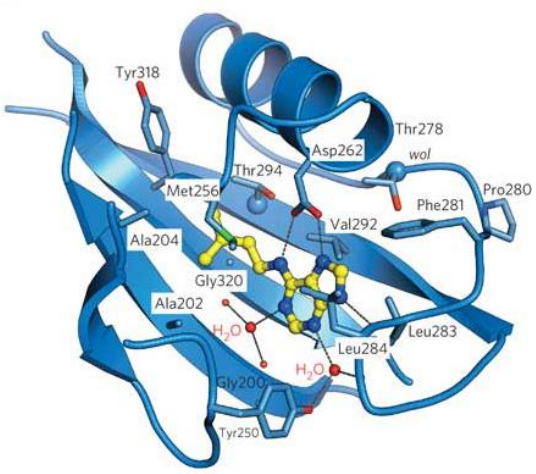


Figure 2: AHK4 binding pocket with ligand. The threedimensional structure shows complex of the membrane-distal PAS domain with iP inside of its binding cavity. Amino acid residues connected via hydrogen bonds to the ligand are displayed (from Hothorn et al., 2011).

Part of the CHASE domain are two PAS (Per-Arnt-Sim) domains (Pas et al., 2004), membrane-proximal and membrane-distal (Steklov et al., 2013). However, the membrane-proximal PAS domain misses one β-strand and hence is called PAS-like or pseudo-PAS (Henry and Crosson, 2011; Steklov et al., 2013). However, PAS domains were primarily recognised as intracellular sensor domain (Ponting and Aravind, 1997; Zhulin et al., 1997). It was shown, that extracellular PAS (PDC)-like domains should have their own superfamily, which was named the Cache superfamily (Upadhyay et al., 2016).

In the binding pocket the cytokinin adenine is bound by hydrogen bonds to Asp262 and Leu284 (Fig. 2). Other interactions are through 3 water molecules: the first water molecule forms hydrogen bond between the hydroxyl of Tyr250, backbone of Leu284 and N3 of adenine. The second water molecule is near N1 of adenine without interaction with the protein, while the third molecule is near the cytokinin side chain and Asp262 and Thr294 (Steklov et al., 2013). Hydrogen bonds between Asp285 and the adenine ring are indispensable for receptor function.

## Cytokinin Physiological Bioassays

The essential attributes that determine the efficacy of a bioassay are as follows – it must be specific to cytokinins, highly sensitive, detecting minute quantities of the more active cytokinins (for detection from plant extracts) and it must be quantitative. It should have a short assay time, to avoid long delays in experiments and to obtain more accurate measurements of activity. The ideal bioassay would last only hours or even minutes as bioassays are not capable of distinguishing between direct cytokinin action and indirect effects due to metabolic conversions. Especially naturally occuring compounds are rapidly metabolised or broken down in plant tissues (Mok and Mok, 1994). The most cytokinin physiological bioassay are based on seed germination and cotyledon expansion, chlorophyl retention, increase of callus biomass and promotion of new buds formation. A unique assay is based on cytokinin-induced synthesis of betacyanin in Amaranthus cotyledons in the dark.

To investigate the role of each cytokinin receptor, various cytokinin-regulated processes were studied in single and higher-order knock-out mutants of AHK2, AHK3 and/or CRE1/AHK4.

All bioassays have their intrinsic shortcomings, often other groups of hormones influence the same biological proceses (Mok and Mok, 1994) and many of the responses are caused by multiple cytokinin receptors in an additive manner. Therefore, it is relevant to study regulatory molecular mechanismus in greater details (Riefler et al., 2006).

## Molecular-based Cytokinin Biotests

The *Arabidopsis* reporter gene assays are based on the ability of cytokinin to induce gene expression. Cytokinins increase the expression of type-A ARRs (D'Agostino et al., 2000). The induction of the type-A *ARR5* gene by exogenous cytokinin is characteristic for cytokinin primary-response genes (Brandstatter and Kieber, 1998; D'Agostino et al., 2000).

In an alternative assay, a two-component output sensor (pTCS) was used to generate a universal cytokinin reporter system in *Arabidopsis*. The reporter system harbours conserved concatemeric 5´-(A/G)GAT(T/C)-3´ type-B ARR-binding motifs (Hosoda et al., 2002; Imamura et al., 2003; Sakai et al., 2000). The *pTCS::LUC* synthetic reporter system was designed for visualization of cytokinin output in an *in vivo Arabidopsis* mesophyll protoplast assay and it showed activation only with cytokinins. Subsequently, a GFP reporter, controlled by the *pTCS* promoter, was generated to determine its expression patern *in planta*. New version of *pTCS::GFP* was published (Zürcher et al., 2013). Variations inside the *TCSn* sequence reflect the range of potential diversity among sites and avoid sequence monotony that could trigger the silencing of GFP in transgenic plants (Zürcher et al., 2013).

### A. Experimental Systems to Study Receptor Activation, Affinity and Ligand Specificity

The first system used to demonstrate that *Arabidopsis* His kinase is capable of functioning as a cytokinin-responsive sensor, was based on the introduction of the receptor into living cells of fission yeast, *Schizosaccharomyces pombe*. The yeast uses a multistep His→Asp phosphorelay system, consisting of three His-kinases (Phk1/2/3), HPt factor (SpyI) and response regulator (Mcs4) to regulate progression of the cell from G2 to mitosis (G2/M). Mutant cells ΔPhk1/2/3 exhibit a changed phenotype – ovoid shape and shorter cell morphology in comparison with WT *S. pombe*. After introducing the AHK4 gene, the phenotype defect of the ΔPhk1/2/3 cells was abolished, when cells were grown on medium with *tZ* (Suzuki et al., 2001).

Another system for detecting receptor function was *E. coli* transformed with the receptor gene. In this prokaryotic eubacterium, many processes are controlled by His→Asp phosphorelay signaling pathways (Suzuki et al., 2001). The Rcs-phosphorelay system in *E. coli* is the most similar pathway to the cytokinin signal transduction system in *A. thaliana* (Suzuki et al., 2001). The RcsC

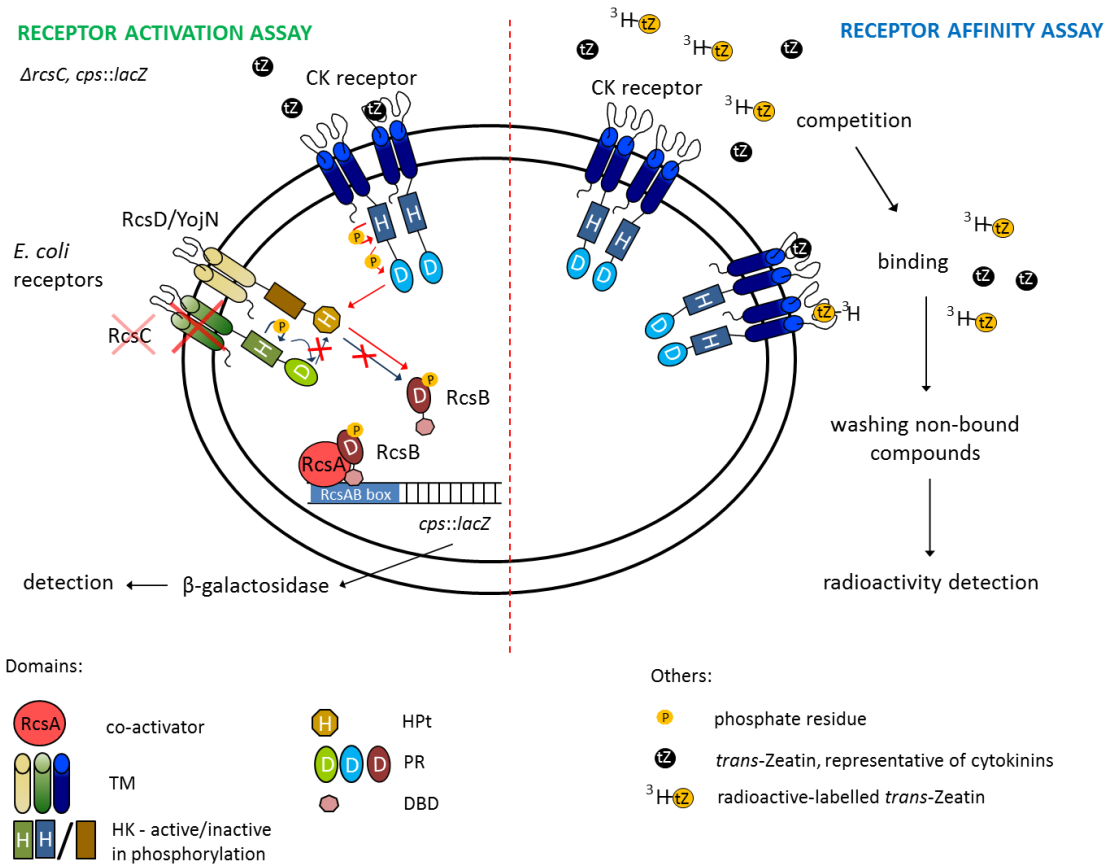


Figure 3: Depiction of two fundamental methods to determinate activity and affinity of cytokinin receptors in *E. coli* cells (modified from Rogov et al., 2006; Spíchal, 2011; Takeda et al., 2001; Wehland and Bernhard, 2000).

hybrid sensor is structurally similar to AHK4, RcsD acts as an AHP factor and RcsB protein operates as a response regulator. The screening system was prepared by cloning *CRE1/AHK4* into the Δ*rcsC* and *cps::lacZ* genetic background, for blue/white test (Fig. 3).

Plant assays with microsomes isolated from tobacco (*Nicotiana benthamiana*) leaves transiently expressing cytokinin receptor genes were preformed by Lomin et al. (2015). Receptor expression was controlled by means of a fluorescence signal.

## B. Point Mutations in the Cytokinin Receptor Sequences

In many cases, point mutations caused modification of activity or binding capacity of the CRE1/AHK4 cytokinin receptor (Fig. 4).

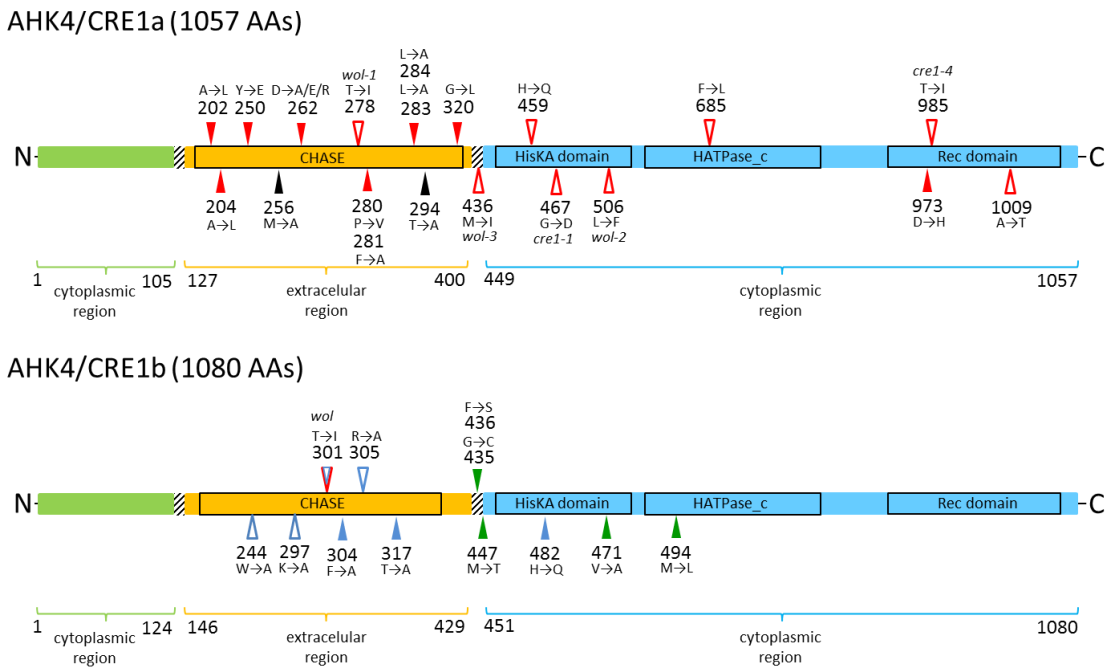


Figure 4: Overview of point mutations that have been tested so far and their influence on physiological effects. Two variants of CRE1/AHK4 were tested: shorter protein version (CRE1a/AHK4 - 1057 amino acids; de León et al., 2004; Franco-Zorrilla et al., 2002; Hothorn et al., 2011; Inoue et al., 2001; Kuroha et al., 2006; Mähönen et al., 2000, 2006b) and the longer version (CRE1b/AHK4 - 1080 amino acids; Heyl et al., 2007; Miwa et al., 2007; Suzuki et al., 2001; Yamada et al., 2001).

## C. Chemical-biology Approaches to Study Cytokinin Response

Cytokinin derivatives are tested in various assays as described above – monitoring their effect on the protein and gene level and their influence on cytokinin-dependent processes in the plant (Doležal et al., 2006).

Antagonist substances, which act as inhibitors of cytokinin action – are called anticytokinins (Nisler et al., 2010). They inhibit the activity of cytokinin receptors. For example,

$N^6$-(benzyloxymethyl)adenosine, showed high specificity to cytokinin receptors of *Arabidopsis*. It inhibits the activation of CRE1/AHK4, but not of the AHK3 receptor (Krivosheev et al., 2012).

Small structural variations, such as the presence and position of a hydroxyl group, also have influence on the activities in bioassays of compounds derived from isoprenoid cytokinins (Mok and Mok, 2001). PI-55, a derivative of BAP with substituents at the *o*- and *m*- positions of the aromatic side chain, caused physiological changes as in the case of receptor loss-of-function mutations in *Arabidopsis*. In addition PI-55 was able to compete with naturally occuring cytokinins for binding space in AHK3 and AHK4 receptors.

Another reported cytokinin antagonist, derived from PI-55 with substitution at the C2-, N7- and N9-position of the adenine ring, were tested in classical cytokinin biotests (Nisler et al., 2010). From 11 tested compounds, 6-(2,5-dihydroxybenzylamino)purine (LGR-991) was found to antagonize cytokinin activity most effectively in transgenic *ARR5::GUS Arabidopsis* plants. It also inhibited CRE1/AHK4 and AHK3 receptors. In addition, a methyl group at the position N9 of the adenine ring in derivtive of PI-55 prevented binding or activation of the receptors, causing loss of activity in all three classical cytokinin bioassays (Nisler et al., 2010). A set of iP derivatives alkylated at the N9-position were also prepared and tested (Mik et al., 2011b). Ethoxyethyl-, acetoxy-, azido-, 4-chlorobutyl- and 3-cyanopropyl-groups negatively influenced recognition by CRE1/AHK4, ZmHK1 and ZmHK3a, but they improved proliferation in the tobacco callus assay (Mik et al., 2011b). N9-derivatives were not recognised by CRE1/AHK4 and in the case of ZmHK3 the effect was observed only at high concentrations.

However, substitutions at the end of the alkyl group appear to be critically determinant during activation of the receptor, probably related to the dimensions of the terminal group. The polarity and length of the spacer in the N9-position of the iP derivatives had significant impact on the activity of compounds in the tobacco assay. Significant cytokinin activity of the N9-derivatives can be caused by the existence of other ways for transmitting the signal into the cell in tobacco callus and *Amaranthus* assays. 2-chloroethyl and 2-bromoethyl groups at the N9-position can prevent negative effects on cell proliferation (Mik et al., 2011b).

N9-substituted kinetin (Kin) derivatives with halogenoalkyl, aliphatic/cyclic ether and/or carboxylic chains enhanced the cytokinin activity of the default compound in the bioassays (Mik et al., 2011a). The effects of the halogenoalkyl derivatives were affected by the specific halogen atom – chlorine showed more effectivity than bromine, probably because of its smaller size. High affinity was measured with Kin derivatives containing aliphatic and/or cyclic ether substituents. These derivatives displayed a negative effect on perception by AHK3 and CRE1/AHK4 receptors but they improved anti-senescence properties and prevented the negative effect on proliferation of cells due to high concentrations of exogenously applied cytokinins. The length of the alkyl chain and the type of halogen atom had influence on delayed degradation of chlorophyll in senescence bioassays. Kinetins with halogenoalkyl substitution at N9 were most effective in the senscence assay (Mik et al., 2011a).

The methods listed below represent author´s contribution to performed experiments.

## Sequence Alignment

Sequences containing the CHASE domain were obtained from the Pfam database (PF03924, http://pfam.xfam.org). Alignment of 1721 domains was generated with ClustalOmega by EMBL-EBI (http://www.ebi.ac.uk;). The alignment was manually modified to 277 CHASE domains originating from 52 different organisms.

## Site-directed Mutagenesis

Site-directed mutagenesis was performed using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, USA).

## Cytokinin Bioassays

The amaranthus assay, tobacco callus growth bioassay and wheat senescence assay were performed according to Holub et al. (1998) with slight modifications.

## β-galactosidase Assay

The assay was performed in accordance to Spíchal (2011).

## Cytokinin Binding Assay

The cytokinin binding assay was performed according to the method described by Romanov et al. (2005, 2006) with slight modifications. GraphPad Prism 5.1 was used for calculation of IC50 and preparation of the graphs (http://www.graphpad.com/scientific-software/prism/).

## *ARR5::GUS* Cytokinin Induction Assay

*ARR5::GUS* cytokinin induction assay was done according to Romanov et al. (2002). Fluorescence was measured using a Fluoroscan Ascent microplate reader (Labsystems, Finland) at excitation and emission wavelengths of 365/450 nm.

## Characterisation of Ligand Specifity of the *Brassica napus* Cytokinin Receptors

Cytokinin receptors were studied first in the model plant *Arabidopsis thaliana* and research on these receptors still continues. Here we have characterised the first cytokinin receptors in the economically important dicotyledonous plant - *Brassica napus* var. Tapidor. Results were summarized in the *Journal of Experimental Botany* (Kuderová et al., 2015). Homologs of AHK2 (BnCHK1 and BnCHK3) and AHK3 (BnCHK5) cytokinin receptors were identified on the basis of phylogenetic analysis (Fig. 5).

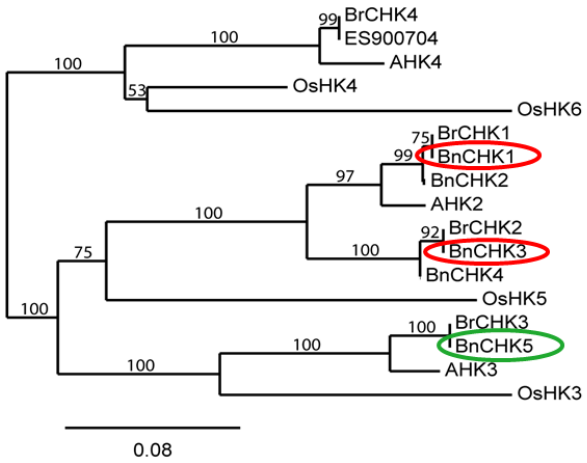


Figure 5. Phylogenetic relationship based on alignment of CHASE-containing His kinases. Tested receptors are marked by elipses.

Cytokinin binding assay showed that they bind specifically cytokinins and it was confirmed that the three *Brassica napus* proteins, BnCHK1, BnCHK3 and BnCHK5, are functional cytokinin receptors. All three receptors showed the strongest recognition in the case of *tZ*, followed by phenylurea-derived cytokinin TDZ. Differences in the ligand preference of BnCHK receptors were obtained for iP and *tZ*R.

## Studies of Cytokinin Receptor Affinity and Activation Using a Chemical Biology Approach

The principle of cytokinin perception and activation of the receptor was recently described (Hlusková et al., submitted). Data were obtained based on the crystal structure of CRE1/AHK4 (3T4L; Hothorn et al., 2011) with combination of computer modelling and simulations. The cytokinin is held by Asp262 in the "arm" helix by hydrogen bonds to $N^6$ and N7 and by a hydrogen bond between backbone of Leu284 and N9. Stabilization of the ligand is primarily through the interaction between Asp262 and $N^6$ and N7 of the cytokinin, together with the cytokinin side chain (Hlusková et al., submitted). .

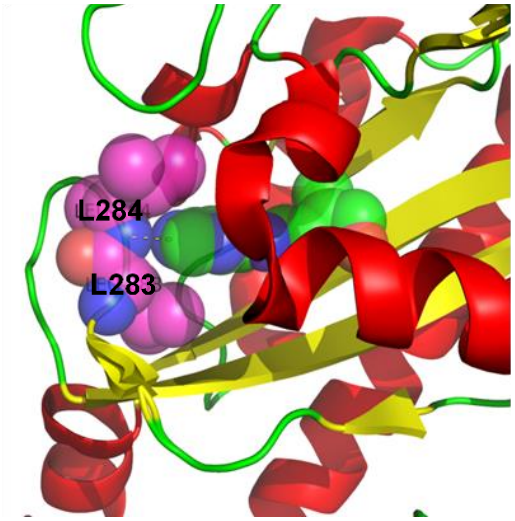


Figure 6. The structure of the "hook" loop. Leu284 and Leu283 (magenta spheres) form a symmetrical C-shaped docking platform stabilizing the ligand *tZ* (green spheres) in a position that enables hydrogen bond formation between *tZ* N9 and the Leu284 backbone amide (visualized by the yellow dashes).

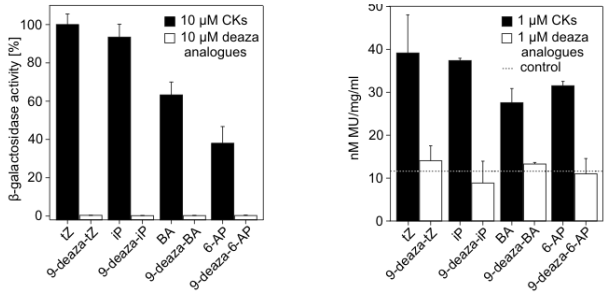


Figure 7. Interaction of 9-deaza-cytokinin analogues with the cytokinin signalling pathway. On the left is graph of activation of the AHK4 cytokinin receptor in the β-galactosidase activity assay. On the right is graph depicting induction of *ARR5::GUS* in transgenic *Arabidopsis* plants. The dashed line represents GUS activity in non-induced plants.

In CRE1/AHK4, a hydrogen bond donor is provided by the main chain amide of Leu284 and N9 serves as a hydrogen bond acceptor. Together with Leu283, these two amino acids form a symmetrical C-shaped docking platform, with the hydrogen bond in the centre and the plane of the cytokinin adenine ring perpendicular to it (Fig. 6).

Further, comparison of 277 amino acid sequences from 52 organisms showed a conserved motif between 261 and 317 amino acids in the PAS domain. There is absolute conservation of key amino acidss – Asp262 and Leu283 and also high conservation of Leu284. This points to the importance of the C-shaped docking platform. The importance of hydrogen bond formation between N9 and Leu284 was confirmed by testing of 9-deaza cytokinin analogues. 9-deaza derivates of *tZ*, iP, BAP and 6-AP were tested in cytokinin assays.

Results from the *Amaranthus* assay and tobacco callus growth assay showed, that N-to-C substitution at position N9 of the adenine ring significantly decreases cytokinin activity. In addition, 9-deaza-cytokinins exibited no activity in the senescence assay.

Indeed, 9-deaza-cytokinins were able to bind to the CRE1/AHK4 receptor, although with lower affinity than their aza-counterparts. Nevertheless, the β-galactosidase activity assay did not show 9-deaza analogues were able to activate the cytokinin pathway (Fig. 7). These results were confirmed also by the *ARR5::GUS* cytokinin induction assay. 9-deaza-cytokinin analogues did not induce the cytokinin signalling pathway even at the highest concentration of 10 μM (Fig. 7).

A 9-deaza compound, 9-deaza-iP, was tested also in previous work by Klimeš et al. (2017). This work tested potential agronomically important substances by the β-galactosidase assay using high-

throughput screening. 19 potential agonists and 12 antagonists of CRE1/AHK4 were identified. 10 potential agonists/antagonists were tested also in a cytokinin binding assay.

## Analysis of the Importance of the Conserved Residues in the Binding Pocket of AHK3 and CRE1/AHK4

The molecular basis of the ligand binding and recognition has been described using co-crystallisation of the CRE1/AHK4 sensor domain with cytokinins (Hothorn et al., 2011). To analyse the importance of conserved residues in the binding pocket, CHASE domains of AHK3 and the CRE1/AHK4 were used. These two receptors have widely contrasting ligand specificities. Four amino acid positions were selected as potentially responsible for the main differences in the ligand specificity of AHK3 and CRE1/AHK4.

Three of the selected amino acids are in the entrance to the binding pocket – Tyr250, Leu251 and Ala322 amino acid. The last amino acid, Ile266, is situated inside the binding pocket, near the end of the cytokinin side chain.

The aim was to change the ligand preference to a more AHK3-like profile. Receptor mutants were subsequently tested for their affinity in a competition assay using *E. coli* cells expressing the wild-type and mutated receptors (Table 1).

Table 1. IC50 values of selected ligands with WT cytokinin receptor CRE1/AHK4 and its mutated forms. IC50 values highlighted in circles show main differences between WT CRE1/AHK4 receptor and its mutant forms; n.d. – not detected.

| Receptor | Cytokinin<br>Apparent IC50 (nM) | | | | | |
|---|---|---|---|---|---|---|
| | *tZ* | iP | *tZR* | *cZ* | DHZ | iPR |
| CRE1/AHK4 | 4.9 ± 1.1 | 15.7 ± 7.8 | 40.4 ± 9.4 | 345 ± 75 | 232 ± 118 | 48.9 ± 17.5 |
| A322V | 6.1 ± 1.7 | 14.9 ± 4.3 | 11.4 ± 1.9 | 403 ± 120 | 402 | 13 |
| I266V | 4.6 ± 1.5 | 42.5 ± 10.6 | 37.0 ± 16.4 | 1002 ± 356 | 83 ± 30 | 118 ± 1.5 |
| Y250H | 5.1 ± 1.6 | 11.3 ± 4.7 | 54.0 ± 19.5 | 300 ± 84 | n.d. | n.d. |
| L251V | 3.5 | 71.2 | 111 | 2388 | n.d. | n.d. |
| I266V_A322V | 4.7 ± 0.6 | 56.0 ± 35.8 | 35.2 ± 22.3 | 1560 ± 237 | n.d. | n.d. |
| Y250H_I266V | 3.4 | 33.4 | 18.6 | 390 | n.d. | n.d. |
| Y250H_A345V | 4.7 ± 2.3 | 12.1 ± 5.6 | 42.8 ± 18.2 | 606 ± 231 | n.d. | n.d. |
| Y250H_L251V | 138 | 540 | n.d. | n.d. | n.d. | n.d. |
| Y250H_L251V_I266V | 1.2 | 13.7 | n.d. | n.d. | n.d. | n.d. |
| Y250H_L251V_A345V | 39.4 | 123 | n.d. | n.d. | n.d. | n.d. |
| Y250H_I266V_A345V | 45.3 | 169 | n.d. | n.d. | n.d. | n.d. |
| quadruple mutant | 56.9 | 201 | n.d. | n.d. | n.d. | n.d. |

The A322V mutation was predicted to influence CRE1/AHK4 affinity to cytokinin ribosides. The mutant CRE1/AHK4-A322V showed more than 3-times higher affinity for ribosides, namely for *t*ZR and iPR, compared to that of AHK3. Substitution of isoleucine for valine at position 266 caused almost 3-times lower affinity for iP and *c*Z. Substitution of valine for L251 brought more than 4-times and almost 6-times lower affinity for iP and *c*Z, respectively, in comparison to the wild-type receptor. Both mutated forms, CRE1/AHK4-L251V and CRE1/AHK4-I266V, showed weak affinity for iP and *c*Z. The single mutation in CRE1/AHK4-Y250H did not influence binding of the tested cytokinins. Combination of mutations in double, triple and quadruple mutants were found to influence the binding capacity of the tested cytokinins (Table 1). However, due to lack of time, these interesting preliminary results could not be followed up and need to be confirmed by repetition of the cytokinin binding assay.

# CONCLUSIONS

This thesis wishes to shed more light on the cytokinin world, especially in the area of their first contact: ligand-receptor interaction. At first, we focused on identification and functional characterization of homologues of sensor histidine kinases homologous to *Arabidopsis* cytokinin receptors in agronomically important winter oilseed rape, *Brassica napus* var. Tapidor. There were identified five CHASE-containing His kinases (BnCHK1–BnCHK5). Four homologues of AHK2 (BnCHK1–BnCHK4) and one homologue of AHK3 (BnCHK5) were defined and their domain structures were determined. Tested receptors, BnCHK1, BnCHK3 and BnCHK5 displayed high affinity for *tZ* (1–3 nM). Implying potential functional divergence, the AHK2 homologues could be divided into two subfamilies (BnCHK1/BnCHK2 and BnCHK3/BnCHK4) that differ in putative transmembrane domain topology and CK binding specificity.

Structural details of cytokinin binding were previously described (Hothorn et al., 2011; Steklov et al., 2013). However, mechanism of receptor activation needed to be elucidated. Computer simulations indicated the importance of hydrogen bond formation between the N9-atom of the adenine ring and peptide backbone of the CRE1/AHK4 binding site. Possible involvement of this interaction in CRE1/AHK4 active site structural rearrangement and its subsequent influence on receptor activation were investigated *in silico* and tested *in vitro* with 9-deaza cytokinin analogues. Lack of ligand binding at N9-position decreased affinity to CRE1/AHK4 receptor and completely blocked its activation. It is likely that missing hydrogen bond in N9-position has influence on destabilization of the dimerization interface of the receptor. Another approach involved mutagenesis of the CRE1/AHK4 receptor and testing of ligand specificity of the mutants. The results suggest that the mutated amino acids are indeed involved in binding specificity as they generally accept cytokinin ribosides with better affinity than WT receptor. However, more testing would be required to confirm these results.

The knowledge of cytokinins and their derivatives' mechanism of action can be also used in agriculture. Compounds that specifically interact with plant hormone pathways have high utility in research and potential agronomic applications for improving crop yields or other aspects of crops' performance. A high throughput method for screening compounds with agonist or antagonist properties toward the CRE1/AHK4 cytokinin receptor was developed.

Thus, the cytokinin receptors in the model plant *Arabidopsis thaliana* and its agrnomically important relative *Brassica napus* were studied. The obtained results moved us further forward in understanding of the interaction between cytokinin receptors and their ligands and of the selectivity of the receptors.

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# LIST OF AUTHOR'S PUBLICATIONS

**Hlusková, L.**, Gándara Barreiro, Z., Berka, K., Bazgier, V., Mik, V., Mazura, P., et al. 9-deaza cytokinins reveal molecular basis of cytokinin receptor ARABIDOPSIS HISTIDINE KINASE 4 signal transmission through membrane. Phytochemistry submitted.

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## Presentations at scientific conferences

**Gallová, L.**, Gándara Barreiro, Z., Bazgier, V., Berka, K., Spíchal, L. (2016) Vplyv 9-deaza Analógov Cytokinínov na Väzbové Vlastnosti Cytokinínového Receptora AHK4 u *Arabidopsis thaliana*, Mezioborové setkání mladých biologů, biochemiků a chemiků, 10. - 12. 5. 2016, Milovy

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