The Roles of Cytokinins and Cytokinin-N-Glucosides in Development and Gene Expression of *Arabidopsis thaliana*

by

Howard Tucker Hallmark

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
August 08, 2020

Keywords: Arabidopsis, cytokinin, trans-Zeatin, isopentenyladenine, glucosides, senescence

Approved by

Aaron M. Rashotte, Chair, Professor of Biological Sciences
Leslie R. Goertzen, Associate Professor of Biological Sciences
Sang-Wook Park, Assistant Professor of Plant Pathology
Haruka Wada, Assistant Professor of Biological Sciences

Abstract

The phytohormone cytokinin (CK) was first discovered over sixty years ago, and the decades following have produced an enormous amount of data about the various roles CK plays in intracellular signaling, gene expression, and plant development. An often-overlooked characteristic of this phytohormone is that it is not one single molecule; “cytokinin” is a generalized term for dozens of molecules which occur naturally in plants. Much of CK research has used synthetic CKs or only a single form of CK because it has long been believed that only a small subset of CKs display biological activity. Much of this dissertation focuses on CK-N-glucosides (CKNGs), long believed to be inactive forms of CK.

Chapter 1 provides a broad overview of CK biology and their functions in plants, with particular focus on the model plant species *Arabidopsis thaliana,* which the rest of this dissertation work is completed in. Chapter 2 reviews recent advances in understanding of Cytokinin Response Factors, a family of transcription factors involved in response to a variety of environmental signals. Chapter 3 focuses on trans-Zeatin-N-glucosides, revealing that this subclass of CKNGs are capable of delaying leaf senescence and altering gene expression. Chapter 4 focuses on another subclass of CKNGs, isopentenyladenine-N-glucosides, revealing one of these compounds is capable of delaying leaf senescence and mildly altering gene expression in an overlapping manner with isopentenyladenine, a highly active form of CK. Chapter 5 explores potential mechanisms by which trans-Zeatin-N-glucosides delay leaf senescence and suggests they are capable of regulating levels of an active CK, trans-Zeatin. Chapter 6 combines CK measurement data from twelve studies, revealing CKNGs are the most abundant forms of CK in young *Arabidopsis thaliana* plants.

Acknowledgments

The process of completing a PhD is an arduous one. There are many people I would like to thank for their personal, professional, and technical assistance. I would first like to thank my Advisor, Dr. Aaron Rashotte, for his guidance, mentorship, and kindness during my undergraduate and graduate work. I must also thank my committee members, Dr. Haruka Wada, Dr. Les Goertzen, and Dr. Sang-Wook Park, for their helpful feedback and assistance over the last few years.

I would not have considered pursuing a PhD if it were not for Dr. Robert Locy, who taught me Genomic Biology and gave me my first lessons on the command line. Dr. Paul Zwack and Dr. Erika Keshishian were also advocates of pursuing a graduate degree. I am grateful for their friendship and mentorship. Dr. Chloe Josefson and Rachel Powell, MS, have been great friends and listening ears throughout this journey. I appreciate the efforts of these individuals for getting me into graduate school in the first place and for helping me navigate academia.

I could not have completed all of these experiments without some help- though there are many undergraduate researchers who served as incredibly helpful hands, I would like to specifically thank Lesley Turner, Lauren Collins, and Kaki Oberman.

Though not an official committee member of mine, I am grateful for the mentorship and guidance of Dr. Courtney Leisner. She has been a great source of advice in the two years since she moved into our "lab neighborhood," and her presence was especially appreciated during Aaron's sabbatical in Czech Republic.

When I first told my parents that I was thinking of going to grad school, my dad asked how I planned on paying for it. I am grateful that both my mother and father were strongly supportive of me embarking into the odd world of academia, which was foreign to them both. My father worked exceptionally hard to support me and help me earn my Bachelor's degree, and my mother's emotional support did not go unnoticed. I am proud to be the first Dr. Hallmark. But based on what I see in some of my nieces and nephews, I won't be the only doctor in the family for long. I am also incredibly grateful for the support of my sister Sallie Hallmark, one of the most well-composed and successful people I know, whose career advice served me well.

Lastly, I cannot begin to express my gratitude for my partner through this and all endeavors, Anthony Hamilton. Your strength and courage through all you faced kept me strong and courageous. I will never be able to surpass your hard-working nature, but you make me work harder, and you taught me to do what I love and to do it well. Thank you for letting me complain and ramble on. Thank you for keeping me together through everything. Thank you for putting your life plans on hold so we could stay together while I finished what I came here for.

I dedicate this Dissertation in memory of two family members. First, this is for my paternal grandfather and namesake, Howard Hallmark, who had a grade school education, yet worked his way out of poverty into owning and operating a successful company still in business today. He passed away months before I was born, but I know he would probably be the proudest of anyone over this achievement. Second, for my sister-in-law, Allyson Hamilton, whose sudden passing served as a reminder that life really is too short and that we must cherish those around us.

Table of Contents

Chapters 2 and 3 have been previously published, and citation information is listed on each title page. Chapter 4 is in peer review at *Plant Direct* as of June 29, 2020.

List of Tables

List of Figures

Chapter 1: Introduction to Cytokinins and Cytokinin Conjugates in Plant Development and Stress Response

H. Tucker Hallmark

Outline

I. Discovery of cytokinin as a plant hormone

II. Cytokinin biosynthesis

    a. History

    b. Current state

III. Cytokinin signaling

    a. History

    b. Cytokinin Response Factors

    c. Current state

IV. Biological activity of cytokinin

    a. Role in development

    b. Role in stress response

V. Degradation and conjugation of cytokinin

    a. History

        i. Degradation

        ii. O-conjugation

        iii. N-conjugation

    b. Current state

Abbreviations and Definitions

AHK2/AHK3	Arabidopsis Histidine Kinase 2 and 3 - cytokinin receptors
AHK4	Arabidopsis Histidine Kinase 4 - cytokinin receptor, also known as WOL and CRE1
AHP	Arabidopsis Histidine-containing Phosphotransfer protein - relay signal from AHKs to ARRs
AMP/ADP/ATP	Adenosine monophosphate/diphosphate/triphosphate
AP2/ERF	APETALA2/Ethylene Response Factor - large family of transcription factors, of which CRFs are a member
ARR	Arabidopsis Response Regulator - transcription factors (type B) and nuclear localized proteins (type A) which act downstream of AHKs and AHPs
CKX	Cytokinin Oxidase - class of cytokinin degrading enzymes
CRE1	Cytokinin Response 1 - cytokinin receptor, also known as AHK4 and WOL
CRF	Cytokinin Response Factor - class of transcription factors, some of which are involved in response to cytokinin
CRISPR-Cas9	Clustered Regularly Interspaced Short Palindromic Repeat - recently developed method of targeted DNA mutagenesis
cv.	Cultivar
cZ	cis-Zeatin - one form of the plant hormone cytokinin
DZ	Dihydrozeatin - one form of the plant hormone cytokinin
HPt	Histidine-containing phosphotransfer protein - general name for non-Arabidopsis AHP
IAA	Indole-3-acetic acid - one form of the plant hormone auxin
iP	Isopentenyl adenine - one form of the plant hormone cytokinin
IPT	Isopentenyltransferase - class of enzymes that catalyze the first step of iP, tZ, and DZ synthesis
LOG	

	Lonely Guy - class of enzymes required to convert cytokinin nucleotides into cytokinin bases
qPCR	Quantitative Polymerase Chain Reaction - A variant of PCR in which transcript abundance can be estimated by using fluorescence as a marker of how much product is generated with each PCR cycle
ROS	Reactive Oxygen Species - oxygen containing compounds such as hydrogen peroxide and superoxide prone to reacting with cellular components such as lipids and DNA
SAM	Shoot Apical Meristem - region in which pluripotent stem cells divide and differentiate to form shoot tissues
T-DNA	Transfer-DNA - Plasmid DNA from the bacterium *Agrobacterium tumafaciens* which integrates into plant DNA and interrupts wildtype DNA sequences
tRNA	transfer RNA
tZ	trans-Zeatin - one form of the plant hormone cytokinin
tZOG	trans-Zeatin-O-glucoside
tZ7G	trans-Zeatin-7-glucoside
tZ9G	trans-Zeatin-9-glucoside
UGT	UDP-glucosyl transferase - enzyme catalyzing the transfer of a glucose molecule from UDP to a substrate
WOL	Wooden Leg - cytokinin receptor, also known as AHK4 and CRE1
WT	Wildtype - Nontransgenic organism

I. Discovery of Cytokinin as a Plant Hormone

Cytokinin is a plant hormone first identified as “kinetin” by Miller and Skoog in the mid-twentieth century through a series of experiments attempting to identify substances that would promote cell division in plant tissue culture (Miller et al., 1955). Based on previous findings that DNA derived compounds may promote cell division, the authors autoclaved DNA-rich herring sperm as well as DNA from calf thymus; these solutions were added to media also containing the then-known plant growth regulator auxin, or IAA (Miller et al., 1955). These DNA derived compounds, which the authors named “kinetin,” promoted cell division and growth of tobacco callus in conjunction with IAA (Miller et al., 1955), and shortly after, kinetin was determined to be a purine-based compound and the class of growth regulators of which it was a member was named “kinins,” later renamed “cytokinins” (Miller et al., 1956).

Over the next decade, Miller and others identified and characterized the first naturally occurring cytokinin in *Zea mays* and noted its structure was similar to adenine (Miller, 1961). This was an important discovery, as it was the first evidence cytokinins were produced endogenously in plants. Using fruitlets from *Prunus domestica*, Letham purified another compound referred to as A1. A1 was noted as having the same cell division promoting activity as kinetin while being a separate compound (Letham, 1963a), one of the early indications that multiple structurally distinct cytokinins exist. Shortly after, Letham isolated a compound from *Zea mays* seeds that was chromatographically and physiologically indistinguishable from A1; he proposed calling this compound Zeatin. Letham noted this was possibly the same compound that Miller partially purified two years earlier, and that it was likely an adenine molecule with an additional chemical group attached at the N6 position (Miller, 1961; Letham, 1963b).

Since their discovery, cytokinins have been implicated in a number of biological processes ranging from promotion of cell proliferation in meristematic regions to delaying senescence in leaf tissues (as reviewed by Mok and Mok, 2001; Sakakibara, 2006; Kieber and Schaller, 2014). Due to advances in molecular biology, biochemistry, and the establishment of the model plant system *Arabidopsis thaliana* (hereafter referred to as Arabidopsis), the cytokinin biosynthetic and signaling pathways have been well dissected genetically.

II. Cytokinin Biosynthesis

II.a. History

Four major classes of cytokinins are known to exist in plants: trans-Zeatin (tZ), cis-Zeatin (cZ), isopentenyl adenine (iP), and dihydrozeatin (DZ) (Sakakibara, 2006). One of the earliest examinations of cytokinin biosynthesis was performed in *Pisum sativum*, where it was found that cytokinin, specifically cis-Zeatin, could be isolated from tRNAs (Vreman et al., 1972). One idea of how cytokinins were synthesized was that cis-Zeatin was released from tRNA and then converted into the more active trans-Zeatin (Bassil et al., 1993). However, it has since been shown that, though interconversion of cis- and trans-Zeatin can occur enzymatically, it does not appear to be a major contributor to the pool of cytokinins other than cis-Zeatin (Gajdošová et al., 2011). Cytokinins are not plant-specific compounds; in fact, one of the first steps of cytokinin biosynthesis, which is conserved in plants, appears to be that first identified in an amoeba system and shortly after identified in the bacterium *Agrobacterium tumafaciens*. The reaction identified in both studies was the addition of an isopentenyl group to an adenosine monophosphate (AMP) to form isopentenyl adenine nucleotide (Taya et al., 1978; Akiyoshi et al., 1984). In the case of *Agrobacterium*, the gene responsible for the conversion of adenosine monophosphate to isopentenyl adenine nucleotide was identified as the IPT gene (Akiyoshi et al., 1984).

The first plant IPT genes were identified at the start of the new millennium using *in silico* analysis to identify genes in the then-newly sequenced Arabidopsis genome that showed homology to the bacterial IPT genes; the activity of several Arabidopsis IPT (AtIPT) genes was confirmed in a bacterial system (Takei et al., 2001). Concurrently, Kakimoto showed that at least one AtIPT used ATP and ADP instead of AMP to form isopentenyl adenine nucleotide (Kakimoto, 2001). These studies demonstrated strong evidence for the formation of isopentenyl adenine nucleotide, but the question of how trans-Zeatin was synthesized as well as how the nucleotide forms were activated remained unanswered.

In 2004, a genetic screen looking to identify genes capable of converting iP nucleotide to tZ nucleotide led to the identification of two genes, CYP735A1 and CYP735A2, which encode for enzymes that catalyze this reaction (Takei et al., 2004). A few years later, the first gene involved in activating these cytokinin nucleotides was identified and named Lonely Guy (LOG); shortly after, several more LOG genes were identified in Arabidopsis (Kurakawa et al., 2007; Kuroha et al., 2009). LOGs cleave the ribophosphate group from the cytokinin nucleotide, converting the molecule into the active base form (Kurakawa et al., 2007; Kuroha et al., 2009).

II.b. Current State

The current understanding of the cytokinin biosynthetic pathway (summarized in Figure 1) is that ATP, ADP, or AMP are converted to iP nucleotide by the addition of an isoprenoid sidechain, and this reaction is performed by a set of genes in Arabidopsis known as IPTs (Kakimoto, 2001; Takei et al., 2001). Isopentenyl adenine nucleotides can be hydroxylated by CYP735A2 to form tZ nucleotides. Both iP and tZ nucleotides can be converted to active cytokinin bases by one of the several LOG genes, which cleave off the ribose and phosphate groups (Kurakawa et al., 2007; Kuroha et al., 2009). Another form of cytokinins, cZ, are

synthesized in a similar manner, but the starting point is tRNA-derived adenosine monophosphate (Vreman et al., 1972). Though conversion between trans- and cis-Zeatin is possible (Bassil et al., 1993), it requires a poorly characterized enzyme, and does not appear to be a major contributor to the overall cytokinin composition of a plant (Gajdošová et al., 2011).

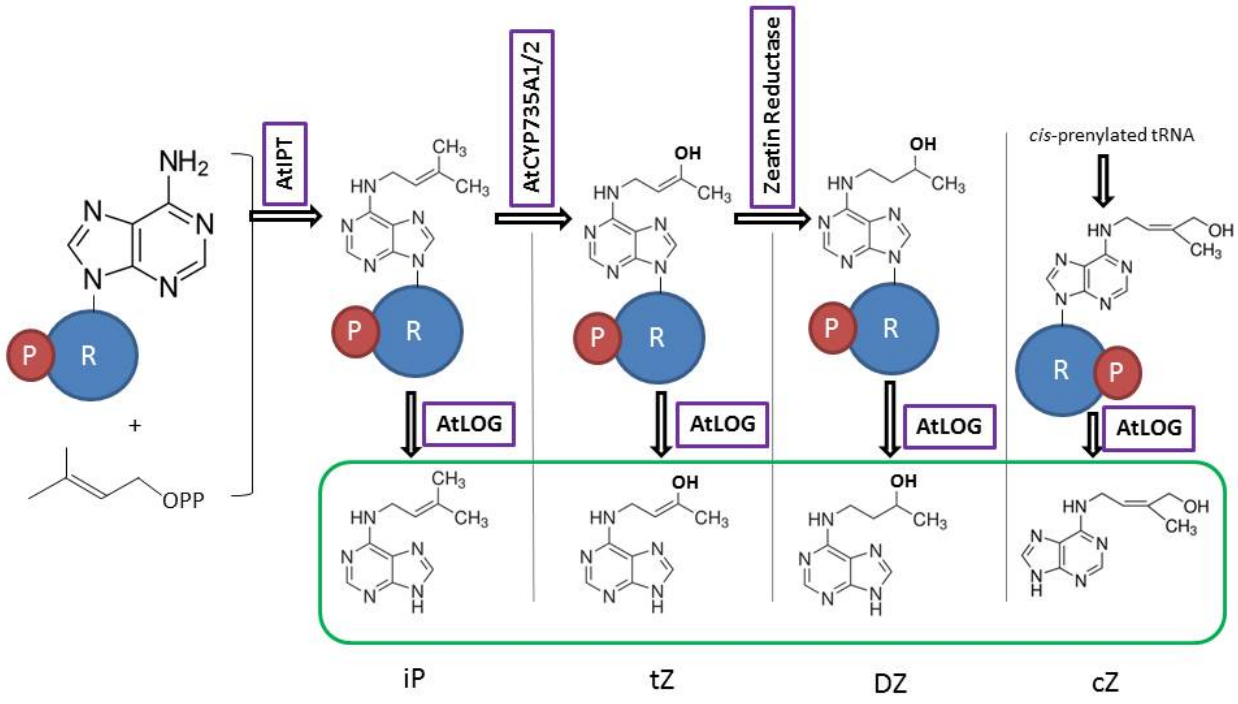


Figure 1. Abbreviated Arabidopsis cytokinin biosynthetic pathway: from precursors to active cytokinin bases. Red circled "P" represents a phosphate group. Blue circled "R" represents a ribose. Text in purple boxes indicates known genes responsible for reactions. Base forms of each cytokinin are boxed in green. Abbreviations are as defined in the text.

III. Cytokinin Signaling

III.a. History

The field of cytokinin signaling began largely with the identification of Arabidopsis genes believed to be cytokinin receptors. The first of these was CKI1, or Cytokinin Independent 1, which was identified in an activation tagging screen in the mid-1990s (Kakimoto, 1996). Kakimoto transformed tens of thousands of Arabidopsis calli with an activation tagging construct and looked for calli that would form shoots even in the absence of cytokinin (Kakimoto, 1996). A handful of transformants fit this phenotype, and when the genes near the activation tags were sequenced, it was found that four of the transformants harbored activation tags near a receptor like gene which the group aptly named Cytokinin Independent 1 for its ability to grow shoot-like structures without exogenously applied cytokinin (Kakimoto, 1996). As exciting as this finding was, the mechanism behind the phenotype remained elusive; it was not until the discovery of another receptor gene that cytokinin signaling became more clear.

Arabidopsis Histidine Kinase 4 (AHK4, also known as Cytokinin Response 1 and Wooden Leg) was identified by several independent groups (Scheres et al., 1995; Mähönen et al., 2000; Inoue et al., 2001; Ueguchi et al., 2001b; Ueguchi et al., 2001a) for its role in vascular tissue development and ultimately cytokinin perception. Scheres et al. reported that a *wooden leg* (WOL) mutant exhibited reduced root growth and disrupted vascular patterning, but this phenotype was not yet correlated with cytokinin (Scheres et al., 1995). WOL was further characterized by Mähönen et al. who showed that WOL acts as a histidine kinase receiver in a two-component signaling system, but did not specify a ligand (Mähönen et al., 2000). Shortly after, Inoue et al. performed a mutagenesis screen similar to that performed by Kakimoto (1996); however, in this screen, researchers grew calli from their mutagenized seed on media containing

cytokinin and isolated individuals that did not respond (Inoue et al., 2001). This screen led to the identification of Cytokinin Response 1 (CRE1); the group performed a number of cytokinin bioassays and determined *cre1* mutants to be insensitive to cytokinin (Inoue et al., 2001). Based on sequence homology, CRE1 was believed to be a receptor kinase, and by expressing CRE1 in yeast and supplying cytokinin, the group was able to show that CRE1 bound cytokinin which activated the receptor (Inoue et al., 2001). Concurrently with the first publication of CRE1, Ueguchi et al. published two papers showing that Arabidopsis has three highly related histidine kinase receptors (AHK2, AHK3, and AHK4) and also reported that AHK3 was capable of rescuing a yeast receptor kinase mutant (Ueguchi et al., 2001a). The second paper connected AHKs to cytokinin signaling by recapitulating many of the results seen by Inoue et al. (2001), including the insensitivity of Arabidopsis *ahk4* mutants to cytokinin as well as the ability of AHK4 to bind cytokinin in a yeast system (Ueguchi et al., 2001b).

There has been further biochemical characterization of the AHKs. Yamada et al. performed a series of binding assays in yeast and showed that AHK4, AHK3, and possibly AHK2 are capable of binding cytokinin and transducing a signal through a membrane (Yamada et al., 2001). Romanov et al. further dissected the biochemical properties of AHK3 and AHK4 through a series of direct binding assays performed in bacteria (2006). They found that pH played an important role in cytokinin binding, and they also tested a variety of cytokinins and demonstrated the ability of AHK3 and AHK4 to bind trans-Zeatin, trans-Zeatin riboside, dihydrozeatin, cis-Zeatin, and isopentenyl adenine; interestingly trans-Zeatin-O-glucosides showed no binding to either receptor (Romanov et al., 2006). There are several caveats to the Romanov et al. study: AHK2 was not tested, no N-conjugated cytokinins were tested, and all binding assays were performed in bacteria expressing only a single receptor at 0°C, which clearly is quite a different

environment than what would be seen in nature. Additionally, the cytokinin benzyladenine, which is often used in cytokinin experiments because of its high potency, showed incredibly low binding to AHK3 and AHK4; this may reflect idiosyncrasies in the experimental system or may indicate there are more important aspects to cytokinin perception than binding alone (Romanov et al., 2006). A more rudimentary study was performed and published as a short communication by the same group years two earlier (Spíchal et al., 2004). Trans-Zeatin-N-glucosides were shown not to bind AHK3 or AHK4 in bacteria, but the compounds did have moderate activity *in planta* as determined by a cytokinin gene-reporter assay; it is also worth noting that this is possibly a different bacterial system than was used by Romanov et al. as no methodology is reported (Spíchal et al., 2004). More recently, it was shown that the cytokinin receptors are localized to the endoplasmic reticulum, not solely the plasma membrane as previously thought (Caesar et al., 2011; Wulfetange et al., 2011).

While some groups were searching for cytokinin receptors, others were identifying additional parts of the signaling pathway. After discovery of CKI1, a histidine kinase receptor (Kakimoto, 1996), some groups hypothesized that two-component signaling similar to that seen in bacterial systems may exist in plants (Imamura et al., 1998). Two-component signaling is a common feature of bacterial signaling and is characterized by a sensor (i.e. receptor) which is activated by an environmental stimulus or signaling molecule; the sensor is phosphorylated and passes its phosphoryl group to a response regulator which then mediates the organism's response to the environmental cue (Parkinson and Kofoid, 1992). After the identification of CKI1, Imamura et al. searched Arabidopsis EST databases for genes encoding proteins with sequence similarity to known response regulators in hopes of identifying a clear two-component signaling pathway in plants (Imamura et al., 1998). After finding five candidate genes which the group named

Arabidopsis Response Regulators (ARRs), the genes were cloned, expressed in bacterial systems, and the proteins were purified; after incubating ARR proteins with a phosphoryl-donating protein, it was evident the ARRs were capable of acting as true response regulators *in vitro* (Imamura et al., 1998). To further strengthen the role of ARRs as true response regulators, the authors also performed an *in vivo* experiment in which they rescued an *E. coli* strain with a mutated two-component signaling system by expressing ARRs (Imamura et al., 1998). Further examination of the ARRs led to the determination that there are two classes of ARRs; type-A response regulators are transcriptionally induced by cytokinin treatment and lack DNA-binding domains while type-B response regulators generally are not transcriptionally induced by cytokinin but do contain a DNA-binding domain (Imamura et al., 1999).

Even after the identification of possible histidine kinase receptors, a missing link in the cytokinin signaling cascade remained: how was the message transduced from an activated receptor to a response regulator? Similar questions were being answered in non-plant organisms in the 1990s (Appleby et al., 1996), and some groups seized this new knowledge and applied it to Arabidopsis. In 1998, two groups published similar works identifying the missing link as histidine-containing phosphotransfer proteins (Miyata et al., 1998; Suzuki et al., 1998), also known as HPts, or AHPs in Arabidopsis. Suzuki et al. used known histidine-containing phosphotransfer domain sequences to search the Arabidopsis EST database for proteins with high sequence similarity to known HPts (1998). This led to the identification of AHP1, AHP2, and AHP3; Northern blot analysis showed unique expression profiles of each of the AHPs and a functional complementation experiment showed the ability of AHP1 to complement a mutant yeast strain lacking a HPt gene required for survival on glucose containing media (Suzuki et al., 1998). These sequence data and *in vivo* experimental data, along with *in vitro* data showing the

ability of AHP1 to receive phosphoryl groups from a donor, are strong evidence that the AHP genes identified are, in fact, capable of relaying a phosphoryl group from a receptor kinase to a response regulator (Suzuki et al., 1998). In an incredibly similar set of experiments, Miyata et al. showed that AHP1, AHP2, and AHP3 were capable of rescuing the same mutant yeast strain as described above (Miyata et al., 1998).

With the major players identified (receptor kinases, histidine-containing phosphotransfer proteins, and response regulators), it became important to prove involvement of these components with cytokinin or with each other. With the receptors, early studies confirmed cytokinin activation of the receptors in yeast systems (Inoue et al., 2001; Ueguchi et al., 2001b). Some of the earliest evidence of the involvement of the AHPs with cytokinin came from a yeast two-hybrid experiment which showed interaction between several AHPs and ARR10, as well as an *in vitro* experiment showing the donation of a phosphoryl group from AHP2 to ARR3 and ARR4 (Imamura et al., 1999). A more extensive yeast two-hybrid screen expanded these findings and determined a larger number of interactions between AHPs and ARRs (Suzuki et al., 2001). To further elucidate the role of AHPs in cytokinin signaling, Suzuki et al. overexpressed AHP2 and performed a number of cytokinin bioassays, revealing that when AHP2 is expressed at high levels, a plant is hypersensitive to cytokinin; this result indicates AHPs act as positive regulators of cytokinin signaling (Suzuki et al., 2002). In the same publication, the authors demonstrated interaction between AHK4 and AHP2 by co-expressing the two genes in *E. coli*; when expressed alone, AHK4 is capable of activating the downstream signaling cascade leading to beta-galactosidase activity, but when AHK4 is co-expressed with AHP2, beta-galactosidase activity is diminished (Suzuki et al., 2002). The authors interpret this result to mean that AHP2 is

competing for interaction with AHK4, preventing AHK4 from activating the downstream signaling cascade (Suzuki et al., 2002).

Hwang and Sheen provided a clear, detailed description of cytokinin two component signaling through a series of gene expression analyses and fluorescence microscopy experiments (Hwang and Sheen, 2001). Generally, upon binding cytokinin, a membrane-bound AHK is activated and phosphorylated at an aspartate residue; this phosphoryl group is then passed to AHPs which then translocate to the nucleus and phosphorylate ARRs, of which type-B ARRs become transcriptionally active upon phosphorylation. Upon activation, these ARRs alter gene expression leading to cytokinin-mediated processes (Hwang and Sheen, 2001).

III.b. Cytokinin Response Factors

AHKs, AHPs, and ARRs are not the only players in cytokinin signaling in Arabidopsis. In the few years after the identification of these major signaling components, a transcriptome study by Rashotte et al. led to the identification of a separate group of transcription factors which act in concert with the other members of the two component signaling pathway (Rashotte et al., 2006). A few genes from the ERF (Ethylene Response Factor) family of transcription factors were shown to be upregulated by cytokinin treatment; phylogenetic analysis revealed a group of six closely related AP2/ERF transcription factors which authors named Cytokinin Response Factors, or CRFs (Rashotte et al., 2006). CRF2 and CRF5 are rapidly induced by cytokinin treatment, however this induction is not seen in an *arr1,arr12* mutant background; since ARR1 and ARR12 are major type-B response regulators, this result indicates CRF2 and CRF5 are direct or indirect targets of ARRs, placing them in the same two component signaling pathway (Rashotte et al., 2006). The authors also showed rapid accumulation of CRFs in the nucleus after treatment with cytokinin; again, this phenomenon was dependent on AHKs and AHPs (i.e. no nuclear

accumulation was observed in a receptor mutant background), placing CRFs in the same signaling pathway as AHKs and AHPs (Rashotte et al., 2006). This connection was strengthened when physical interaction between CRFs and AHPs was detected using bimolecular fluorescence complementation (Cutcliffe et al., 2011). Phenotypic analysis revealed weakly penetrant phenotypes for single *crf* loss of function mutants, however the *crf5,6* double mutant is embryo lethal indicating these factors are required for proper development and may act redundantly (Rashotte et al., 2006).

Further characterization of CRFs led to the determination of a canonical CRF protein structure and expression pattern. A CRF is an AP2/ERF protein which contains, at its N terminus, a highly conserved amino acid sequence known as the CRF domain which has known roles in protein-protein interaction (Cutcliffe et al., 2011). The AP2/ERF domain toward the center of the protein sequence is a DNA binding domain, and the C terminus of most CRFs contains a clade-specific sequence which groups CRFs into five clades based on C terminus similarity (Zwack et al., 2012). In the same publication, reporter constructs were generated for the CRFs, and it was shown that CRFs are primarily expressed in the vasculature (Zwack et al., 2012).

Perhaps the most well studied member of this gene family is CRF6. In Arabidopsis, CRF6 has been shown to play a role in leaf senescence as well as response to reactive oxygen species (Zwack et al., 2013; Zwack et al., 2016b). CRF5 is a member of the same clade as CRF6 (Zwack et al., 2012), and CRF5 has also been tied to stress response. Overexpression of CRF5 in Arabidopsis has also been shown to increase resistance to a bacterial pathogen by increasing expression of pathogen defense related genes (Liang et al., 2010). Both CRF5 and CRF6 were identified as being direct targets of the key retrograde signaling transcription factor ANAC017, which relocates to the nucleus to affect gene expression during mitochondrial dysfunction (Ng et

al., 2013). Recently, a deletion series was performed and it was determined that the C-terminal end of the CRF5 protein is responsible for its transcriptional activity (Striberny et al., 2017). CRF5 has also been shown to be repressed by the evening complex, a protein complex which modulates gene expression in response to light and temperature to regulate growth (Ezer et al., 2017). Together these results may point to a role of CRF5 in altering gene expression in response to environmental cues such as oxidative stress.

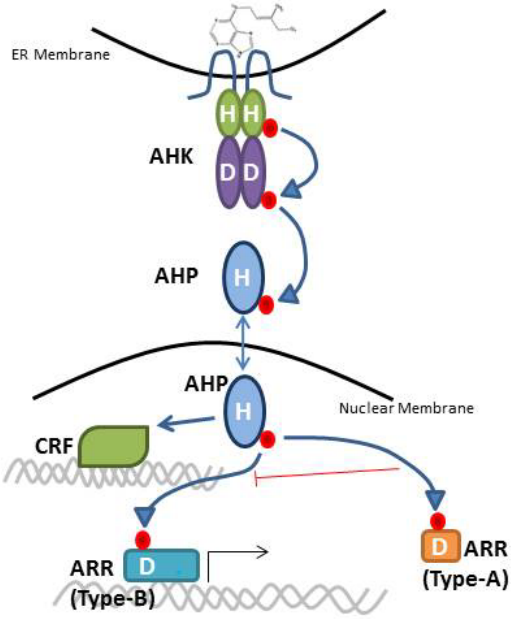


Figure 2. Simplified Arabidopsis cytokinin signaling pathway. Cytokinin binds the ER-bound AHK receptor which causes a phosphoryl group (red circle) to be transferred from a Histidine (H) residue to an Aspartate (D). The phosphoryl group is then passed to an AHP which translocates to the nucleus to phosphorylate type-B ARRs. This activates type-B ARRs to induce the transcription of type-A ARRs which are phosphorylated by AHPs but are not capable of binding DNA to alter gene expression. Downstream of AHPs are CRFs, which accumulate in the nucleus upon cytokinin signaling to alter gene expression.

III.c. Current State

The current model of cytokinin signaling is that Arabidopsis has three major cytokinin receptors, AHKs, which are capable of binding cytokinin bases and transducing a signal in a manner similar to bacterial two component signaling pathways (Inoue et al., 2001; Ueguchi et al., 2001a; Ueguchi et al., 2001b). Arabidopsis also has a set of histidine-containing phosphotransfer proteins (AHPs) (Miyata et al., 1998; Suzuki et al., 1998) which transduce the signal originating at the activated AHK to response regulators (Imamura et al., 1999; Suzuki et

al., 2001). These response regulators (ARRs) are transcription factors that mediate transcriptional response to cytokinin stimulus (Hwang and Sheen, 2001). In addition to the signal being transduced from AHK to AHP to ARR, cytokinin signaling can lead to the nuclear localization and activity of Cytokinin Response Factors, a set of transcription factors which act downstream of AHPs and AHKs (Rashotte et al., 2006). A simplified model of the cytokinin signaling pathway is presented in Figure 2.

IV. Biological Activity of Cytokinin

IV.a. Roles in Development

The earliest function attributed to cytokinins was their ability to promote cell division in plant tissue cultures (Miller et al., 1955). Shortly after, it was noted that cytokinins could promote shoot formation in plant tissue culture (Skoog and Miller, 1957). These remained the key features of cytokinin for many years before further investigation of plant physiology in connection to the hormone. It was not until Miller determined cytokinins were endogenously produced in plants (Miller, 1961) that more cytokinin-related physiology was explored. One early function attributed to cytokinin was its role in vascular development. By excising cotyledons and culturing them in different concentrations of hormones, it was shown that cytokinin promoted xylem differentiation (Aloni, 1982). The Arabidopsis mutant *wooden leg* displays vascular disorganization in the root meristem (Scheres et al., 1995) which was later attributed to the positive role of WOL in asymmetric cell divisions (Mähönen et al., 2000); *wol* was actually a mutant of the AHK4/CRE1 cytokinin receptor (Inoue et al., 2001), and examination of *ahp* mutants as well as plants overproducing cytokinin degrading enzymes also displayed disorganized vasculature (Mähönen et al., 2006), further strengthening the relationship between cytokinin and vascular development.

Another early study focused on chlorophyll and protein degradation and suggested cytokinin could delay senescence in detached leaves (Richmond and Lang, 1957). To show that this phenomenon was not specific to exogenously applied cytokinins, one group genetically engineered *Nicotiana tabacum* (tobacco) to increase production of cytokinins during senescence; this successfully delayed senescence, allowing plants to live and photosynthesize longer than tobacco containing normal levels of cytokinins (Gan and Amasino, 1995). However, the molecular mechanism of cytokinin retarding senescence was still relatively unknown. In a mutagenesis screen looking for regulators of senescence, Kim et al. identified ORE12, later determined to be AHK3, as being a key protein involved in leaf senescence (Kim et al., 2006). The authors showed ORE12 was a gain of function mutant of AHK3, and that it constitutively phosphorylated the response regulator ARR2; by using both loss- and gain-of-function mutants, the group determined that phosphorylation of ARR2 by AHK3 played a central role in delaying senescence (Kim et al., 2006).

A set of experiments by Werner et al. used transgenic *Nicotiana tabacum* overexpressing cytokinin oxidases, the enzymes responsible for cytokinin degradation (Werner et al., 2001). The transgenic plants displayed an array of phenotypes: plant height, total number of leaves, leaf surface area, leaf cell number, xylem and phloem size, and shoot apical meristem size were all decreased relative to wildtype (Werner et al., 2001), indicating a positive role for cytokinin in these processes. Interestingly, root tissue showed the opposite phenotypes: increased overall root growth and root meristem size relative to wildtype (Werner et al., 2001), suggesting cytokinin may have opposing effects on shoots and roots. The majority of the phenotypes seen in the transgenic tobacco lines were seen again in transgenic Arabidopsis overexpressing cytokinin oxidase genes (Werner et al., 2003).

A detailed study of the shoot apical meristem (SAM) revealed that a number of type-A ARRs are downregulated by the transcription factor WUSCHEL (WUS), a positive regulator of shoot meristem development (Leibfried et al., 2005). Because the SAM functions as the source of cells for shoot tissues, it is vital that the SAM maintains regular cell proliferation and division, of which WUS is a key regulator. A type-A response regulator multiple mutant showed reduced expression of WUS, indicating there may be a feedback loop in which type-A ARRs positively regulate WUS which in turn negatively regulates type-A ARRs; because cytokinin induces type-A ARRs, cytokinin may positively regulate WUS (Leibfried et al., 2005), suggesting cytokinin is indispensable for proper shoot formation.

IV.b. Role in Stress Response

Over the past decade or more, a number of studies have found connections between cytokinin, cytokinin signaling, and stress response (O'Brien and Benkova, 2013; Zwack and Rashotte, 2015; Pavlů et al., 2018). CRF6, a clade III member of the Cytokinin Response Factors, has been shown to be connected to oxidative stress response in Arabidopsis (Zwack et al., 2016b). Upon treatment with a general oxidative stress agent such as hydrogen peroxide, expression of CRF6 is induced and *crf6* loss of function mutants are less capable of dealing with oxidative stress than wildtype as revealed by their significant decrease in chlorophyll content, fluorescence, and root growth (Zwack et al., 2016b). CRF6 overexpressing plants display the opposite phenotype and are significantly more resistant to oxidative stress using the same parameters (Zwack et al., 2016b). A transcriptome study was performed in which wildtype and *crf6* mutants were treated with or without hydrogen peroxide for six hours. Analysis revealed that 147 genes affected by the treatment did so in a CRF6-dependent manner. GO term enrichment analysis revealed that, of genes downregulated in a CRF6-dependent manner during oxidative stress, genes involved in

"response to cytokinin stimulus" and "cytokinin mediated signaling" were overrepresented (Zwack et al., 2016b). Among the genes repressed in a CRF6-dependent manner are one AHP, multiple ARRs, one LOG, and ABCG14 (Zwack et al., 2016b), the latter of which has been implicated in transport of cytokinins (Ko et al., 2014; Zhang et al., 2014). Some of these targets were confirmed by yeast one-hybrid and protoplast luciferase assays, and the general model the authors propose is that CRF6 downregulates cytokinin-associated genes in order to promote tolerance to oxidative stress (Zwack et al., 2016b).

This is far from the only study that demonstrates a connection between cytokinin and stress. Cytokinin signaling was also connected to salt and drought stress by Tran et al. (2007). Arabidopsis mutants lacking AHK2, AHK3, or both receptors have significantly higher survival rates than wildtype under both drought and salt stress, suggesting cytokinin signaling is involved with salt and drought stress response (Tran et al., 2007). It is unclear whether the increased survival rate of these mutants is due directly to cytokinin signaling or due to the pleiotropic developmental effects seen in these mutants.

Another cytokinin-stress connection was shown by Jeon et al. who examined the role of ARR7 in cold and freezing response (Jeon et al., 2010). By measuring survival rate and electrolyte leakage, the authors showed that *arr7* loss of function mutants fared better than wildtype under freezing stress, while ARR7 overexpressing plants were more susceptible to freezing stress (Jeon et al., 2010). Surprisingly, overall cytokinin levels were not altered by this treatment, which indicates there may be crosstalk between the cytokinin signaling pathway and temperature signaling networks (Jeon et al., 2010).

Wang et al. demonstrated a connection between cytokinin and reactive oxygen species (ROS) through a series of salt stress experiments (Wang et al., 2015). The authors generated a

conditional IPT overexpressor; when these transgenics were treated with dexamethasone, IPT expression increases, leading to increased cytokinin levels. When plants were co-treated with dexamethasone and salt, the transgenics exhibited significantly reduced growth, decreased chlorophyll, and increased mortality relative to wildtype plants which were also co-treated with salt and dexamethasone (Wang et al., 2015). The authors determined that the transgenics accumulated more ROS than wildtype during the co-treatment, and that the transgenics also had decreased activity of antioxidant enzymes catalase and superoxide dismutase (Wang et al., 2015), signifying a potential role for cytokinin in regulating these enzymes.

Though it is not regulated by cytokinin, CRF4, a member of the Cytokinin Response Factors, has been shown to mediate cold response; *crf4* loss of function mutants have significantly lower survival rate during freezing stress than wildtype, while plants overexpressing CRF4 have significantly higher survival rate (Zwack et al., 2016a). A survey of CRFs in *Solanum lycopersicum* (tomato) showed that a number of SlCRFs have altered expression during various stresses including heat, cold, salt, osmotic, flooding, and oxidative stress, as well as with treatment with stress-related hormones (Shi et al., 2012; Gupta and Rashotte, 2014; Shi et al., 2014).

In all, these data suggest that cytokinin signaling may be closely associated with stress signaling, and further investigation of this connection is necessary to fully understand the role of cytokinin in stress response.

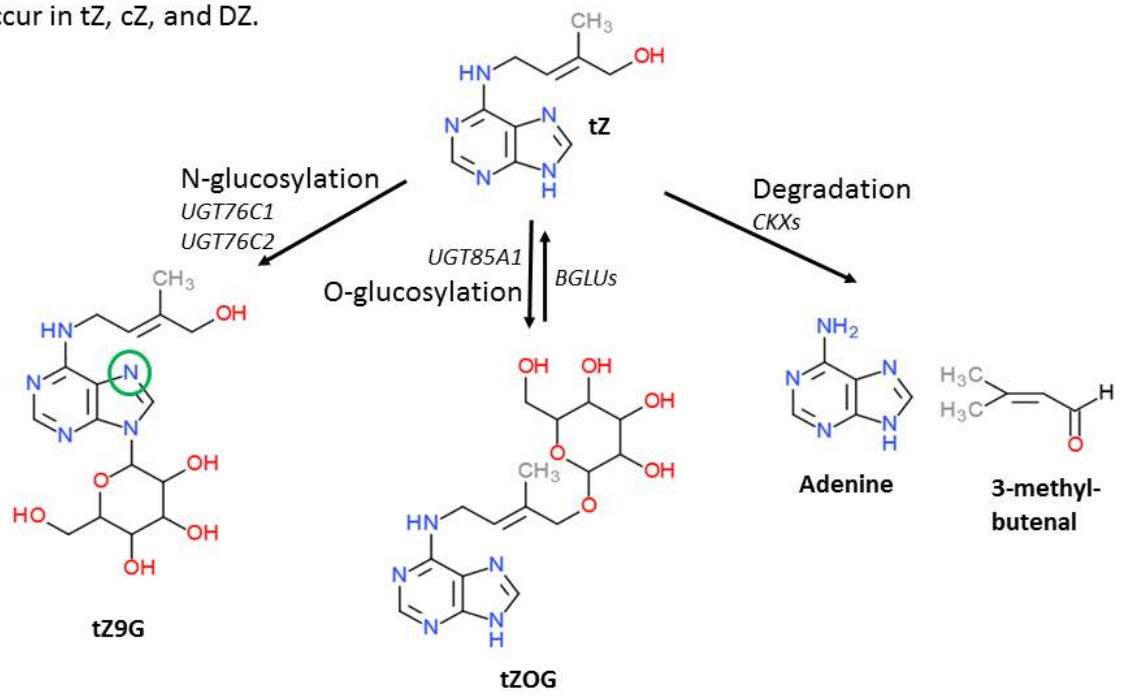
V. Cytokinin Degradation and Conjugation

V.a. History

After a cytokinin base (e.g. tZ) is synthesized, it may bind a receptor and signal for downstream changes in gene expression. Alternatively the molecule can enter one of three main fates:

degradation, conjugation at the O position in the sidechain, or conjugation at an N position in the adenine ring (Figure 3).

Figure 3. Fates of cytokinin bases. A trans-Zeatin molecule is shown entering three potential pathways: N-glucosylation (trans-Zeatin-9-glucoside is shown, but N7 circled in green may be glucosylated to form trans-Zeatin-7-glucoside), O-glucosylation which can be reversed by beta-glucosidase, and degradation. Genes responsible for these reactions are provided in italics. Abbreviations are as defined in the text. N-glucosylation and degradation are possible in all four main forms of cytokinin bases; O-glucosylation can occur in tZ, cZ, and DZ.



V.a.i. Cytokinin Degradation

The observation that cytokinins are degraded by oxidation was first noted decades ago (Whitty and Hall, 1974; Brownlee et al., 1975; McGaw and Horgan, 1983). Cytokinin oxidation results in the terminal degradation of cytokinin bases or ribosides into adenine or adenosine and a side chain derivative (Whitty and Hall, 1974). The first cytokinin oxidase cloned was from maize (Houba-Hérin et al., 1999; Morris et al., 1999). After the sequencing of the Arabidopsis genome, seven cytokinin oxidase (CKX) genes were identified and unique characteristics including

subcellular localization and preference for different types of cytokinins was examined (Schmülling et al., 2003; Werner et al., 2003).

V.a.ii. O-Conjugation

Early evidence of cytokinin-O-glucosides became known when Kende chromatographically identified two factors from *Helianthus annus*, one which acted like cytokinin in bioassays and one which showed weak cytokinin activity until hydrolysis, at which point the compound promoted cell division and chlorophyll retention like other cytokinins (Kende, 1965). Similarly, Yoshida and Oritani isolated a compound from roots of *Oryza sativa* that, when boiled in acid or exposed to beta-glucosidase, released zeatin and a glucose molecule (Yoshida and Oritani, 1972). The compound itself had some cytokinin activity in chlorophyll retention assays but little activity in callus growth assays until the glucose molecule was cleaved by acid hydrolysis or a beta-glucosidase (Yoshida and Oritani, 1972). Structurally, cytokinin conjugation at the O position in the sidechains of zeatin molecules was first identified using mass spectrometry in the late 1970s (Morris, 1977; Duke et al., 1979). The principle molecule attached at this position appears to be a glucose, though some studies have indicated xylose may also be conjugated at this position (Dixon et al., 1989; Martin et al., 1999). These cytokinin-O-glucosides may represent a storage form for cytokinin, as it has been demonstrated that the glucose residue can be cleaved off by beta-glucosidase enzymes to release a glucose and a free base cytokinin (Yoshida and Oritani, 1972; Brzobohaty et al., 1993; Falk and Rask, 1995).

V.a.iii. N-Conjugation

Another form of conjugation that can occur is glucosylation at one of the N positions in the adenine ring of the cytokinin molecule. This type of conjugation was first reported by Parker et al. who supplied *Raphanus raphanistrum* with radiolabeled zeatin and hours later performed

chromatographic analysis to determine if the supplied zeatin had been metabolized; results showed a large portion of the zeatin had been converted to a zeatin molecule with a sugar moiety attached to the purine ring, most likely at the N7 position, which they named Raphanatin (Parker et al., 1972). The next year, the same group performed a highly similar assay in *Zea mays* seedling and showed that the majority of the radiolabeled zeatin was converted to an N9-glucoside form, though N7-glucosides were also formed (Parker et al., 1973). Deleuze et al. also isolated an N7-glucosylated cytokinin and characterized it by performing a series of enzymatic assays; it was shown that the N7-glucosylated cytokinin molecule was not readily converted back to cytokinin and glucose when treated with either an alpha- or beta-glucosidase (Deleuze et al., 1972).

In the early 1980s, the Letham group began physiological examination of the relevance of the N-conjugated cytokinins. One early attempt at physiological characterization of these compounds was a *Glycine max* callus assay in which radiolabeled cytokinins were supplied to soybean callus; it was determined that the N-conjugates zeatin-7-glucoside and zeatin-9-glucoside had minimal activity in promoting callus growth, but it was also shown that these calli did not appreciably take up the compounds (Palni et al., 1984). The small amount of N-conjugates that were taken up were not metabolized to other cytokinin metabolites or degradation products (Palni et al., 1984), indicating these compounds are highly stable. Perhaps the most comprehensive study of the biological activity of these compounds suggested that N-conjugates have similar activity to cytokinin bases in some physiological contexts but not all (Letham et al., 1983). N9-glucosylated benzyladenine was stronger than benzyladenine in delaying senescence in *Raphanus raphanistrum* leaves, and N9-glucosylated zeatin displayed some activity in *Raphanus* callus assay and an *Amaranthus* betacyanin assay (Letham et al., 1983).

Decades later, a screen was performed to identify the Arabidopsis genes responsible for conjugating trans-Zeatin into trans-Zeatin-N-glucosides (Hou et al., 2004). The group cloned more than 100 UDP-glucosyl transferase (UGT) genes from Arabidopsis, recombinantly expressed them in bacteria, purified the enzyme, and assayed the enzymes for their ability to conjugate cytokinins and glucose; this *in vitro* study implicated UGT76C2 and UGT76C1 as the cytokinin N-conjugating enzymes (Hou et al., 2004). This hypothesis was strengthened by a study in which cytokinin levels were measured in single mutants of *ugt76c2* and *ugt76c1*, and it was shown that *ugt76c2*, and to some extent *ugt76c1*, mutants displayed significantly decreased cytokinin-N-glucoside levels (Šmehilová et al., 2016). Analysis of the *ugt76c2* single mutant revealed some developmental phenotypes such as greatly decreased N-conjugated cytokinin levels (approximately 10% of wildtype), slightly increased chlorophyll and anthocyanin content relative to wildtype, and modestly decreased seed weight (Wang et al., 2011). Recently, the Werner group from Free University of Berlin generated a double mutant, *ugt76c2,ugt76c1*, using both traditional T-DNA mutagenesis and CRISPR-Cas9. Cytokinin measurements revealed these plants have less than 1% of N-conjugate levels as compared to wildtype (Werner group, unpublished data); these plants will be indispensable for further examination of the physiological roles of N-conjugates.

V.b. Current State

Free cytokinin bases are subject to one of three main fates: degradation, conjugation at the O position in the side chain, or conjugation at an N position in the adenine ring. Cytokinin degradation has been a known occurrence since the mid-1970s (Whitty and Hall, 1974), and a number of cytokinin oxidase genes have been identified in Arabidopsis (Schmülling et al., 2003).

A possible storage form of cytokinin comes in the form of cytokinin-O-glucosides, where a sugar moiety is conjugated to the O atom in the cytokinin side chain (Morris, 1977; Duke et al., 1979). Evidence for these compounds being a storage form includes their minimal cytokinin activity (Yoshida and Oritani, 1972) which is dramatically increased when a glucose molecule is cleaved by a beta-glucosidase enzyme (Yoshida and Oritani, 1972; Brzobohaty et al., 1993; Falk and Rask, 1995).

Though understanding of cytokinin oxidases has greatly increased in the past two decades and it seems evident O-conjugates are reversible storage forms of cytokinins, the majority of what is known about N-conjugates is limited to the work from more than three decades ago. Thus, conjugation of cytokinins at an N position in the adenine ring represents a poorly understood metabolic path for the plant hormone. N-conjugated cytokinins have been identified in a number of species (Deleuze et al., 1972; Parker et al., 1972; Parker et al., 1973; Letham et al., 1975; Letham and Palni, 1983) but their function has remained elusive due to inconsistencies in activity between species and bioassays. Despite many contemporary reviews making overreaching statements that these compounds are inactive (Sakakibara, 2006; Kieber and Schaller, 2014), evidence for at least some cytokinin activity has been previously shown (Letham et al., 1983; Palni et al., 1984). N-conjugated cytokinins are resistant to cleavage by alpha- or beta-glucosidase enzymes, indicating the glucose moiety may be irreversibly attached to the cytokinin molecule (Deleuze et al., 1972) eliminating the likelihood that N-conjugates are a storage form of cytokinin. An examination of cytokinin stability revealed that N-conjugated cytokinins are significantly more stable than other forms of the hormone (Palni et al., 1984), raising into question what the function of these irreversibly conjugated, highly stable compounds may be. Most recently, two Arabidopsis genes, UGT76C1 and UGT76C2, have been identified

as encoding the enzymes that likely perform the glycosylation of cytokinins (Hou et al., 2004; Šmehilová et al., 2016). Phenotypic analysis of an Arabidopsis *ugt76c2* mutant that produces approximately one-tenth the N-conjugates of wildtype revealed potential roles for N-conjugates in maintaining chlorophyll and anthocyanin levels as well as regulating seed mass (Wang et al., 2011).

References

Akiyoshi DE, Klee H, Amasino RM, Nester EW, Gordon MP (1984) T-DNA of Agrobacterium tumefaciens encodes an enzyme of cytokinin biosynthesis. PNAS **81**: 5994–5998

Aloni R (1982) Role of Cytokinin in Differentiation of Secondary Xylem Fibers. Plant Physiology **70**: 1631–1633

Appleby JL, Parkinson JS, Bourret RB (1996) Signal Transduction via the Multi-Step Phosphorelay: Not Necessarily a Road Less Traveled. Cell **86**: 845–848

Bassil NV, Mok D, Mok MC (1993) Partial Purification of a cis-trans-Isomerase of Zeatin from Immature Seed of Phaseolus vulgaris L. Plant Physiology **102**: 867–872

Brownlee BG, Hall RH, Whitty CD (1975) 3-Methyl-2-butenal: An Enzymatic Degradation Product of the Cytokinin, N6-(Δ2-Isopentenyl)adenine. Can J Biochem **53**: 37–41

Brzobohaty B, Moore I, Kristoffersen P, Bako L, Campos N, Schell J, Palme K (1993) Release of active cytokinin by a beta-glucosidase localized to the maize root meristem. Science **262**: 1051–1054

Caesar K, Thamm AMK, Witthöft J, Elgass K, Huppenberger P, Grefen C, Horak J, Harter K (2011) Evidence for the localization of the Arabidopsis cytokinin receptors AHK3 and AHK4 in the endoplasmic reticulum. J Exp Bot **62**: 5571–5580

Cutcliffe JW, Hellmann E, Heyl A, Rashotte AM (2011) CRFs form protein–protein interactions with each other and with members of the cytokinin signalling pathway in Arabidopsis via the CRF domain. J Exp Bot **62**: 4995–5002

Deleuze GG, McChesney JD, Fox JE (1972) Identification of a stable cytokinin metabolite. Biochemical and Biophysical Research Communications **48**: 1426–1432

Dixon SC, Martin RC, Mok MC, Shaw G, Mok DWS (1989) Zeatin Glycosylation Enzymes in Phaseolus: Isolation of O-Glucosyltransferase from P. lunatus and Comparison to O-Xylosyltransferase from P. vulgaris. Plant Physiology **90**: 1316–1321

Duke CC, Letham DS, Parker CW, MacLeod JK, Summons RE (1979) The complex of O-glucosylzeatin derivatives formed in Populus species. Phytochemistry **18**: 819–824

Ezer D, Jung JH, Lan H, Biswas S, Gregoire L, Box MS, Charoensawan V, Cortijo S, Lai X, Stöckle D, et al (2017) The evening complex coordinates environmental and endogenous signals in Arabidopsis., The Evening Complex coordinates environmental and endogenous signals in Arabidopsis. Nat Plants **3, 3**: 17087, 17087–17087

Falk A, Rask L (1995) Expression of a Zeatin-O-Glucoside-Degrading [beta]-Glucosidase in Brassica napus. Plant Physiology **108**: 1369–1377

Gajdošová S, Spíchal L, Kamínek M, Hoyerová K, Novák O, Dobrev PI, Galuszka P, Klíma P, Gaudinová A, Žižková E, et al (2011) Distribution, biological activities, metabolism, and the conceivable function of cis-zeatin-type cytokinins in plants. J Exp Bot **62**: 2827–2840

Gan S, Amasino RM (1995) Inhibition of Leaf Senescence by Autoregulated Production of Cytokinin. Science **270**: 1986–1988

Gupta S, Rashotte AM (2014) Expression patterns and regulation of SlCRF3 and SlCRF5 in response to cytokinin and abiotic stresses in tomato (Solanum lycopersicum). Journal of Plant Physiology **171**: 349–358

Hou B, Lim E-K, Higgins GS, Bowles DJ (2004) N-Glucosylation of Cytokinins by Glycosyltransferases of Arabidopsis thaliana. J Biol Chem **279**: 47822–47832

Houba-Hérin N, Pethe C, D'Alayer J, Laloue M (1999) Cytokinin oxidase from Zea mays: purification, cDNA cloning and expression in moss protoplasts. The Plant Journal **17**: 615–626

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

Imamura A, Hanaki N, Nakamura A, Suzuki T, Taniguchi M, Kiba T, Ueguchi C, Sugiyama T, Mizuno T (1999) Compilation and Characterization of Arabiopsis thaliana Response Regulators Implicated in His-Asp Phosphorelay Signal Transduction. Plant Cell Physiol **40**: 733–742

Imamura A, Hanaki N, Umeda H, Nakamura A, Suzuki T, Ueguchi C, Mizuno T (1998) Response regulators implicated in His-to-Asp phosphotransfer signaling in Arabidopsis. Proceedings of the National Academy of Sciences **95**: 2691–2696

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

Jeon J, Kim NY, Kim S, Kang NY, Novák O, Ku S-J, Cho C, Lee DJ, Lee E-J, Strnad M, et al (2010) A Subset of Cytokinin Two-component Signaling System Plays a Role in Cold Temperature Stress Response in Arabidopsis. J Biol Chem **285**: 23371–23386

Kakimoto T (2001) Identification of Plant Cytokinin Biosynthetic Enzymes as Dimethylallyl Diphosphate:ATP/ADP Isopentenyltransferases. Plant Cell Physiol **42**: 677–685

Kakimoto T (1996) CKI1, a Histidine Kinase Homolog Implicated in Cytokinin Signal Transduction. Science **274**: 982–985

Kende H (1965) Kinetinlike Factors in the Root Exudate of Sunflowers. PNAS **53**: 1302–1307

Kieber JJ, Schaller GE (2014) Cytokinins. Arabidopsis Book. doi: 10.1199/tab.0168

Kim HJ, Ryu H, Hong SH, Woo HR, Lim PO, Lee IC, Sheen J, Nam HG, Hwang I (2006) Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in Arabidopsis. PNAS **103**: 814–819

Ko D, Kang J, Kiba T, Park J, Kojima M, Do J, Kim KY, Kwon M, Endler A, Song W-Y, et al (2014) Arabidopsis ABCG14 is essential for the root-to-shoot translocation of cytokinin. Proceedings of the National Academy of Sciences **111**: 7150–7155

Kurakawa T, Ueda N, Maekawa M, Kobayashi K, Kojima M, Nagato Y, Sakakibara H, Kyozuka J (2007) Direct control of shoot meristem activity by a cytokinin-activating enzyme. Nature **445**: 652–655

Kuroha T, Tokunaga H, Kojima M, Ueda N, Ishida T, Nagawa S, Fukuda H, Sugimoto K, Sakakibara H (2009) Functional Analyses of LONELY GUY Cytokinin-Activating Enzymes Reveal the Importance of the Direct Activation Pathway in Arabidopsis. The Plant Cell **21**: 3152–3169

Leibfried A, To JPC, Busch W, Stehling S, Kehle A, Demar M, Kieber JJ, Lohmann JU (2005) WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. Nature **438**: 1172–1175

Letham DS (1963a) Purification of factors inducing cell division extracted from plum fruitlets. Life Sciences **2**: 152–157

Letham DS (1963b) Zeatin, a Factor Inducing Cell Division Isolated From Zea mays. Life Sci **8**: 569–573

Letham DS, Palni LMS (1983) The Biosynthesis and Metabolism of Cytokinins. Annual Review of Plant Physiology **34**: 163–197

Letham DS, Palni LMS, Tao G-Q, Gollnow BI, Bates CM (1983) Regulators of cell division in plant tissues XXIX. The activities of cytokinin glucosides and alanine conjugates in cytokinin bioassays. J Plant Growth Regul **2**: 103–115

Letham DS, Wilson MM, Parker CW, Jenkins ID, Macleod JK, Summons RE (1975) Regulators of cell division in plant tissues: XXIII. The identity of an unusual metabolite of 6-benzylaminopurine. Biochimica et Biophysica Acta (BBA) - General Subjects **399**: 61–70

Liang YS, Ermawati N, Cha J-Y, Jung MH, Su'udi M, Kim MG, Ha S-H, Park C-G, Son D (2010) Overexpression of an AP2/ERF-type transcription factor CRF5 confers pathogen resistance to Arabidopsis plants. J Korean Soc Appl Biol Chem **53**: 142–148

Mähönen AP, Bishopp A, Higuchi M, Nieminen KM, Kinoshita K, Törmäkangas K, Ikeda Y, Oka A, Kakimoto T, Helariutta Y (2006) Cytokinin Signaling and Its Inhibitor AHP6 Regulate Cell Fate During Vascular Development. Science **311**: 94–98

Mähönen AP, Bonke M, Kauppinen L, Riikonen M, Benfey PN, Helariutta Y (2000) A novel two-component hybrid molecule regulates vascular morphogenesis of the Arabidopsis root. Genes Dev **14**: 2938–2943

Martin RC, Mok MC, Mok DWS (1999) A Gene Encoding the Cytokinin Enzyme ZeatinO-Xylosyltransferase of Phaseolus vulgaris. Plant Physiology **120**: 553–558

McGaw BA, Horgan R (1983) Cytokinin catabolism and cytokinin oxidase. Phytochemistry **22**: 1103–1105

Miller CO (1961) A Kinetin-Like Compound in Maize. PNAS **47**: 170–174

Miller CO, Skoog F, Okumura FS, Von Saltza MH, Strong FM (1956) Isolation, Structure and Synthesis of Kinetin, a Substance Promoting Cell Division1,2. J Am Chem Soc **78**: 1375–1380

Miller CO, Skoog F, Von Saltza MH, Strong FM (1955) KINETIN, A CELL DIVISION FACTOR FROM DEOXYRIBONUCLEIC ACID1. J Am Chem Soc **77**: 1392–1392

Miyata S, Urao T, Yamaguchi-Shinozaki K, Shinozaki K (1998) Characterization of genes for two-component phosphorelay mediators with a single HPt domain in Arabidopsis thaliana. FEBS Letters **437**: 11–14

Mok DW, Mok and MC (2001) Cytokinin Metabolism and Action. Annual Review of Plant Physiology and Plant Molecular Biology **52**: 89–118

Morris RO (1977) Mass Spectroscopic Identification of Cytokinins: Glucosyl Zeatin and Glucosyl Ribosylzeatin from Vinca rosea Crown Gall. Plant Physiology **59**: 1029–1033

Morris RO, Bilyeu KD, Laskey JG, Cheikh NN (1999) Isolation of a Gene Encoding a Glycosylated Cytokinin Oxidase from Maize. Biochemical and Biophysical Research Communications **255**: 328–333

Ng S, Ivanova A, Duncan O, Law SR, Aken OV, Clercq ID, Wang Y, Carrie C, Xu L, Kmiec B, et al (2013) A Membrane-Bound NAC Transcription Factor, ANAC017, Mediates Mitochondrial Retrograde Signaling in Arabidopsis. The Plant Cell tpc.113.113985

O'Brien JA, Benkova E (2013) Cytokinin cross-talking during biotic and abiotic stress responses. Front Plant Sci. doi: 10.3389/fpls.2013.00451

Palni LMS, Palmer MV, Letham DS (1984) The stability and biological activity of cytokinin metabolites in soybean callus tissue. Planta **160**: 242–249

Parker CW, Letham DS, Cowley DE, MacLeod JK (1972) Raphanatin, an unusual purine derivative and a metabolite of zeatin. Biochemical and Biophysical Research Communications **49**: 460–466

Parker CW, Wilson MM, Letham DS, Cowley DE, MacLeod JK (1973) The glucosylation of cytokinins. Biochemical and Biophysical Research Communications **55**: 1370–1376

Parkinson JS, Kofoid EC (1992) Communication Modules in Bacterial Signaling Proteins. Annual Review of Genetics **26**: 71–112

Pavlů J, Novák J, Koukalová V, Luklová M, Brzobohatý B, Černý M (2018) Cytokinin at the Crossroads of Abiotic Stress Signalling Pathways. Int J Mol Sci. doi: 10.3390/ijms19082450

Rashotte AM, Mason MG, Hutchison CE, Ferreira FJ, Schaller GE, Kieber JJ (2006) A subset of Arabidopsis AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. PNAS **103**: 11081–11085

Richmond AE, Lang A (1957) Effect of Kinetin on Protein Content and Survival of Detached Xanthium Leaves. Science **125**: 650–651

Romanov GA, Lomin SN, Schmülling T (2006) Biochemical characteristics and ligand-binding properties of Arabidopsis cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. J Exp Bot **57**: 4051–4058

Sakakibara H (2006) CYTOKININS: Activity, Biosynthesis, and Translocation. Annual Review of Plant Biology **57**: 431–449

Scheres B, Laurenzio LD, Willemsen V, Hauser MT, Janmaat K, Weisbeek P, Benfey PN (1995) Mutations affecting the radial organisation of the Arabidopsis root display specific defects throughout the embryonic axis. Development **121**: 53–62

Schmülling T, Werner T, Riefler M, Krupková E, Manns IB y (2003) Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, Arabidopsis and other species. J Plant Res **116**: 241–252

Shi X, Gupta S, Rashotte AM (2014) Characterization of two tomato AP2/ERF genes, SlCRF1 and SlCRF2 in hormone and stress responses. Plant Cell Rep **33**: 35–45

Shi X, Gupta S, Rashotte AM (2012) Solanum lycopersicum cytokinin response factor (SlCRF) genes: characterization of CRF domain-containing ERF genes in tomato. J Exp Bot **63**: 973–982

Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symposia of the Society of Experimental Biology **54**: 118–130

Šmehilová M, Dobrůšková J, Novák O, Takáč T, Galuszka P (2016) Cytokinin-Specific Glycosyltransferases Possess Different Roles in Cytokinin Homeostasis Maintenance. Front Plant Sci. doi: 10.3389/fpls.2016.01264

Spíchal L, Rakova NY, Riefler M, Mizuno T, Romanov GA, Strnad M, Schmülling T (2004) Two Cytokinin Receptors of Arabidopsis thaliana, CRE1/AHK4 and AHK3, Differ in their Ligand Specificity in a Bacterial Assay. Plant Cell Physiol **45**: 1299–1305

Striberny B, Melton AE, Schwacke R, Krause K, Fischer K, Goertzen LR, Rashotte AM (2017) Cytokinin Response Factor 5 has transcriptional activity governed by its C-terminal domain. Plant Signaling & Behavior **12**: e1276684

Suzuki T, Imamura A, Ueguchi C, Mizuno T (1998) Histidine-Containing Phosphotransfer (HPt) Signal Transducers Implicated in His-to-Asp Phosphorelay in Arabidopsis. Plant Cell Physiol **39**: 1258–1268

Suzuki T, Ishikawa K, Yamashino T, Mizuno T (2002) An Arabidopsis Histidine-Containing Phosphotransfer (HPt) Factor Implicated in Phosphorelay Signal Transduction: Overexpression of AHP2 in Plants Results in Hypersensitiveness to Cytokinin. Plant Cell Physiol **43**: 123–129

Suzuki T, Sakurai K, Ueguchi C, Mizuno T (2001) Two Types of Putative Nuclear Factors that Physically Interactwith Histidine-Containing Phosphotransfer (Hpt) Domains,

SignalingMediators in His-to-Asp Phosphorelay, in Arabidopsisthaliana. Plant Cell Physiol **42**: 37–45

Takei K, Sakakibara H, Sugiyama T (2001) Identification of Genes Encoding Adenylate Isopentenyltransferase, a Cytokinin Biosynthesis Enzyme, inArabidopsis thaliana. J Biol Chem **276**: 26405–26410

Takei K, Yamaya T, Sakakibara H (2004) Arabidopsis CYP735A1 and CYP735A2 Encode Cytokinin Hydroxylases That Catalyze the Biosynthesis of trans-Zeatin. J Biol Chem **279**: 41866–41872

Taya Y, Tanaka Y, Nishimura S (1978) 5′-AMP is a direct precursor of cytokinin in Dictyostelium discoideum. Nature **271**: 545–547

Tran L-SP, Urao T, Qin F, Maruyama K, Kakimoto T, Shinozaki K, Yamaguchi-Shinozaki K (2007) Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in Arabidopsis. Proceedings of the National Academy of Sciences **104**: 20623–20628

Ueguchi C, Koizumi H, Suzuki T, Mizuno T (2001a) Novel Family of Sensor Histidine Kinase Genes in Arabidopsis thaliana. Plant Cell Physiol **42**: 231–235

Ueguchi C, Sato S, Kato T, Tabata S (2001b) The AHK4 Gene Involved in the Cytokinin-Signaling Pathway as a Direct Receptor Molecule in Arabidopsis thaliana. Plant Cell Physiol **42**: 751–755

Vreman HJ, Skoog F, Frihart CR, Leonard NJ (1972) Cytokinins in Pisum Transfer Ribonucleic Acid. Plant Physiology **49**: 848–851

Wang J, Ma X-M, Kojima M, Sakakibara H, Hou B-K (2011) N-Glucosyltransferase UGT76C2 is Involved in Cytokinin Homeostasis and Cytokinin Response in Arabidopsis thaliana. Plant Cell Physiol **52**: 2200–2213

Wang Y, Shen W, Chan Z, Wu Y (2015) Endogenous Cytokinin Overproduction Modulates ROS Homeostasis and Decreases Salt Stress Resistance in Arabidopsis Thaliana. Front Plant Sci. doi: 10.3389/fpls.2015.01004

Werner T, Motyka V, Laucou V, Smets R, Onckelen HV, Schmülling T (2003) Cytokinin-Deficient Transgenic Arabidopsis Plants Show Multiple Developmental Alterations Indicating Opposite Functions of Cytokinins in the Regulation of Shoot and Root Meristem Activity. The Plant Cell **15**: 2532–2550

Werner T, Motyka V, Strnad M, Schmülling T (2001) Regulation of plant growth by cytokinin. PNAS **98**: 10487–10492

Whitty CD, Hall RH (1974) A Cytokinin Oxidase in Zea mays. Can J Biochem **52**: 789–799

Wulfetange K, Lomin SN, Romanov GA, Stolz A, Heyl A, Schmulling T (2011) The Cytokinin Receptors of Arabidopsis Are Located Mainly to the Endoplasmic Reticulum. PLANT PHYSIOLOGY **156**: 1808–1818

Yamada H, Suzuki T, Terada K, Takei K, Ishikawa K, Miwa K, Yamashino T, Mizuno T (2001) The Arabidopsis AHK4 Histidine Kinase is a Cytokinin-Binding Receptor that Transduces Cytokinin Signals Across the Membrane. Plant Cell Physiol **42**: 1017–1023

Yoshida R, Oritani T (1972) Cytokinin glucoside in roots of the rice plant. Plant Cell Physiol **13**: 337–343

Zhang K, Novak O, Wei Z, Gou M, Zhang X, Yu Y, Yang H, Cai Y, Strnad M, Liu C-J (2014) Arabidopsis ABCG14 protein controls the acropetal translocation of root-synthesized cytokinins. Nature Communications. doi: 10.1038/ncomms4274

Zwack PJ, Compton MA, Adams CI, Rashotte AM (2016a) Cytokinin response factor 4 (CRF4) is induced by cold and involved in freezing tolerance. Plant Cell Rep **35**: 573–584

Zwack PJ, De Clercq I, Howton TC, Hallmark HT, Hurny A, Keshishian EA, Parish AM, Benkova E, Mukhtar MS, Van Breusegem F, et al (2016b) Cytokinin Response Factor 6 Represses Cytokinin-Associated Genes during Oxidative Stress. Plant Physiol **172**: 1249–1258

Zwack PJ, Rashotte AM (2015) Interactions between cytokinin signalling and abiotic stress responses. J Exp Bot **66**: 4863–4871

Zwack PJ, Robinson BR, Risley MG, Rashotte AM (2013) Cytokinin Response Factor 6 Negatively Regulates Leaf Senescence and is Induced in Response to Cytokinin and Numerous Abiotic Stresses. Plant Cell Physiol **54**: 971–981

Zwack PJ, Shi X, Robinson BR, Gupta S, Compton MA, Gerken DM, Goertzen LR, Rashotte AM (2012) Vascular Expression and C-Terminal Sequence Divergence of Cytokinin Response Factors in Flowering Plants. Plant Cell Physiol **53**: 1683–1695

Chapter 2: Review – Cytokinin Response Factors: Responding to More Than Cytokinin

H. Tucker Hallmark, Aaron M. Rashotte*

101 Rouse Life Sciences, Department of Biological Sciences, Auburn University, USA

*Corresponding Author: Aaron M. Rashotte 101 Rouse Life Sciences, Department of Biological Sciences, Auburn University, USA. E-mail: rashotte@auburn.edu

Declarations of interest: none

Citation: Hallmark HT and Rashotte AM (2019) Review – Cytokinin Response Factors: Responding to More Than Cytokinin. Plant Science 289: 110251.

https://doi.org/10.1016/j.plantsci.2019.110251

Abstract

Cytokinin Response Factors (CRFs) are a family of transcription factors which make up a side branch of the classical cytokinin two-component signaling pathway. CRFs were originally identified and have been primarily studied in Arabidopsis thaliana, although orthologs have be found throughout all land plants. Research into the evolution of CRFs as sub-group members of the larger APETALA2/Ethylene Response Factor (AP2/ERF) family has yielded interesting and useful insights related to the functional roles of CRFs in plants. Recent studies of CRFs suggest that these transcription factors are a lot more than just a group of cytokinin related genes and play important roles in both plant development and environmental stress response. This review focuses on recent advances in understanding the roles of CRFs beyond cytokinin, in reproductive development and abiotic stress response, as well as to other environmental cues.

Keywords: Cytokinin Response Factors; CRF; abiotic stress; development; cytokinin

Table of Contents:

1. Introduction

First described in 2006, Cytokinin Response Factors (CRFs) are a small gene family found ubiquitously in land plants as part of the larger AP2/ERF transcription factor (TF) family [1,2]. CRFs are composed of a single AP2/ERF TF DNA binding domain, a highly conserved CRF domain, involved in protein-protein interaction, that distinguishes CRFs from other AP2/ERF members, and a variable C-terminal region [1,2]. In addition, the roughly 10-12 CRF genes found in most Angiosperms are not a monolithic group; instead they can be further divided into 5 distinct groups or clades (I-V), mostly easily identified by conservation of their C-terminal protein regions [3]. CRFs serve as a side-branch of a two-component signaling system, in which cytokinin binds to a HISTIDINE KINASE receptor which becomes autophosphorylated, then passes is phosphoryl group to a HISTIDINE PHOSPHOTRANSFER PROTEIN (HPT), which passes its phosphoryl group to a TF, a TYPE-B RESPONSE REGULATOR (RRB). Phosphorylation of these TFs leads to their activation, causing them to bind DNA and activate transcription of cytokinin-related genes. The exact relationship of CRFs with the rest of the two-component pathway is complex, as some CRFs appear to be targets of RRBs and some appear to interact directly with HPTs [reviewed in 4]. Yet despite the CRF designation, not all CRFs are cytokinin-responsive; instead, expression of CRFs is broadly regulated by a range of environmental cues and even other hormones such as auxin and abscisic acid [5-10]. The diverse signals that lead to induction of CRF expression suggest CRFs play pleiotropic roles in plant development and stress response as opposed to solely mediating cytokinin-regulated processes. Recent studies, primarily in the model species Arabidopsis thaliana (hereafter referred to as Arabidopsis), have indicated broader functional roles for CRFs in processes related to reproductive development and embryogenesis [11-14], as well as abiotic stress response,

including salt [12,15,16], oxidative [6,10,17], and cold stress [9,18,24]. Additionally, the mechanisms behind different CRF functions are starting to be revealed, largely from detailed molecular studies, but also from computational studies implicating CRF4 as a key part of nitrogen responsive signaling [10,19-21,33]. Together, these findings suggest CRFs contribute significantly to proper regulation of plant development and stress response.

2. Cytokinin Response Factors and Plant Reproductive Development

Reproductive development including proper embryo formation is critical to formation of seeds in the next generation. The first evidence for a functional role of CRFs in reproduction came from the inability to obtain a viable double homozygous knockout of *crf5* and *crf6* in Arabidopsis, despite other double and triple CRF mutant combinations showing no such defects [1]. Approximately 25% of seeds abort in the F2 generation of a *crf5* x *crf6* cross, and microscopy revealed a similar 25% of embryos were unable to develop beyond heart or early globular stage [1]. This indicated that at least some CRFs might be required for normal embryo development. This issue was further explored in a later study [14], where it was shown that three Arabidopsis CRFs (CRF2, CRF3, and CRF6) are embryonically expressed and that their loss-of-function mutants display embryo phenotypes, such as atypical cell divisions and even the formation of two embryos from a single fertilization event [14]. Even though the penetrance of these embryo phenotypes is relatively low, they still occur in *crf2*, *crf3*, and *crf3crf6* at a frequency which is approximately ten times greater than in wildtype [14]. The authors note this phenotype resembles that found in the auxin transporter mutants, *pin1* and *pin7*, two genes which the authors show are under transcriptional control of CRFs [14]. An additional study in cork oak, *Quercus suber*, identified an ortholog of CRF3 as having a role in embryogenesis, and the authors suggest it may

be important for cytokinin-mediated embryonic development [22]. These studies reveal CRFs play a role, at times with the auxin-related PIN genes, in regulating embryo development [14, 22].

The importance of CRFs for proper seed development was further established when it was revealed that some multiple or higher order *crf* mutant combinations (*crf2,5,6*, *crf1,3,5,6*, and the segregating *crf1-/+,2,5,6*) have smaller siliques with fewer seeds than that of wildtype [13]. In this study [13], several multiple mutants examined contain *crf5,6* combinations that were previously noted as embryo lethal [1]. However, in this more recent study [13], only a knockdown *crf5* mutant allele was used as opposed to the *crf5* knockout allele used previously [1], potentially explaining how the authors were able to isolate viable plants with both *crf5* and *crf6* mutations. There is lethality in the attempt to generate a homozygous knockout mutant *crf1,2,5,6* containing both *crf1* and *crf2* knockouts and data from controlled crosses involving the *crf1-/+,2,5,6* mutant suggest a sporophytic effect as the cause of infertility [13]. Recent work in a similar mutant, *crf2,3,6*, has provided interesting insights into what phases of reproductive development these TFs are involved [11]. The triple mutant, which displays fewer ovules per pistil, decreased placental length, and decreased ovule density relative to wildtype, lacks functional copies of the three CRFs expressed during embryo development [11,14].

While the mechanism behind this reproductive embryo lethality it is not specifically clear, our best hints at this are based on evolutionary relationships within the CRFs. In Arabidopsis, mutant lines have been examined in three distinct evolutionary CRF clades, each with two members: Clade I – CRF1, CRF2, Clade II – CRF3, CRF4, and Clade III – CRF5, CRF6 [3; Figure 1]. At no point is there a multiple mutant knockout combination that completely eliminates CRF expression from one clade. The *crf2,3,6* triple mutant is missing one CRF from

Clades I, II, and III [11]. A homozygous quadruple *crf1,2,5,6* mutant was never obtained; CRF1 and CRF2 are both Clade I members, and CRF5 and CRF6 are Clade III members, yet the mutant allele of crf5 used in this higher order was not a full knockout [13]. A full double knockout of *crf5,6* or elimination if Clade III is embryo lethal [1]. This may suggest that Arabidopsis requires at least one copy of a CRF from each clade to remain viable. These studies would minimally indicate that both Clade I and III are important for normal reproductive development.

3. Cytokinin Response Factors and Abiotic Stress

Though first discovered as being a part of the cytokinin signaling cascade, CRFs have been increasingly identified as being key TFs in abiotic stress response, including response to oxidative, cold, salt, drought, and flooding stress [5,7,9,10,12,23; Fig. 1]. A study on mitochondrial retrograde signaling found that, after treatment of Arabidopsis with the mitochondrial inhibitor Antimycin A, a membrane-bound TF, NAC017, relocalizes to the nucleus and physically binds the promoters of CRF5 and CRF6 to activate their transcription [6]. A detailed study of CRF6 revealed that, in addition to negatively regulating senescence as previously shown [23], this TF is capable of negatively regulating oxidative stress independently of the cytokinin signaling pathway [10]. Arabidopsis plants lacking a functional copy of CRF6 have decreased photosystem II efficiency and chlorophyll content after treatment with oxidative stress agents than wildtype, and the opposite is true of plants overexpressing CRF6 [10]. Interestingly, CRF6 downregulates several cytokinin associated genes during stress response, possibly as a protective measure against oxidative stress [10]. There has been some previous work in tomato connecting other orthologous CRFs (other Clade III CRFs) to oxidative stress

[5]. Recently, expression of another Clade III CRF from the Asteraceae in *Marshallia* also shows induction by oxidative stress, possibly suggesting Clade-specific functions for CRFs across Angiosperms [17].

Cold and freezing stress response has also been linked to CRFs in Arabidopsis, where the majority of work focused on Clade II (CRF3 and CRF4), primarily CRF4 [9]. After noticing both CRF3 and CRF4 expression increased after exposure to cold, the authors focused on comparing *crf4* knockout mutants and CRF4 overexpressing plants to wildtype and found that CRF4 overexpressing plants have significantly decreased germination at 4°C [9]. Interestingly, a different effect was noted when plants were exposed to freezing stress: *crf4* mutants and CRF4 overexpressing plants showed decreased and increased survival, respectively, compared to wildtype after exposure to freezing temperatures [9]. This work also placed CRF4 downstream of the standard cold response pathway as potential targets of CBF TF proteins [9]. CRF4 is not the only CRF connected to cold stress; transcriptome analysis performed as part of a natural variation study revealed that CRF2 and CRF3 are among the “first-wave transcription factors” whose expression is induced in response to exposure to 4°C for twenty-four hours [18]. This study further connects these CRFs into the cold stress response pathway like CRF4 [18]. Cold-induction of CRF2 expression is dependent on the two-component signaling pathway, specifically ARR1, whereas cold-induction of CRF3 is not [24]. Furthermore, mutants of *crf2* and *crf3* grow fewer lateral roots than wildtype under cold conditions, and the effect of these two mutations is additive, suggesting a role for these TFs in cold-regulated root development [24].

Salt stress is an increasing issue in agriculture, which occurs when plants are exposed to salt that builds up over time in irrigated fields [25]. Both CRF1 and CRF2 have been connected to salt stress; in Arabidopsis, *crf1* and *crf2* loss of function mutants have higher photosystem II

efficiency than wildtype after treatment with salt [12]. Since plants lacking a functional CRF1 or CRF2 appear more resistant to the damage caused by salt stress, one may hypothesize that these TFs negatively regulate response to salt stress. Interestingly, another CRF (ThCRF1) from *Tamarix hispida* has also been connected to salt stress [16]. Electrolyte leakage measurement and Evans blue staining reveal that plants overexpressing ThCRF1 are more resistant to negative effects of salt stress [16]. A potential mechanism for this stress resistance is an increase in antioxidant capacity; ThCRF1 overexpression lines have increased expression of superoxide dismutase and peroxidase genes as compared to wildtype under both normal and salt stress conditions [16]. This oxidative stress resistant phenotype also resembles that seen in Arabidopsis plants overexpressing CRF6 [10]. ThCRF1 is an example of a CRF from a non-model species having a role in stress response, similar to the tomato CRFs (SlCRF1, SlCRF2, SlCRF3, and SlCRF5) which have been connected to heat, cold, drought, flooding, salt, and osmotic stress response [5,7,8, reviewed in 26; Table 1]. These data seem to suggest that CRFs are not only important for development under standard conditions, but that CRFs are important regulators of plant-environment interactions.

4. Cytokinin Response Factors and Auxin

It has long been known that auxin and cytokinin have significant often opposing interactions [27]. In some cases, genes primarily responsive to one of these hormones, often are also secondarily responsive to the other hormone as part of a feedback loop, making it difficult to determine such interactions are direct or indirect [27]. Recent studies of CRFs and the auxin transporters, PINs, suggest a more direct role for CRFs in regulating auxin distribution throughout the plant [11,14]. PINs are responsible for the polar transport of auxin from cell to

cell, and some PINs, such as PIN1 and PIN7, have been shown to be regulated by cytokinin [14]. To further understand the way in which cytokinin regulates PINs, serial deletions of the PIN7 promoter from Arabidopsis were performed, and the cytokinin responsive element in the promoter was tested in a yeast one-hybrid assay to determine what TFs might bind to it [14]. Results of the yeast one-hybrid revealed CRF2, CRF3, and CRF6 are capable of binding the cytokinin responsive elements found in some PIN promoters [14]. Chromatin immunoprecipitation and luciferase reporter assays provided further proof that both CRF2 and CRF6 are capable of binding to elements found in the promoters of some PIN genes, suggesting roles for CRFs in controlling auxin distribution [14]. Analysis of the cytokinin responsive elements revealed that the CRF-regulated PIN promoters lack the canonical GCC box previously thought to be the target of AP2/ERF TFs; instead, it is suggested that CRF2 and CRF6 recognize motifs with a conserved G2, G5, and C7 core bases [14]. The occurrence of double embryos, as well as decreased size of roots and root meristems in certain *crf* mutants are similar to that of auxin transport mutants, further indicating CRFs play a key role in regulating auxin concentrations [14]. These findings were followed up with physiological analysis of the *crf2,3,6* mutant (discussed above), revealing that this mutant has significantly decreased PIN1 expression in inflorescences even after exogenous cytokinin treatment, establishing these three CRFs are involved in proper cytokinin-dependent PIN1 expression [11].

Earlier work on auxin signaling connected CRF2 to auxin, as it was identified as being a downstream target of MONOPTEROS (CRF2 = TARGET OF MONOPTEROS 3), a key auxin-regulated TF [28]. MONOPTEROS and CRF2 both affect organogenesis, and a functional copy of CRF2 is required for de novo shoot formation in plants with a gain-of-function allele of MONOPTEROS [29]. Furthermore, both genes are expressed in inflorescence shoot apices, with

CRF2 expression requiring MONOPTEROS and two chromatin remodeling ATPases [30]. Altogether, these data suggest CRF2 acts as a central bridge between cytokinin and auxin signaling with effects on development, and, more generally, CRFs play roles in auxin signaling as well as auxin transport via PINs.

A recent publication further connects auxin and cytokinin responses through some CRFs with their involvement in response to gravity during root development [31]. Results of a genome-wide association study in Arabidopsis implicated cytokinin metabolism as having an important role in gravitropic setpoint angle, the angle at which lateral roots develop relative to the main root, and the authors investigated multiple cytokinin mutants [31]. CRF2 and CRF3 were found to be expressed in lateral roots from emergence to maturity, so *crf2* and *crf3* loss-of-function mutants were examined [31]. Both loss-of-function mutants displayed more lateral root bending than wildtype, while plants overexpressing these genes had lateral roots more perpendicular to the main root [31]. This implicates CRF2 and CRF3 as having some effect on the development of lateral roots with regards to gravity, usually thought of as a process controlled by auxin transport. However, the lack of asymmetrical distribution of CRF2 and CRF3 as observed by GFP promoter lines in gravity responding lateral roots indicates that other unknown factors are involved in the connection between CRFs, PIN auxin transport, and gravity response.

5. Other advances in understanding Cytokinin Response Factors: Nitrogen signaling, Root development, Biotic stress, CUC2-interactions

Nitrogen is indispensable for plant growth and development, and plants have developed a variety of methods to ensure their nitrogen needs are satisfied [32]. A recent study using computational modeling to identify the most important genes involved in plant nitrogen response found that the

earliest nitrogen responsive TF in Arabidopsis is CRF4 [21]. The authors grew plants on media containing minimal amounts of nitrogen before transferring the plants to media containing standard levels of nitrogen, and RNA was collected at ten timepoints between zero and 120 minutes after transfer for transcriptome analysis [21]. Through machine learning and computational analyses, the authors showed that CRF4 is rapidly induced after exposure to the nitrogen rich media, and CRF4, along with two other TFs (SCHNARCHZAPFEN and CYCLING DOF FACTOR 1), regulates more than half of genes associated with nitrogen uptake and assimilation [21]. The importance of CRF4 in nitrogen response was confirmed in planta by overexpressing CRF4, which led to decreased nitrate uptake and decreased shoot biomass relative to wildtype [21]. Further analysis of CRF4 and the nitrogen signaling network validated the TF as being a key factor in nitrogen response, controlling five key nitrogen-responsive TFs, and revealed that a large number of CRF4-induced genes were regulated by a cis-regulatory element in the 5'UTR [33].

Though all connections between CRFs and environmental cues described above involve abiotic factors, there is also evidence for CRFs having roles in biotic stress response. Arabidopsis plants overexpressing CRF2 show increased expression of pathogenesis related genes, and, as a result, are more resistant to bacterial infection by the pathogen *Pseudomonas syringae* than wildtype Arabidopsis [34]. CRF2 overexpressing plants also show accelerated senescence when compared with wildtype; this finding was further examined by using Trypan blue staining and measuring expression of senescence-associated genes, both of which indicate high expression of CRF2 is correlated with early senescence [34]. These results are similar to a previous study in which Arabidopsis plants overexpressing CRF5 display increased resistance to the pathogen *Pst*

DC3000 as well as upregulation of pathogenesis related genes [35]. It would seem that a more thorough examination of CRFs to biotic stress is warranted.

Several advances in the understanding of CRFs at the molecular level have recently been published. A study on gene expression in axillary meristems and organ boundaries cells revealed that the translatome, i.e. transcripts that are bound by a ribosome, in boundary regions was enriched for cytokinin related genes [20]. To confirm their translatome results and further clarify the gene regulatory network associated with boundary regions, a yeast one-hybrid screen was performed which showed that CRF5 binds upstream of CUP-SHAPED COTYLEDON 2 (CUC2), a key boundary formation transcription factor, which then binds upstream of LATERAL SUPPRESSOR, a key axillary meristem transcription factor [20]. These data suggest an important role for CRF5 in boundary and organ formation, though it is also worth noting that CUC2 regulates PIN1 and formation of ovules and other organs [36, 37]; this may implicate CRF5 as having a role in reproductive development similar to CRF2, 3, and 6 (discussed above) or connections to auxin that are as yet unknown. A separate study of CRF5 utilized a modified yeast two-hybrid assay and a series of amino acid deletions to demonstrate that specific regions the C-terminal of the protein are responsible for its TF activity [19], supporting previous findings that the C-terminal was important for activation [35].

6. Conclusions and Future Directions

Despite the name, Cytokinin Response Factors have roles that extend far beyond response to their namesake phytohormone, as summarized in Figure 1. As evidenced by the many studies reviewed in this article, CRFs have clearly been established as having roles in response to abiotic and biotic stressors, nitrogen uptake and assimilation, and reproductive development in

Arabidopsis. Yet there are still outstanding questions with regards to the functional significance of CRFs. One key unresolved question is why CRFs have evolved as important players at the interface of plant-environment interactions. Though first discovered as being players in cytokinin signaling [1], it may stand to reason that CRFs originally evolved as TFs regulating stress responses, and millions of years of duplication and divergence led to new roles for CRFs, including cytokinin response. However, the opposing argument may be equally valid; perhaps CRFs originally evolved as a part of the cytokinin signaling pathway but eventually became players in stress response. Regardless of which of these theories is accurate, one question still lingers. Is the function of CRFs to integrate cytokinin response and stress response to control development, or are some CRFs performing strictly cytokinin duties and other strictly stress response? It is worth noting that CRFs are expressed throughout plant vasculature [3], which may be an ideal location to integrate hormone signals with environmental conditions.

Moving forward, CRF researchers might consider incorporating some of the following ideas into their research. First, researchers should be mindful of hormone crosstalk; as discussed in this review, cytokinin and CRFs regulate components of other hormone pathways as well particularly auxin. Therefore, processes which involve CRFs may not directly involve cytokinin, but other hormones instead. Second, phylogenetic analyses have revealed interesting information about the evolution of CRFs, in particular the existence of five distinct clades. More exploration of CRFs across taxa, particularly from an evolutionary standpoint, could be highly informative and may lead to the discovery clade-specific functions of CRFs. Deeper analysis of the cis-regulatory elements controlling CRF expression may also shed light on functional similarities between members of the same CRF clade. This subject is particularly interesting considering that there has yet to be an Arabidopsis mutant line that has fully lost an entire clade of CRFs (e.g. full

knockout of both CRF1 and CRF2 or both CRF5 and CRF6), suggesting there may be functional redundancy within the clades. Finally, despite only some CRFs being directly regulated by cytokinin, pairing stress experiments with hormone measurements could be illuminating since little is known about how phytohormones not typically associated with stress, like cytokinin, are affected by various stressors.

References

[1] A.M. Rashotte, et al.

A subset of Arabidopsis AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway.

Proc. Natl. Acad. Sci., 103 (2006), pp. 11081-5, 10.1073/pnas.0602038103

[2] A.M. Rashotte, L.R. Goertzen

The CRF domain defines Cytokinin Response Factor proteins in plants.

BMC Plant Biol., 10 (2010), 10.1186/1471-2229-10-74

[3] P.J. Zwack, et al.

Vascular Expression and C-Terminal Sequence Divergence of Cytokinin Response Factors in Flower Plants.

Plant Cell Physiol., 53 (2012), pp. 1683-95, 10.1093/pcp/pcs110

[4] E.A. Keshishian, A.M. Rashotte

Plant cytokinin signaling.

Essays Biochem., 58 (2015), pp. 13-27, 10.1042/bse0580013

[5] S. Gupta, A.M. Rashotte

Expression patterns and regulation of SlCRF3 and SlCRF5 in response to cytokinin and abiotic stresses in tomato (Solanum lycopersicum).

J. Plant Physiol., 171 (2014), pp. 349-358, 10.1016/j.jplph.2013.09.003

[6] S. Ng, et al.

A Membrane-Bound NAC Transcription Factor, ANAC017, Mediates Mitochondrial Retrograde Signaling in Arabidopsis.

Plant Cell, 25 (2013), pp. 3450-71, 10.1105/tpc.113.113985

[7] X. Shi, S. Gupta, A.M. Rashotte

Characterization of two tomato AP2/ERF genes, SlCRF1 and SlCRF2 in hormone and stress responses.

Plant Cell Rep., 33 (2014), pp. 35-45, 10.1007/s00299-013-1510-6

[8] X. Shi, S. Gupta, A.M. Rashotte

Solanum lycopersicum cytokinin response factor (SlCRF) genes: characterization of CRF domain-containing ERF genes in tomato.

J. Exp. Bot., 63 (2012), pp. 973-82, 10.1093/jxb/err325

[9] P.J. Zwack, M.A. Compton, C.I. Adams, A.M. Rashotte

Cytokinin response factor 4 (CRF4) is induced by cold and involved in freezing tolerance.

Plant Cell Rep., 35 (2016), pp. 573-84, 10.1007/s00299-015-1904-8

[10] P.J. Zwack, et al.

Cytokinin Response Factor 6 Represses Cytokinin-Associated Genes during Oxidative Stress.

Plant Physiol., 172 (2016), pp. 1249-58, 10.1104/pp.16.00415

[11] M. Cucinotta, et al.

Cytokinin response factors integrate auxin and cytokinin pathways for female reproductive organ development.

Development, 143 (2016), pp. 4419-24, 10.1242/dev.143545

[12] E.A. Keshishian

CRF2 and its role in cytokinin response and abiotic stress.

PhD Dissertation, Auburn University (2018), https://etd.auburn.edu/handle/10415/6369

[13] T. Raines, et al.

The cytokinin response factors modulate root and shoot growth and promote leaf senescence in Arabidopsis.

Plant J., 85 (2016), pp. 134–147, 10.1111/tpj.13097

[14] M. Šimášková, et al.

Cytokinin response factors regulate PIN-FORMED auxin transporters.

Nat. Commun., 6 (2015), 8717, 10.1038/ncomms9717

[15] L. Kong, et al.

Comparative analysis of cytokinin response factors in Brassica diploids and amphidiploids and insights into the evolution of Brassica species.

BMC Genomics, 19 (2018), 728, 10.1186/s12864-018-5114-y

[16] L. Qin, et al.

An ERF transcription factor from Tamarix hispida, ThCRF1, can adjust osmotic potential and reactive oxygen species scavenging capability to improve salt tolerance.

Plant Sci., 265 (2017), pp. 154–166, 10.1016/j.plantsci.2017.10.006

[17] A.E. Melton, P.J. Zwack, A.M. Rashotte, L.R. Goertzen

Identification and functional characterization of the Marshallia (Asteraceae) Clade III Cytokinin Response Factor (CRF).

Plant Signal. Behav., (2019), 10.1080/15592324.2019.1633886

[18] S. Park, S.J. Gilmour, R. Grumet, M.F. Thomashow

CBF-dependent and CBF-independent regulatory pathways contribute to the differences in freezing tolerance and cold-regulated gene expression of two Arabidopsis ecotypes locally adapted to sites in Sweden and Italy.

PLOS ONE, 13 (2018), e0207723, 10.1371/journal.pone.0207723

[19] B. Striberny, et al.

Cytokinin Response Factor 5 has transcriptional activity governed by its C-terminal domain.

Plant Signal. Behav., 12 (2017), e1276684, 10.1080/15592324.2016.1276684

[20] C. Tian, et al.

An organ boundary-enriched gene regulatory network uncovers regulatory hierarchies underlying axillary meristem initiation.

Mol. Syst. Biol., 10 (2014), 755, 10.15252/msb.20145470

[21] K. Varala, et al.

Temporal transcriptional logic of dynamic regulatory networks underlying nitrogen signaling and use in plants.

Proc. Natl. Acad. Sci., 115 (2018), pp. 6494-99, 10.1073/pnas.1721487115

[22] T. Capote, et al.

Transcriptome dynamics of cork oak (Quercus suber) somatic embryogenesis reveals active gene players in transcription regulation and phytohormone homeostasis of embryo development.

Tree Genet. Genomes, 15:52 (2019), 10.1007/s11295-019-1353-6

[23] P.J. Zwack, B.R. Robinson, M.G. Risley, A.M. Rashotte

Cytokinin Response Factor 6 Negatively Regulates Leaf Senescence and is Induced in Response to Cytokinin and Numerous Abiotic Stresses.

Plant Cell Physiol., 54 (2013), pp. 971–981, 10.1093/pcp/pct049

[24] J. Jeon, C. Cho, M.R. Lee, N.V. Binh, J. Kim

CYTOKININ RESPONSE FACTOR 2 (CRF2) and CRF3 regulate lateral root development in response to cold stress in Arabidopsis

Plant Cell, 28 (2016), 10.1105/tpc.15.00909

[25] J.R. Acosata-Motos, et al.

Plant Responses to Salt Stress: Adaptive Mechanisms.

Agronomy, 7 (2017), 10.3390/agronomy7010018

[26] P.J. Zwack, A.M. Rashotte

Interactions between cytokinin signaling and abiotic stress responses.

J. Exp. Bot., 66 (2015), pp. 4863-4871, 10.1093/jxb/erv172

[27] G.E. Schaller, A. Bishopp, J.J. Kieber

The Yin-Yang of Hormones: Cytokinin and Auxin Interactions in Plant Development.

Plant Cell, 27 (2015), pp. 44-63, 10.1105/tpc.114.133595

[28] A. Schlereth, et al.

MONOPTEROS controls embryonic root initiation by regulating a mobile transcription factor.

Nature, 464 (2010), pp. 913-916, 10.1038/nature08836

[29] W. Ckurshumova, et al.

Irrepressible MONOPTEROS/ARF5 promotes de novo shoot formation.

New Phytol., 204 (2014), pp. 556-566, 10.1111/nph.13014

[30] M.F. Wu, et al.

Auxin-regulated chromatin switch directs acquisition of flower primordium founder fate.

eLife, 4 (2015), 10.7554/eLife.09269

[31] S. Waidmann, et al.

Cytokinin functions as an asymmetric and anti-gravitropic signal in lateral roots.

Nat. Commun., 10 (2019), pp. 1-14, 10.1038/s41467-019-11483-4

[32] G. Xu, X. Fan, A.J. Miller

Plant Nitrogen Assimilation and Use Efficiency.

Annu. Rev. Plant Biol., 63 (2012), pp. 153-82, 10.1146/annurev-arplant-042811-105532

[33] M.D. Brooks, et al.

Network Walking charts transcriptional dynamics of nitrogen signaling by integrating validated and predicted genome-wide interactions.

Nat. Commun., 10 (2019), 10.1038/s41467-019-09522-1

[34] T. Kwon

Cytokinin Response Factor 2 positively regulates salicylic acid-mediated plant immunity in Arabidopsis thaliana.

Plant Biotechnol., 33 (2016), pp. 207–210, 10.5511/plantbiotechnology.16.0711a

[35] Y.S. Liang, et al.

Overexpression of an AP2/ERF-type transcription factor CRF5 confers pathogen resistance to Arabidopsis plants.

J. Korean Soc. App. Bi., 53 (2010), pp. 142-148, 10.3839/jksabc.2010.024

[36] G.D. Bilsborough, et al.

Model for the regulation of Arabidopsis thaliana leaf margin development.

PNAS, 108 (2011), 3424-3429, 10.1073/pnas.1015162108

[37] F. Galbiati, et al.

An integrative model of the control of ovule primordia formation.

Plant J., 76 (2013), pp. 446-455, 10.1111/tpj.12309

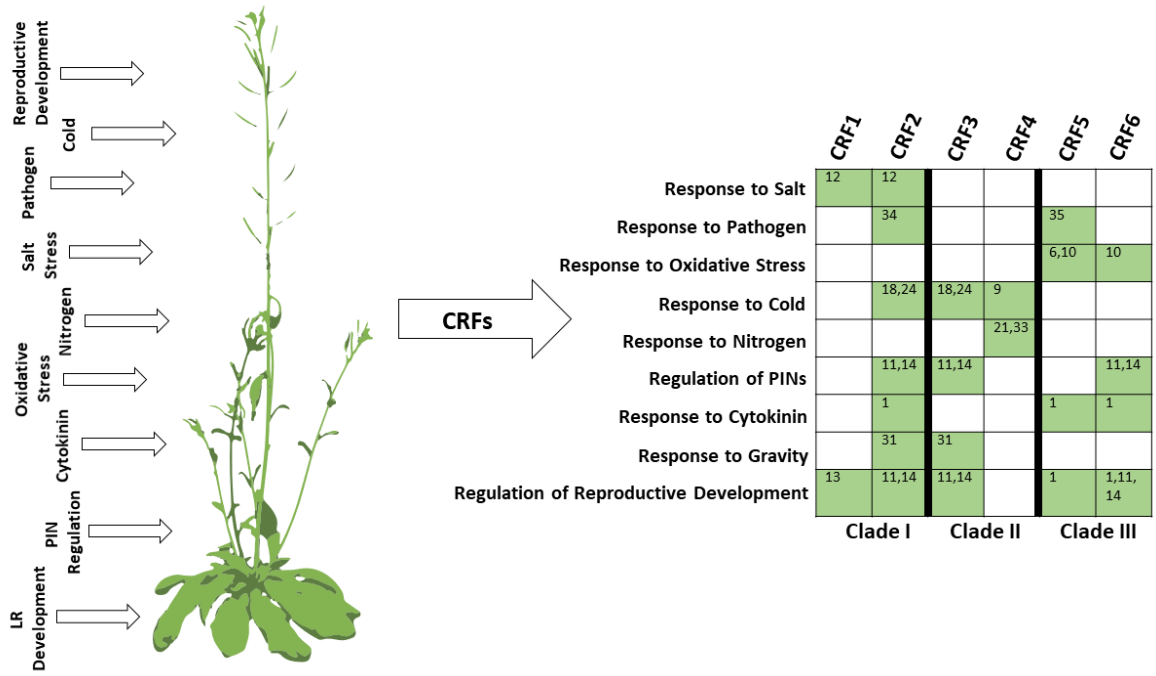


Figure 1. Cytokinin Response Factors (CRFs) are involved in a variety of environmental responses and developmental processes. Arabidopsis CRFs are divided into five clades, three of which have been investigated for roles in physiological processes. Clade I (CRF 1 and 2), Clade II (CRF3 and 4), and Clade III (CRF5 and 6) show somewhat distinct patterns of regulation. Darkened boxes indicate involvement of that CRF in the noted biological process, and numbers indicate the associated reference.

Species	Gene name	Clade	Role	Reference
Solanum lycopersicum	SlCRF1	IV	Response to cytokinin, cold, heat, salt, and drought	7,8
	SlCRF2	I	Response to cytokinin, oxidative stress, and cold	7,8
	SlCRF3	Uncladed	Response to cytokinin, drought, and oxidative stress	5,8
	SlCRF5	III	Response to cytokinin, abscisic acid, flooding, drought, cold, and oxidative stress Root, leaf, and flower development	5,8
Marshallia caespitosa	McCRF1	III	Response to cytokinin and oxidative stress	17
Marshallia mohrii	MmCRF1	III	-	17
Tamarix hispida	ThCRF1	-	Response to salt stress	16
Quercus suber	QsCRF3	II	Embryonic development	22

Table 1. Cytokinin Response Factors from non-Arabidopsis species.

Chapter 3: trans-Zeatin-N-glucosides have biological activity in *Arabidopsis thaliana*

H. Tucker Hallmark[1], Martin Černý[2], Břetislav Brzobohatý[2], Aaron M. Rashotte[1,2*]

[1] Department of Biological Sciences, Auburn University, Auburn, AL USA

[2] Department of Molecular Biology and Radiobiology, Faculty of AgriSciences, Mendel University in Brno, Brno, Czech Republic

*Corresponding author

E-mail: rashotte@auburn.edu (AMR)

Citation: Hallmark HT, Černy´ M, Brzobohaty´ B, Rashotte AM (2020) trans-Zeatin-N-glucosides have biological activity in *Arabidopsis thaliana*. PLoS ONE 15(5): e0232762. https://doi.org/10.1371/journal.pone.0232762

Abstract

Cytokinin is an indispensable phytohormone responsible for physiological processes ranging from root development to leaf senescence. The term “cytokinin” refers to several dozen adenine-derived compounds occurring naturally in plants. Cytokinins (CKs) can be divided into various classes and forms; base forms are generally considered to be active while highly abundant cytokinin-N-glucosides (CKNGs), composed of a CK base irreversibly conjugated to a glucose molecule, are considered inactive. However, results from early CK studies suggest CKNGs do not always lack activity despite the perpetuation over several decades in the literature that they are inactive. Here we show that exogenous application of trans-Zeatin-N-glucosides (tZNGs, a specific class of CKNGs) to Arabidopsis results in CK response comparable to the application of an active CK base. These results are most apparent in senescence assays where both a CK base (tZ) and tZNGs (tZ7G, tZ9G) delay senescence in cotyledons. Further experiments involving root growth and shoot regeneration revealed tZNGs do not always have the same effects as tZ, and have largely distinct effects on the transcriptome and proteome. These data are in contrast to previous reports of CKNGs being inactive and raise questions about the function of these compounds as well as their mechanism of action.

Introduction

Cytokinin (CK) is an adenine derivative which acts as a phytohormone and has roles in a variety of developmental processes, including shoot growth, senescence, and root growth [1]. However, the word “cytokinin” is an umbrella term which includes dozens of compounds naturally occurring in plants [2]. In *Arabidopsis thaliana* (hereafter referred to as Arabidopsis), abundant isoprenoid CKs fall into one of four classes: trans-Zeatin (tZ), isopentenyladenine (iP), cis-Zeatin (cZ), or dihydrozeatin (DZ) These four classes of CK are distinguished by their N6 side chains

[2]. The side chains of tZ, cZ, and DZ are hydroxylated forms of the iP side chain; tZ and cZ differ from one another in their side chain stereochemistry, and DZ lacks a double bond in its side chain [2].

Within each class of CKs, several forms exist. For example, tZ exists in a ribophosphate form (tZRP), a riboside form (tZR), a base form (tZ), an O-glucoside form (tZOG), and N-glucoside forms (tZ7G, tZ9G) [2]. Each form of CKs has a different function: generally speaking, the ribophosphate form is a precursor to bioactive CKs [2], the riboside form is a transported form with some bioactivity [3,4], the base form is the canonical active form [3–5], the O-glucoside form serves as an inactive but convertible storage form [6–9], and N-glucosides have historically been referred to as being permanently inactive and irreversibly conjugated to a glucose molecule [2,7,10–12]. One exception to this is kinetin-N3-glucoside, which has been demonstrated to be reversibly conjugated [6]. Though these compounds are generally present in minuscule amounts, recent advances have allowed researchers to accurately measure CKs, and many studies in Arabidopsis have revealed that the majority of whole-plant CK is in the N-glucoside form [13–19].

Early work on cytokinin-N-glucosides (CKNG) revealed mixed results about the activity of these molecules; some bioassays performed using soybean suggested these compounds lacked activity, while other studies in *Raphanus* and *Amaranthus* suggested otherwise [20–22]. Perhaps the most insightful finding from these studies was that CKNGs have high metabolic stability and do not seem to be converted to bases, ribosides, or other CK forms typically considered to have biological activity [21–23].

In recent years, two genes from Arabidopsis, UGT76C2 and UGT76C1, have been identified as being glucosyltransferases responsible for conversion of CK bases to CKNGs [24]. Mutation of

UGT76C2 leads to a significant reduction in CKNG, though they are not fully abolished, and plants harboring this mutation have increased sensitivity to the CK benzyladenine (BA), decreased seed size, and sensitivity to drought [25,26].

Recently, a metabolic study suggested that tZNGs are converted to active tZ in Arabidopsis within minutes of exogenous application [27] but it is important to note the fact that Hošek *et al*. used Arabidopsis cell lines for some of their work. CKNGs are largely localized to the extracellular space [28], so using a liquid-grown cell line may have significant impacts on CK homeostasis.

In this study, we examined the biological effects of trans-Zeatin-7-glucoside (tZ7G) and trans-Zeatin-9-glucoside (tZ9G), referred to collectively as tZNGs. We show that exogenous application of two highly abundant tZNGs can mimic exogenous application of a canonically active CK base, tZ, in senescence assays, where tZ7G and tZ9G delay senescence in detached leaves. However, these compounds do not appear to function as active CK in the inhibition of root growth or as a major factor in shoot regeneration like tZ. Interestingly, tZ7G and tZ9G appear to have many distinct transcriptional and proteomic regulatory targets separate from tZ. Since CKNGs have been previously shown to be non-convertible to CK base forms, this would indicate a different mechanism is responsible for the activity of tZ7G and tZ9G in CK bioassays. Together, these data suggest that tZNGs display context-dependent CK bioactivity, as well as having their own effects on gene expression.

Materials and Methods

Plant material and growth conditions

Unless otherwise stated, all plants were wildtype Col-0 Arabidopsis. Seeds were surface sterilized using 70% ethanol and 20% bleach with Tween prior to plating on full strength MS

agar plates. Seeds were stratified for 2-4 days before being moved into the growth chamber, which uses a 16h/8h light cycle (100µE) with temperatures of 22°C/18°C. Mutant *cypDM* seed was provided by RIKEN BRC through the National BioResource Project of the MEXT/AMED, Japan.

Cotyledon senescence assay

The cotyledon senescence assays were modified for use with Arabidopsis from previously established CK bioassays [29,30] and were performed at least three times. Cotyledons (15 per treatment) of wildtype Col-0 Arabidopsis seedlings were excised 12 day after germination at the petiole and floated on 3mM MES buffer at pH 5.7. Cytokinin (tZ, tZ7G, tZ9G) was added to a final concentration of 1µM (with the exception of in the dose curve experiments in which the indicated concentration was used) or an equivalent volume of DMSO was added as a solvent control. CKNGs used in this study were obtained from OlChemIm (Olomouc, Czech Republic) as analytical standards with >95% purity guaranteed; no CK bases contaminated these standards. Previous work with CKNGs obtained from OlChemIm has revealed there is no conversion to active CKs during chlorophyll retention assays [31]. Once in solution, the cotyledons were placed in the dark at 20°C for six days, at which point chlorophyll was extracted and quantified according to a previously established protocol [32]. Briefly, cotyledons were transferred to twenty volumes of methanol and were placed at 4°C overnight. The next morning, 100µL of each solution was added to 900µL methanol and absorbance was measured at A652 and A665 to quantify chlorophyll concentration.

Root growth inhibition assay

Wildtype Col-0 Arabidopsis seedlings were germinated on full strength MS agar. Four days after germination, seedlings of uniform size were transferred to full strength MS plates supplemented

with 1µM cytokinin (tZ, tZ7G, tZ9G) or an equivalent volume (0.1%) DMSO. The transferred seedlings were allowed to continue to grow under standard conditions until day 9. Root growth from day 4 to day 9 was measured using ImageJ (NIH). Three biological replicates consisting of at least ten plants per treatment were performed.

Shoot regeneration assay

Shoot regeneration assay was completed similarly to previous assays [33]. Wildtype Col-0 Arabidopsis seedlings were grown for five days in the dark for the seedlings to become etiolated. The hypocotyl was excised, avoiding tissue within 2mm of the root-shoot junction and cotyledons. Hypocotyls were then placed on full strength MS agar supplemented with 5µM cytokinin (tZ, tZ7G, tZ9G) or an equivalent volume of DMSO (0.5%) and 5µM NAA. Development was tracked over 45 days and final callus masses were measured. Three biological replicates consisting of at least four calli per treatment were performed.

Transcriptomic analysis

Whole Col-0 Arabidopsis seedlings, ten days after germination, were transferred from MS agar plates to petri dishes containing 3mM MES buffer at pH 5.7 supplemented with either 1µM cytokinin (tZ, tZ7G, tZ9G) or an equivalent volume of DMSO (0.1%). Plant were gently shaken during a two-hour treatment before seedlings were removed and snap-frozen in liquid nitrogen. RNA was extracted using a Qiagen RNeasy Plant Mini Kit, according to manufacturer's instructions. RNA was sent to Novogene, Inc. for quality check, library preparation, and 150x paired end sequencing on an Illumina HiSeq X. At least 20 million paired end reads were generated per sample. Each treatment had three biological replicates with at least 5 seedlings per treatment. Read quality was evaluated with FastQC [34]. Trimming was performed using Trimmomatic [35]. Read mapping, transcript assembly, and differential expression analysis was

performed using HISAT2, StringTie, and DESeq2 [36,37]. The significance threshold was padj < 0.05. GO analysis was performed using AgriGO 2.0 [38]. Raw sequence data is available for download at NCBI Sequence Read Archive under the BioProject ID PRJNA588257. qRT-PCR confirmation of RNAseq data was completed using SYBR-green and sequence-specific primers on an Eppendorf Realplex2 as previously described [39]. Subcellular localization analysis was performed by running DEG lists through SUBA4 and using the SUBA consensus location [40]. Full transcriptome results and primer sequences can be found in Supplementary Information.

Protein extraction and LC-MS analysis

Plants for proteomic analysis were grown and treated in a manner similar to that done for transcriptomic analysis, then flash frozen in liquid analysis for further sample preparation as detailed above. Each treatment had four biological replicates. Total protein extracts were prepared as described previously [41] with some modifications. Briefly, 100 mg of homogenized tissue was precipitated with 1 ml of methanol/methyl tert-butyl ether/water (1:3:1), pellets were solubilized [2% (w/v) SDS, 30% (w/v) sucrose, 5% (v/v) β-mercaptoethanol, 5 mM EDTA, 100 mM Tris, pH 8.0], proteins were extracted with phenol, precipitated, and the resulting pellets were solubilized. Next, aliquots corresponding to 100 µg of protein were reduced, alkylated with iodoacetamide, digested with trypsin (1:100, Promega) and desalted by C18 SPE. Finally, aliquots corresponding to 2.5 µg of peptide were analyzed by nanoflow C18 reverse-phase liquid chromatography using a 15 cm column (Zorbax, Agilent), a Dionex Ultimate 3000 RSLC nano-UPLC system (Thermo) and the Orbitrap Fusion Lumos Tribrid Mass Spectrometer. Peptides were eluted with up to a 120-min, 4% to 40% acetonitrile gradient. Spectra were acquired using the default settings for peptide identification, employing HCD activation, resolution 60000 (MS) and 15000 (MS2), and 60 s dynamic exclusion. The measured spectra were recalibrated and

searched against Araport 11 protein database by Proteome Discoverer 2.3, employing Sequest HT, Mascot 2.4 and MS Amanda 2.0 with the following parameters: Enzyme - trypsin, max two missed cleavage sites; MS1 tolerance - 5 ppm; MS2 tolerance - 15 ppm (MS Amanda), 0.1 Da (Sequest, Mascot); Modifications - carbamidomethyl (Cys) and up to three dynamic modifications including Met oxidation, Asn/Gln deamidation, N-terminal acetylation. Only proteins with at least two unique peptides were considered for the quantitative analysis. The quantitative differences were determined by Minora, employing precursor ion quantification followed by normalization and background based t-test, and the resulting data were evaluated in Skyline 19.1 (MacCossLab Software, https://skyline.gs.washington.edu).The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcanzio et al., 2016) with the dataset identifier PXD016087.

Results

trans-Zeatin-N-glucosides delay senescence in detached cotyledons

Active cytokinins are known to delay senescence in detached cotyledons [21,29,42,43]. Chlorophyll content can be a proxy to determine if a leaf has senesced or if senescence has been delayed because chlorophyll degradation occurs during senescence [44]. In this assay, cotyledons from 12 day after germination (12dag) seedlings were floated in buffer supplemented with 1µM tZ, tZ7G, or tZ9G. Hormones were dissolved in DMSO (0.1% DMSO treatment was included as a solvent control). The treated cotyledons were placed in the dark for six days to allow senescence to take place. As expected, cotyledons treated with the known active cytokinin tZ retained significantly more chlorophyll than the solvent control (Fig 1). Interestingly, both tZ7G-

and tZ9G-treated samples also retained similar levels of chlorophyll (Fig 1). To test if this effect was dose dependent, a range of hormone concentrations were tested, starting at 10pM (Fig 2). Both tZ7G and tZ9G retained more chlorophyll than the DMSO control even at 1nM concentration (Fig 2). This indicates that tZ7G and tZ9G are effective in this bioassay at similar levels as tZ.

trans-Zeatin-N-glucosides do not affect root elongation

Active cytokinins are known to inhibit root elongation [45,46]. Here, seedlings were germinated on standard media and transferred to the same media supplemented with 1µM tZ, tZ7G, tZ9G, or a 0.1% DMSO solvent control. Root growth was measured from day 4, when the seedlings were transferred to the new media, until day 9. The tZ-treated seedlings had significantly inhibited root growth relative to the DMSO control, whereas the tZNG-treated seedlings saw no difference from the control at the tested concentration (Fig 3). This suggests tZ7G and tZ9G do not significantly influence root elongation in seedlings of this stage.

trans-Zeatin-7-glucoside modestly influences shoot regeneration

A classical test of cytokinin activity is the shoot regeneration assay in which hypocotyls are exposed to cytokinin and auxin. If the cytokinin is “active” and present in proper concentration, then a callus should develop, begin to turn green, and produce shoot-like structures such as leaves [47]. In this assay, hypocotyls were excised from the etiolated Arabidopsis seedlings and were subjected to a treatment with the synthetic auxin NAA and an equal amount of tZ, tZ7G, tZ9G, or DMSO as a negative control. Hormone concentrations used in this experiment were chosen based on Rashotte et al., 2006. To quantify shoot regeneration, callus weight was

determined after 45 days on the hormone-containing media. Samples treated with NAA and tZ produced significantly higher callus weight than those with NAA and DMSO (Fig 4). Though the difference is modest, samples treated with NAA and tZ7G also had a slight but statistically significant increase in weight, relative to the control treatment. No significant change was seen in tZ9G samples.

trans-Zeatin-N-glucosides alter the transcriptome distinctly from trans-Zeatin

To determine if tZNGs affect the transcriptome of Arabidopsis, RNA sequencing was performed. Ten-day old seedlings were floated in buffer supplemented with 1μM tZ, tZ7G, tZ9G, or a 0.1% DMSO control treatment for two hours. After the two-hour treatment, tissue was flash frozen in liquid nitrogen and RNA was extracted and sequenced. The sequencing data was mapped to the Arabidopsis reference genome, and transcript level differential expression analysis was performed to determine Differentially Expressed Gene (DEG) lists for comparison. Each treatment group had hundreds of differentially expressed transcripts, but overlap between treatments was minimal (Fig 5A). Treatment with tZ, tZ7G, and tZ9G led to 340, 190, and 216 transcripts being uniquely regulated, respectively (Fig 5A). The regulation of cytokinin-related DEGs are presented in Table 1, revealing tZ to induce most of these. To validate the overall findings of the RNAseq analysis, qRT-PCR was performed and results generally mirrored transcriptome findings (Fig 5C). These results suggest tZNGs do not tend to regulate CK-related genes in a similar manner to tZ, though there are some notable exceptions such as CKX4 (Table 1, Fig 5C).

To better understand the generated gene lists, Gene Ontology (GO) term enrichment was performed using agriGO v2.0 [38]. As expected, the tZ-treated group had several GO terms

associated with cytokinin (Fig 5B). The tZ7G and tZ9G groups did not have any directly cytokinin-related GO terms, but did include “Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process,” and “Adenyl nucleotide binding” (Fig 5B). It is worth noting that tZ, tZ7G, and tZ9G are all adenine-derived compounds. Although DEGs from neither tZ7G nor tZ9G were enriched for CK-related GO terms, it is of particular interest that tZ9G induced the CK receptor AHK4, specifically a transcript isoform with an extended 5’UTR, compared to the isoform induced by tZ (Table 1). Both tZNGs induced CKH1, a histidine kinase that regulates CK response [48,49], and tZ7G induced CKX4, a cytokinin degrading enzyme which has been shown to degrade CK bases and ribosides, but likely not CKNGs [50,51] (Table 1). Several other potentially interesting transcripts were found as DEGs after tZ7G and tZ9G treatments (Table S2). These include SEN2/AtCAT3, a catalase gene connected to senescence response, which was repressed in tZ7G and tZ9G as well as in tZ; PAC (Pale Cress), a gene involved in chloroplast and leaf development, which was repressed in tZ7G and tZ9G only; and SOD1 (superoxide dismutase 1) that was induced only in tZ9G treatment (Table S2) [52–54]. While subcellular localization of DEGs appears to be quite similar between all CK treatments, there is a slight increase in plastid localization for tZNG treatment, relative to tZ treatment (Figure S1).

Gene Identifier	**Gene Name**	**tZ**		**tZ7G**		**tZ9G**		**CK Function**
		log2FC	padj	log2FC	padj	log2FC	padj	

AT2G01830.4	AHK4	**1.31**	**1.05E-07**	0.77	0.99	0.53	1	Receptor
AT2G01830.6	AHK4	20.78	NA	1.87	NA	**9.75**	**9.27E-11**	Receptor
AT1G59940.1	ARR3	**6.39**	**0.004**	1.47	NA	3.76	1	Response Regulator
AT1G10470.2	ARR4	**2.37**	**2.74E-33**	0.09	1	0.29	1	Response Regulator
AT3G48100.1	ARR5	**3.17**	**5.60E-14**	0.17	1	0.15	1	Response Regulator
AT5G62920.1	ARR6	**3.01**	**1.31E-17**	0.64	1	0.37	1	Response Regulator
AT1G19050.1	ARR7	**2.8**	**3.79E-14**	-0.21	1	0.01	1	Response Regulator
AT2G41310.1	ARR8	**2.1**	**2.46E-05**	0.2	1	0.4	1	Response Regulator
AT1G74890.1	ARR15	**4.05**	**1.34E-09**	-0.29	1	0.01	1	Response Regulator
AT2G40670.1	ARR16	**1.52**	**1.83E-06**	-0.6	1	-0.13	1	Response Regulator
AT3G44326.1	CFB	**2.07**	**0.001**	-0.04	1	0.27	1	Signaling F-Box
AT2G46310.1	CRF5	**1.56**	**0.002**	-0.13	1	-0.02	1	Signaling CRF
AT1G17440.1	CKH1	1.05	0.366	**1.75**	**0.002**	**1.53**	**0.048**	Signaling Response
AT2G17820.1	AHK1	**1.36**	**6.17E-13**	-0.1	1	0.01	1	Ortholog of Receptor
AT5G05860.1	UGT76C2	**1.56**	**3.34E-07**	-0.27	1	-0.01	1	N-Conjugation
AT1G67110.1	CYP735A2	**3.01**	**3.96E-13**	0.19	1	0	1	Biosynthesis
AT5G56970.1	CKX3	**3.13**	**0.029**	0.52	1	0	1	Degradation
AT4G29740.3	CKX4	**6.59**	**5.94E-16**	**4.16**	**0.027**	3.01	0.907	Degradation

Table 1. List of select differentially expressed CK-related transcripts after two-hour treatment with tZ, tZ7G, and tZ9G relative to the DMSO control. Values in bold indicate a significant difference (adjusted p-value < 0.05) between the CK-treated and control-treated samples. NA indicates read counts that were too low to establish statistical significance.

trans-Zeatin-N-glucosides alter the proteome distinctly from trans-Zeatin

In order to determine if tZNGs might be connected to gene regulation beyond the transcriptional level, a proteomic analysis was performed. Seedlings were treated with tZ, tZ7G, tZ9G, or a DMSO control in the same manner as for the transcriptome analysis, then frozen in liquid nitrogen for additional preparation and examination. Once prepared, 2119 protein families were identified, and the relative abundances of 14,412 peptides representing 1,629 proteins were quantified. The liquid chromatography/mass spectrometry (LC/MS) shotgun proteomic analysis yielded 81 proteins with a significant ($P < 0.05$; background based t-test) difference from DMSO-treated control samples in three biological replicates. In detail, 49, 58, and 36 proteins were identified to be regulated by tZ, tZ7G, and tZ9G, respectively (Fig 6). There was a nearly even split between induced (42) and repressed (39) proteins (Table S1). The list of tZ-regulated proteins shares some overlap with previously noted CK-regulated proteins from other proteomic analyses (Table 2) [55–60]. While there is only moderate overlap between the significantly regulated proteins between tZ7G and tZ9G (only 16 shared between the two treatments), both treatments overlap heavily with tZ (Fig 6). Each treatment had several uniquely regulated proteins.

Interestingly, tZ, tZ7G, and tZ9G treatment all led to the differential regulation of ACP4, ERD14, PAE2, AT3G26450, AT4G10300, which have all previously been shown to be CK responsive proteins [55–58] (Table 2). The three proteins regulated by tZ, but not tZNGs, were MAPR2, AT3G15450, AT2G45180, none of which appear to be functionally characterized to date. Among the protein regulated by tZ7G are EDD1, TUB5, NADP1;1, which have been shown to be CK-regulated in previous studies [55,59,60] (Table 2). Proteins regulated by tZ9G included RPL4 and AT5G43830, which have also previously shown CK-regulation [55,57,58]

(Table 2). These data indicate that while tZNGs appear to have some distinct effects on the proteome, treatment with tZNGs may affect the proteome in a manner consistent with previously tested CKs (Table 2). Novel targets of the tZNGs include multiple ribosomal proteins for tZ9G (Fig 6C) and several amino acid metabolism proteins for tZ7G (Fig 6B).

Accession	Response	Protein name (UniProt)	Relative abundance tZ vs DMSO	Relative abundance tZ7G vs DMSO	Relative abundance tZ9G vs DMSO	Gene Symbol	# Unique Peptides	Previous studies showing CK-regulation	UniProt Accession
AT3G26450	tZ- | tZ7G- | tZ9G-	*Polyketide cyclase/dehydrase and lipid transport superfamily protein*	*0.3*	*0.5*	*0.5*	AT3G26450	6	Lochmanová et al., 2008; Černý et al., 2013; down	Q9LIN0

AT4G10300	**tZ- | tZ7G- | tZ9G-**	***RmlC-like cupins superfamily protein***	***0.2***	***0.2***	***0.4***	**AT4G10300**	**2**	**Černý et al., 2014; down**	**Q9SV91**
AT4G25050	**tZ- | tZ7G- | tZ9G-**	**Acyl carrier protein 4, chloroplastic**	***0.5***	***0.6***	***0.3***	**ACP4**	**3**	**Černý et al., 2013; up**	**Q9SW21**
AT1G76180	**tZ- | tZ7G- | tZ9G-**	**Dehydrin ERD14**	***0.3***	***0.2***	***0.2***	**ERD14**	**5**	**Černý et al., 2011; Černý et al., 2014; down / up**	**P42763**
AT3G14290	**tZ+ | tZ7G+ | tZ9G+**	**Proteasome subunit alpha type-5-B, EC 3.4.25.1 (20S proteasome alpha subunit E-2) (Proteasome component Z)**	***2.2***	***2.2***	***1.4***	**PAE2**	**3**	**Černý et al., 2011; down**	**Q42134**
AT2G45180	**tZ+**	***At2g45180 (At2g45180/T14P1.1) (Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein) (Expressed protein) (Putative proline-rich protein)***	***2.9***	**1.6**	**0.8**	**AT2G45180**	**2**		**Q42044**
AT3G15450	**tZ-**	***Aluminum induced protein with YGL and LRDR motifs***	***0.1***	**0.5**	**0.3**	**AT3G15450**	**2**		**F4IYS4**
AT2G24940	**tZ-**	**Probable steroid-binding protein 3, AtMP3 (Membrane-associated progesterone-binding protein 2, AtMAPR2)**	***0.2***	**0.6**	**0.7**	**MAPR2**	**2**		**Q9SK39**
AT3G48110	**tZ7G+**	**Glycine--tRNA ligase, chloroplastic/mitochondrial 2, EC 6.1.1.14 (Glycyl-tRNA synthetase 2, GlyRS-2) (Protein EMBRYO-DEFECTIVE-DEVELOPMENT 1)**	**2.8**	***4.5***	**0.7**	**EDD1**	**2**	**Žďárská et al., 2013; up**	**Q8L785**
AT4G26110	**tZ7G+**	**Nucleosome assembly protein 1;1, AtNAP1;1 (Nucleosome/chromatin assembly factor group A1)**	**1.1**	***0.5***	**0.8**	**NAP1;1**	**4**	**Černý et al., 2011; Zhang et al., 2012; up**	**Q9SZI2**
AT1G20010	**tZ7G-**	**Tubulin beta-5 chain (Beta-5-tubulin)**	**0.7**	***0.3***	**0.7**	**TUB5**	**5**	**Černý et al., 2011; up**	**P29513**
AT5G43830	**tZ9G-**	***Aluminum induced protein with YGL and LRDR motifs***	**0.9**	**1.1**	***0.4***	**AT5G43830**	**5**	**Černý et al., 2011; Černý et al., 2014; down**	**Q9FG81**

Table 2. List of select differentially expressed proteins after two-hour treatment with tZ, tZ7G, and tZ9G relative to the DMSO control. Numbers in bold and italicized indicate a statistically significant difference in the abundance of the protein between the CK treatment and the DMSO control, background based t-test p-value < 0.05.

trans-Zeatin-N-glucosides do not appear to be hydrolyzed to trans-Zeatin in cotyledons

It was recently reported that within minutes of exogenous application, tZNGs were hydrolyzed to tZ base [27]. While the physiological, transcriptomic, and proteomic data above appear to refute

this claim, or at least suggest tZNGs are not fully converted to tZ, we decided to also use a genetic approach to test this hypothesis. Mutants which lack functional copies of CYP735A1 and CYP735A2, the enzymes responsible for hydroxylation of iP-type cytokinins and thus production of tZ-type cytokinins, have been previously generated and described [16]. Use of these mutants in the cotyledon senescence assay, in which tZNGs display the effect most similar to that of tZ, revealed that tZNGs are not capable of delaying senescence in the *cypDM* mutant background, while tZ is (Fig 7). While this result has many implications, it suggests tZNGs are not converted back to tZ in an appreciable amount during this assay. Additionally, a previous study utilized tZ9G obtained from OlChemIm (same manufacturer as the CKs used in this study) to perform a chlorophyll retention assay in wheat; HPLC analysis confirmed no conversion of tZ9G was detected during the assay [31].

Discussion

trans-Zeatin-N-glucosides have physiological activity in Arabidopsis

Though often over-generalized as inactivated forms of cytokinin [2,20], findings here indicate that tZNGs appear to have some cytokinin-like capabilities when exogenously applied to Arabidopsis tissues. What is particularly interesting is that the tZNGs tested do not mimic tZ in every assay (Figs 1-4), suggesting that while there is overlap between the activities of these compounds, they are not identical in their biological roles.

One important prerequisite to examining effects of exogenously applied tZNGs is ensuring purity of the compounds. While no synthesized compounds are 100% pure, as noted in the Methods, the tZNGs used in this study were purchased as analytical standards from OlChemIm (Olomouc, Czech Republic) and are >95% pure with no detectable tZ. Previous analysis of these standards

found that no tZ was detectable in tZ9G solution before, during, or after conducting chlorophyll retention assays similar to what is described in this study [31]. This, along with the overall results of our study, strongly suggest that any effects observed were due to the exogenous application of untainted tZNGs.

Results from the senescence assay suggest exogenous application of tZ7G and tZ9G is capable of delaying senescence to a similar degree as tZ (Fig 1), and the dose curve similarly reveals it is not only at a micromolar concentration that these compounds display activity (Fig 2). The similar trend of the dose curve for tZ, tZ7G, and tZ9G may suggest the same anti-senescent pathway is being activated in each treatment, though separate mechanisms cannot be ruled out. When the same bioassay was performed using mutants which lack endogenous tZ, tZ7G and tZ9G were not capable of delaying senescence, suggesting activity of exogenously applied tZNGs relies on the presence of endogenous tZ (Fig 7).

In the root growth assay, tZ7G and tZ9G displayed effects different from that of tZ. While tZ significantly inhibited root growth in seedlings, neither tZ7G nor tZ9G altered root length (Fig 3). The difference in effects of tZ and the tZNGs suggests that conversion back to tZ, which has previously been shown to not occur [6,22], is also not a reason for the active effects seen in the senescence assay. While tZ7G and tZ9G mimic tZ during cotyledon senescence, it is possible that these compounds have no effect, or a different effect, on roots. Life stage of the plants may also play a role; the pathway in which tZ7G and tZ9G are perceived may not be active early on during seedling development.

During shoot initiation, tZ7G displayed only minor ability to increase callus mass as opposed to the robust increase in mass seen with tZ (Fig 4). Regeneration did not occur in the tZ9G treatment, nor with the negative auxin-only control. Similar to the root growth assay, these data

suggest tZ, tZ7G, and tZ9G do not function identically when exogenously applied to tissue; it is possible each compound only exerts notable effects under particular conditions, i.e. during a specific developmental stage or in a particular organ or tissue.

These bioassay results are strong evidence that tZNGs are not simply converted to tZ as recently reported [27]; one would expect nearly identical results in all bioassays if this were the case. Instead, these data suggest some type of context-dependent cytokinin activity for tZNGs, though the exact nature of this is not yet understood.

trans-Zeatin-N-glucosides have differing effects on gene expression in Arabidopsis

In the transcriptomic analysis (Fig 5), tZ treatment resulted in gene expression changes quite similar to those seen in other CK transcriptome studies, as many genes from the "Golden list" were affected, and cytokinin-related GO terms were strongly enriched [61,62]. The lack of overlap between tZ with tZ7G and tZ9G, as well as the lack of overlap between the two tZNGs, suggests distinct transcriptional roles for all three compounds. There are some similar targets, however, such as CYTOKININ OXIDASE 4 (CKX4, an extracellular CK degrading enzyme) which is induced by both tZ and tZ7G (Table 1). Though it may seem that induction of CKX4 by tZ7G is possible evidence that CKX4 is capable of degrading CKNGs, previous *in vitro* studies suggest CKNGs are largely resistant to degradation by the AtCKX enzymes, with tZ9G only being degraded by two of seven tested CKXs under acidic conditions and at much lower rates than tZ and tZR [63]. A study in which radiolabeled tZNGs were exogenously applied to soybean tissue revealed that tZNGs do not appear to be degraded *in planta* [22]. Also, ARABIDOPSIS HISTIDINE KINASE 4 (AHK4, CK receptor) is regulated by both tZ and tZ9G, although the two induce different transcript isoforms which differ in their 5'UTR, but not

in their protein coding region (Table 1). These data suggest some overlap between the tZ and tZNG perception and/or signaling pathways, despite the largely different transcriptional effects. One characteristic of tZNGs that should be noted is their size. As mentioned earlier, tZNGs are composed of tZ and a glucose molecule. Therefore, tZNGs are significantly larger than tZ which is likely to impact their transportability. While few CK transporters are known and have not been studied with regards to CKNG transport [15,19,64–66], it may stand to reason that tZNGs are not able to cross membranes into cells as efficiently as tZ. This may be one reason for the different transcriptional effects observed.

An interesting difference between treatments is a difference in subcellular localization of the DEGs. While the subcellular distribution of DEGs between treatments is largely the same, there is a visible increase in percentage of DEGs which localize to the plastid during tZ7G (15% of DEGs) and tZ9G (12.3%) treatment relative to tZ treatment (8.6%) (Fig S1); this is reflected in GO Enrichment Analysis, as the term “plastid” was significantly enriched in tZ7G DEGs (FDR < 0.014) but was not significant in tZ or tZ9G DEGs. Treatment with tZ7G also has decreased extracellular-localized DEGs (3.7%) relative to treatment with tZ9G (7%) (Fig S1).

Little overlap was seen between results of the transcriptome and proteome, though this may be unsurprising due to the short treatment time (2h), during which the compounds had to be taken up by the plants, activate signaling pathways, and activate or repress transcription before translation of induced transcripts could take place. The two hour timepoint was chosen as it has previously shown to be effective for determining CK response at the transcriptional level [61,62]. Transcriptional control is of course only one of many levels of gene expression regulation. As noted in other studies of both plant transcriptomes and proteomes, correlation between mRNA and protein levels is influenced by a large number of factors including

biological reasons, such as protein turnover, as well as technical reasons, such as difficulty extracting low molecular weight and hydrophobic proteins [67,68]. Future studies will pair early transcriptomes of tZNG-treated plants with proteomes from later timepoints, possibly increasing the overlap between the two datasets, as two hours may not be enough time for the transcriptional changes observed to be observed at the protein level as well. What is striking between the transcriptome and proteome results is how neither show fully unique, nor entirely distinct, effects of the tZNGs; both sets of results show some degree of overlap as well as some unique targets (Fig 6). As noted with the subcellular localization in the transcriptome findings, there are also some differences in subcellular localization of differentially affected proteins identified as being CK-regulated; the proportion of CK-affected proteins which localize to the mitochondria is decreased relative to the total number of proteins identified in the analysis, possibly suggesting organelle-specific responses to CK treatment (Fig S2).

Many possible mechanisms may be responsible for CKNG activity

Though CKNGs have not been shown to bind to any tested CK receptors, there may be a number of reasons for this: not all CKNGs have been tested, not all CK receptors have been tested, and the binding experiments have been carried out in microbes which may not be an ideal system for detecting these interactions [5,69,70]. These receptor binding studies have been invaluable to our understanding of CK signaling, but it is noteworthy that BA, a highly potent CK, has shown low binding potential in these assays [69,70] while showing high activity *in planta*. When tested, tZ7G and tZ9G showed no AHK3 or AHK4 binding but still displayed minimal activity *in planta* [70], perhaps pointing toward a role for AHK2 in tZNG perception. This suggests at least the possibility that CKNGs may be acting through the established CK signaling pathway despite no

receptor binding assay detecting interaction. However, the lack of similar transcriptional effects between tZ, tZ7G, and tZ9G (Fig 5) may suggest tZNGs, at least at the developmental stages tested, act through different signaling pathways.

It may be possible that the mechanism of CKNG action is a yet undiscovered signaling pathway. Perhaps tZ7G and tZ9G have their own receptors separate from the canonical CK receptors (AHK2, AHK3, and AHK4), though admittedly there is no evidence supporting this hypothesis. However, other histidine kinases such as CYTOKININ INDEPENDENT 1 are known to activate the cytokinin two component signaling pathway despite not being shown to bind to the cytokinins tested [5,71,72], further suggesting our current understanding of the CK signaling pathway is incomplete.

Perhaps the most likely mechanism for the activity of CKNGs is that they act to regulate cytokinin levels. The key cytokinin-N-glucosylating enzyme UGT76C2 has been previously reported to play a role in cytokinin homeostasis [26], and it may stand to reason that exogenous application of CKNGs makes it unfavorable for UGT76C2 to convert CK bases to CKNGs, causing a buildup of CK bases which are responsible for the CK-like phenotypes observed in the senescence assays. One study utilizing an inducible cytokinin oxidase whose preferred substrate is tZ demonstrated that 6h after induction of the enzyme, tZ levels significantly decreased; however, levels of tZ7G surprisingly increased [56]. While it is not clear how increasing degradation of tZ leads to an increase of tZ7G, these data reflect the complex relationship between CK bases and CKNGs and may suggest some type of regulatory role for CKNGs. The authors hypothesize that induction of cytokinin oxidase may have led to an increase in CK biosynthesis; newly synthesized tZ would either be quickly degraded by cytokinin oxidase or converted to the degradation-resistant tZ7G form [56].This may also raise the question of

whether CKNGs play some role in stimulating CK biosynthesis, which could be one explanation for the tZ-like effects seen in chlorophyll retention assays (Figs 1, 2). One approach that could clarify the mechanism by which tZNGs exert effects would be through pairing the CK bioassays with LC-MS measurements to determine CK levels after treatment.

While it is unclear how tZNG might activate an anti-senescent pathway and lead to the delay of senescence in detached cotyledons, it should be noted that DEGs connected to senescence, chloroplast development, and oxidative stress were found to be regulated in the transcriptome analysis (Table S2). These include some DEGs that overlap with tZ treatment like SEN2/AtCAT3 as well as unique tZNG targets such as PAC and SOD1.

Senescence assays involving the tZ-deficient *cypDM* mutants revealed that tZNGs are not capable of delaying senescence in cotyledons which do not produce endogenous tZ (Fig 7). This suggests that exogenous application of tZNGs alone is not sufficient to delay senescence. This may allude to the possibility that exogenous tZNG application increases endogenous levels of tZ. This possible tZ increase does not appear to be the result of hydrolysis of tZNGs to tZ base, as this would likely result in an effect similar to that of exogenous tZ application, which is not observed (Fig 7). This result also appears to provide genetic evidence countering that described in a recent metabolic study showing conversion of tZNGs to tZ [27]. It must be noted, however, that this assay only included cotyledon tissue; while it does not appear that tZNGs are hydrolyzed to tZ in cotyledons, this data cannot be generalized other organs.

It is important to note that the data presented here do not suggest that the addition of tZNGs always leads to an increase in tZ, as one would expect nearly identical results between all assays if this were the case; tZNG treatment appears to affect plants quite differently from tZ treatment in most experiments (Figs 3-6).

Further study of CKNG function is necessary

Whatever the mechanism, it is apparent that CKNGs merit further investigation and should not always be assumed to be inactive compounds. Past and current work in cytokinin measurement and phylogenetics has revealed CKNGs are absent or present at extremely low levels in algae and cyanobacteria [73], present at low or moderate levels in some Byrophytes [74], and present at high levels in vascular plants, suggesting an important role for these compounds in the evolution of higher plants [75]. There is almost certainly an evolutionary advantage to explain why plants expend a valuable resource like glucose in making CKNGs, but further investigation is warranted into what exactly these advantages are.

Acknowledgements

We thank Lesley Turner and Valeriia Kopytko for their technical assistance throughout the project, as well as Dr. Chloe Josefson for her assistance with statistical analyses.

Funding

Funding support for this work comes from an Auburn University IGP grant 190561 to A.M.R. and H.T.H., a Mendel University/EU International Development grant CZ.02.2.69/0.0/0.0/16_027/0007953 to A.M.R., and the Ministry of Education, Youth and Sports of CR project from European Regional Development Fund "Centre for Experimental Plant Biology" No. CZ.02.1.01/0.0/0.0/16_019/0000738 to M.C. and B.B. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests

The authors have declared that no competing interests exist.

References

1. Kieber JJ, Schaller GE. Cytokinins. Arab Book Am Soc Plant Biol. 2014 Jan 2;12. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3894907/

2. Sakakibara H. CYTOKININS: Activity, Biosynthesis, and Translocation. Annu Rev Plant Biol. 2006;57(1):431–49.

3. Schmitz RY, Skoog F, Hecht SM, Bock RM, Leonard NJ. Comparison of cytokinin activities of naturally occurring ribonucleosides and corresponding bases. Phytochemistry. 1972 May 1;11(5):1603–10.

4. Spiess LD. Comparative Activity of Isomers of Zeatin and Ribosyl-Zeatin on Funaria hygrometrica. Plant Physiol. 1975 Mar 1;55(3):583–5.

5. Yamada H, Suzuki T, Terada K, Takei K, Ishikawa K, Miwa K, et al. The Arabidopsis AHK4 Histidine Kinase is a Cytokinin-Binding Receptor that Transduces Cytokinin Signals Across the Membrane. Plant Cell Physiol. 2001 Sep 15;42(9):1017–23.

6. Brzobohaty B, Moore I, Kristoffersen P, Bako L, Campos N, Schell J, et al. Release of active cytokinin by a beta-glucosidase localized to the maize root meristem. Science. 1993 Nov 12;262(5136):1051–4.

7. Entsch B, Letham DS. Enzymic glucosylation of the cytokinin, 6-benzylaminopurine. Plant Sci Lett. 1979 Feb 1;14(2):205–12.

8. Falk A, Rask L. Expression of a Zeatin-O-Glucoside-Degrading [beta]-Glucosidase in Brassica napus. Plant Physiol. 1995 Aug 1;108(4):1369–77.

9. Yoshida R, Oritani T. Cytokinin glucoside in roots of the rice plant. Plant Cell Physiol. 1972 Apr 1;13(2):337–43.

10. Bairu MW, Novák O, Doležal K, Van Staden J. Changes in endogenous cytokinin profiles in micropropagated Harpagophytum procumbens in relation to shoot-tip necrosis and cytokinin treatments. Plant Growth Regul. 2011 Mar 1;63(2):105–14.

11. Fox JE, Cornette J, Deleuze G, Dyson W, Giersak C, Niu P, et al. The Formation, Isolation, and Biological Activity of a Cytokinin 7-Glucoside. Plant Physiol. 1973 Dec 1;52(6):627–32.

12. Spíchal L. Cytokinins – recent news and views of evolutionally old molecules. Funct Plant Biol. 2012 May 15;39(4):267–84.

13. Kiba T, Takebayashi Y, Kojima M, Sakakibara H. Sugar-induced de novo cytokinin biosynthesis contributes to Arabidopsis growth under elevated CO 2. Sci Rep. 2019 May 23;9(1):1–15.

14. Tokunaga H, Kojima M, Kuroha T, Ishida T, Sugimoto K, Kiba T, et al. Arabidopsis lonely guy (LOG) multiple mutants reveal a central role of the LOG-dependent pathway in cytokinin activation. Plant J. 2012;69(2):355–65.

15. Ko D, Kang J, Kiba T, Park J, Kojima M, Do J, et al. Arabidopsis ABCG14 is essential for the root-to-shoot translocation of cytokinin. Proc Natl Acad Sci. 2014 May 13;111(19):7150–5.

16. Kiba T, Takei K, Kojima M, Sakakibara H. Side-Chain Modification of Cytokinins Controls Shoot Growth in Arabidopsis. Dev Cell. 2013 Nov 25;27(4):452–61.

17. Nishiyama R, Watanabe Y, Fujita Y, Le DT, Kojima M, Werner T, et al. Analysis of Cytokinin Mutants and Regulation of Cytokinin Metabolic Genes Reveals Important Regulatory Roles of Cytokinins in Drought, Salt and Abscisic Acid Responses, and Abscisic Acid Biosynthesis. Plant Cell. 2011 Jun 1;23(6):2169–83.

18. Sakakibara H, Kasahara H, Ueda N, Kojima M, Takei K, Hishiyama S, et al. Agrobacterium tumefaciens increases cytokinin production in plastids by modifying the biosynthetic pathway in the host plant. Proc Natl Acad Sci. 2005 Jul 12;102(28):9972–7.

19. Zhang K, Novak O, Wei Z, Gou M, Zhang X, Yu Y, et al. Arabidopsis ABCG14 protein controls the acropetal translocation of root-synthesized cytokinins. Nat Commun. 2014 Dec;5(1).

20. Letham DS, Palni LMS. The Biosynthesis and Metabolism of Cytokinins. Annu Rev Plant Physiol. 1983;34(1):163–97.

21. Letham DS, Palni LMS, Tao G-Q, Gollnow BI, Bates CM. Regulators of cell division in plant tissues XXIX. The activities of cytokinin glucosides and alanine conjugates in cytokinin bioassays. J Plant Growth Regul. 1983 Aug 1;2(1):103–15.

22. Palni LMS, Palmer MV, Letham DS. The stability and biological activity of cytokinin metabolites in soybean callus tissue. Planta. 1984 Mar 1;160(3):242–9.

23. Deleuze GG, McChesney JD, Fox JE. Identification of a stable cytokinin metabolite. Biochem Biophys Res Commun. 1972 Sep 26;48(6):1426–32.

24. Hou B, Lim E-K, Higgins GS, Bowles DJ. N-Glucosylation of Cytokinins by Glycosyltransferases of Arabidopsis thaliana. J Biol Chem. 2004 Nov 12;279(46):47822–32.

25. Li Y, Wang B, Dong R, Hou B. AtUGT76C2, an Arabidopsis cytokinin glycosyltransferase is involved in drought stress adaptation. Plant Sci. 2015 Jul 1;236(Supplement C):157–67.

26. Wang J, Ma X-M, Kojima M, Sakakibara H, Hou B-K. N-Glucosyltransferase UGT76C2 is Involved in Cytokinin Homeostasis and Cytokinin Response in Arabidopsis thaliana. Plant Cell Physiol. 2011 Dec 1;52(12):2200–13.

27. Hošek P, Hoyerová K, Kiran NS, Dobrev PI, Zahajská L, Filepová R, et al. Distinct metabolism of N-glucosides of isopentenyladenine and trans-zeatin determines cytokinin metabolic spectrum in Arabidopsis. New Phytol. 2019; Available from: https://nph.onlinelibrary.wiley.com/doi/abs/10.1111/nph.16310

28. Jiskrová E, Novák O, Pospíšilová H, Holubová K, Karády M, Galuszka P, et al. Extra- and intracellular distribution of cytokinins in the leaves of monocots and dicots. New Biotechnol. 2016 Sep;33(5):735–42.

29. Fletcher RA, McCullagh D. Cytokinin-induced chlorophyll formation in cucumber cotyledons. Planta. 1971 Mar 1;101(1):88–90.

30. Letham DS. Chemistry and Physiology of Kinetin-Like Compounds. Annu Rev Plant Physiol. 1967;18(1):349–64.

31. Gajdošová S. Biological effects and metabolism of cis-Zeatin-type cytokinins in plants. Prague: Charles University; 2011. Available from: https://dspace.cuni.cz/handle/20.500.11956/47271

32. Sumanta N, Haque CI, Nishika J, Suprakash R. Spectrophotometric Analysis of Chlorophylls and Carotenoids from Commonly Grown Fern Species by Using Various Extracting Solvents. Res J Chem Sci. 2014;9(4).

33. Rashotte AM, Mason MG, Hutchison CE, Ferreira FJ, Schaller GE, Kieber JJ. A subset of Arabidopsis AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. Proc Natl Acad Sci. 2006 Jul 18;103(29):11081–5.

34. Andrews S. FastQC: A quality control tool for high throughput sequence data.. 2010. Available from: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

35. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014 Aug 1;30(15):2114–20.

36. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014 Dec 5;15(12):550.

37. Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. Nat Protoc. 2016 Sep;11(9):1650–67.

38. Tian T, Liu Y, Yan H, You Q, Yi X, Du Z, et al. agriGO v2.0: a GO analysis toolkit for the agricultural community, 2017 update. Nucleic Acids Res. 2017 Jul 3;45:W122–9.

39. Zwack PJ, Robinson BR, Risley MG, Rashotte AM. Cytokinin Response Factor 6 Negatively Regulates Leaf Senescence and is Induced in Response to Cytokinin and Numerous Abiotic Stresses. Plant Cell Physiol. 2013 Jun 1;54(6):971–81.

40. Hooper CM, Castleden IR, Tanz SK, Aryamanesh N, Millar AH. SUBA4: the interactive data analysis centre for Arabidopsis subcellular protein locations. Nucleic Acids Res. 2017 Jan 4;45(D1):D1064–74.

41. Cerna H, Černý M, Habánová H, Šafářová D, Abushamsiya K, Navrátil M, et al. Proteomics offers insight to the mechanism behind Pisum sativum L. response to pea seed-borne mosaic virus (PSbMV). J Proteomics. 2017 Feb 5;153:78–88.

42. Letham DS. Regulators of Cell Division in Plant Tissues XII. A Cytokinin Bioassay Using Excised Radish Cotyledons. Physiol Plant. 1971 Dec 1;25(3):391–6.

43. Zwack PJ, Rashotte AM. Cytokinin inhibition of leaf senescence. Plant Signal Behav. 2013 Jul 1;8(7). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3908980/

44. Hörtensteiner S. Chlorophyll Degradation During Senescence. Annu Rev Plant Biol. 2006;57(1):55–77.

45. Stenlid G. Cytokinins as inhibitors of root growth. Physiol Plant. 1982;56(4):500–6.

46. Werner T, Motyka V, Strnad M, Schmülling T. Regulation of plant growth by cytokinin. Proc Natl Acad Sci. 2001 Aug 28;98(18):10487–92.

47. Skoog F, Miller CO. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp Soc Exp Biol. 1957;54:118–30.

48. Kubo M, Kakimoto T. The CYTOKININ-HYPERSENSITIVE genes of Arabidopsis negatively regulate the cytokinin-signaling pathway for cell division and chloroplast development. Plant J. 2000;23(3):385–94.

49. Suzuki T, Ishikawa K, Yamashino T, Mizuno T. An Arabidopsis Histidine-Containing Phosphotransfer (HPt) Factor Implicated in Phosphorelay Signal Transduction: Overexpression of AHP2 in Plants Results in Hypersensitiveness to Cytokinin. Plant Cell Physiol. 2002 Jan 15;43(1):123–9.

50. Jones RJ, Schreiber BMN. Role and function of cytokinin oxidase in plants. Plant Growth Regul. 1997 Oct 1;23(1):123–34.

51. McGaw BA, Horgan R. Cytokinin catabolism and cytokinin oxidase. Phytochemistry. 1983 Jan 1;22(5):1103–5.

52. Gepstein S, Sabehi G, Carp M-J, Hajouj T, Nesher MFO, Yariv I, et al. Large-scale identification of leaf senescence-associated genes. Plant J. 2003;36(5):629–42.

53. Kliebenstein DJ, Monde R-A, Last RL. Superoxide Dismutase in Arabidopsis: An Eclectic Enzyme Family with Disparate Regulation and Protein Localization. Plant Physiol. 1998 Oct 1;118(2):637–50.

54. Reiter RS, Coomber SA, Bourett TM, Bartley GE, Scolnik PA. Control of leaf and chloroplast development by the Arabidopsis gene pale cress. Plant Cell. 1994 Sep 1;6(9):1253–64.

55. Černý M, Dyčka F, Bobál'ová J, Brzobohatý B. Early cytokinin response proteins and phosphoproteins of Arabidopsis thaliana identified by proteome and phosphoproteome profiling. J Exp Bot. 2011 Jan 1;62(3):921–37.

56. Černý M, Kuklová A, Hoehenwarter W, Fragner L, Novák O, Rotková G, et al. Proteome and metabolome profiling of cytokinin action in Arabidopsis identifying both distinct and similar responses to cytokinin down- and up-regulation. J Exp Bot. 2013 Nov 1;64(14):4193–206.

57. Černý M, Jedelský PL, Novák J, Schlosser A, Brzobohatý B. Cytokinin modulates proteomic, transcriptomic and growth responses to temperature shocks in Arabidopsis. Plant Cell Environ. 2014 Jul 1;37(7):1641–55.

58. Lochmanová G, Zdráhal Z, Konečná H, Koukalová Š, Malbeck J, Souček P, et al. Cytokinin-induced photomorphogenesis in dark-grown Arabidopsis: a proteomic analysis. J Exp Bot. 2008 Oct 1;59(13):3705–19.

59. Žd'árská M, Zatloukalová P, Benítez M, Šedo O, Potěšil D, Novák O, et al. Proteome Analysis in Arabidopsis Reveals Shoot- and Root-Specific Targets of Cytokinin Action and Differential Regulation of Hormonal Homeostasis. Plant Physiol. 2013 Feb 1;161(2):918–30.

60. Zhang Y, Liu S, Dai SY, Yuan JS. Integration of shot-gun proteomics and bioinformatics analysis to explore plant hormone responses. BMC Bioinformatics. 2012 Sep 11;13(15):S8.

61. Bhargava A, Clabaugh I, To JP, Maxwell BB, Chiang Y-H, Schaller EG, et al. Identification of Cytokinin Responsive Genes Using Microarray Meta-analysis and RNA-seq in Arabidopsis thaliana. Plant Physiol. 2013 Jan 1;pp.113.217026.

62. Rashotte AM, Carson SDB, To JPC, Kieber JJ. Expression Profiling of Cytokinin Action in Arabidopsis. Plant Physiol. 2003 Aug 1;132(4):1998–2011.

63. Galuszka P, Popelková H, Werner T, Frébortová J, Pospíšilová H, Mik V, et al. Biochemical Characterization of Cytokinin Oxidases/Dehydrogenases from Arabidopsis thaliana Expressed in Nicotiana tabacum L. J Plant Growth Regul. 2007 Sep 1;26(3):255–67.

64. Bürkle L, Cedzich A, Döpke C, Stransky H, Okumoto S, Gillissen B, et al. Transport of cytokinins mediated by purine transporters of the PUP family expressed in phloem, hydathodes, and pollen of Arabidopsis. Plant J. 2003;34(1):13–26.

65. Hirose N, Makita N, Yamaya T, Sakakibara H. Functional Characterization and Expression Analysis of a Gene, OsENT2, Encoding an Equilibrative Nucleoside Transporter in Rice Suggest a Function in Cytokinin Transport. Plant Physiol. 2005 May 1;138(1):196–206.

66. Sun J, Hirose N, Wang X, Wen P, Xue L, Sakakibara H, et al. Arabidopsis SOI33/AtENT8 Gene Encodes a Putative Equilibrative Nucleoside Transporter That Is Involved in Cytokinin Transport In Planta. J Integr Plant Biol. 2005;47(5):588–603.

67. Graf A, Coman D, Uhrig RG, Walsh S, Flis A, Stitt M, et al. Parallel analysis of Arabidopsis circadian clock mutants reveals different scales of transcriptome and proteome regulation. Open Biol. 7(3):160333.

68. Ponnala L, Wang Y, Sun Q, Wijk KJ van. Correlation of mRNA and protein abundance in the developing maize leaf. Plant J. 2014;78(3):424–40.

69. Romanov GA, Lomin SN, Schmülling T. Biochemical characteristics and ligand-binding properties of Arabidopsis cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. J Exp Bot. 2006 Dec 1;57(15):4051–8.

70. Spíchal L, Rakova NY, Riefler M, Mizuno T, Romanov GA, Strnad M, et al. Two Cytokinin Receptors of Arabidopsis thaliana, CRE1/AHK4 and AHK3, Differ in their Ligand Specificity in a Bacterial Assay. Plant Cell Physiol. 2004 Sep 15;45(9):1299–305.

71. Deng Y, Dong H, Mu J, Ren B, Zheng B, Ji Z, et al. Arabidopsis Histidine Kinase CKI1 Acts Upstream of HISTIDINE PHOSPHOTRANSFER PROTEINS to Regulate Female Gametophyte Development and Vegetative Growth. Plant Cell. 2010 Apr 1;22(4):1232–48.

72. Kakimoto T. CKI1, a Histidine Kinase Homolog Implicated in Cytokinin Signal Transduction. Science. 1996 Nov 8;274(5289):982–5.

73. Žižková E, Kubeš M, Dobrev PI, Přibyl P, Šimura J, Zahajská L, et al. Control of cytokinin and auxin homeostasis in cyanobacteria and algae. Ann Bot. 2017 Jan 1;119(1):151–66.

74. Drábková LZ, Dobrev PI, Motyka V. Phytohormone Profiling across the Bryophytes. PLOS ONE. 2015 May 14;10(5):e0125411.

75. Auer CA. Cytokinin conjugation: recent advances and patterns in plant evolution. Plant Growth Regul. 1997;23.

Figures

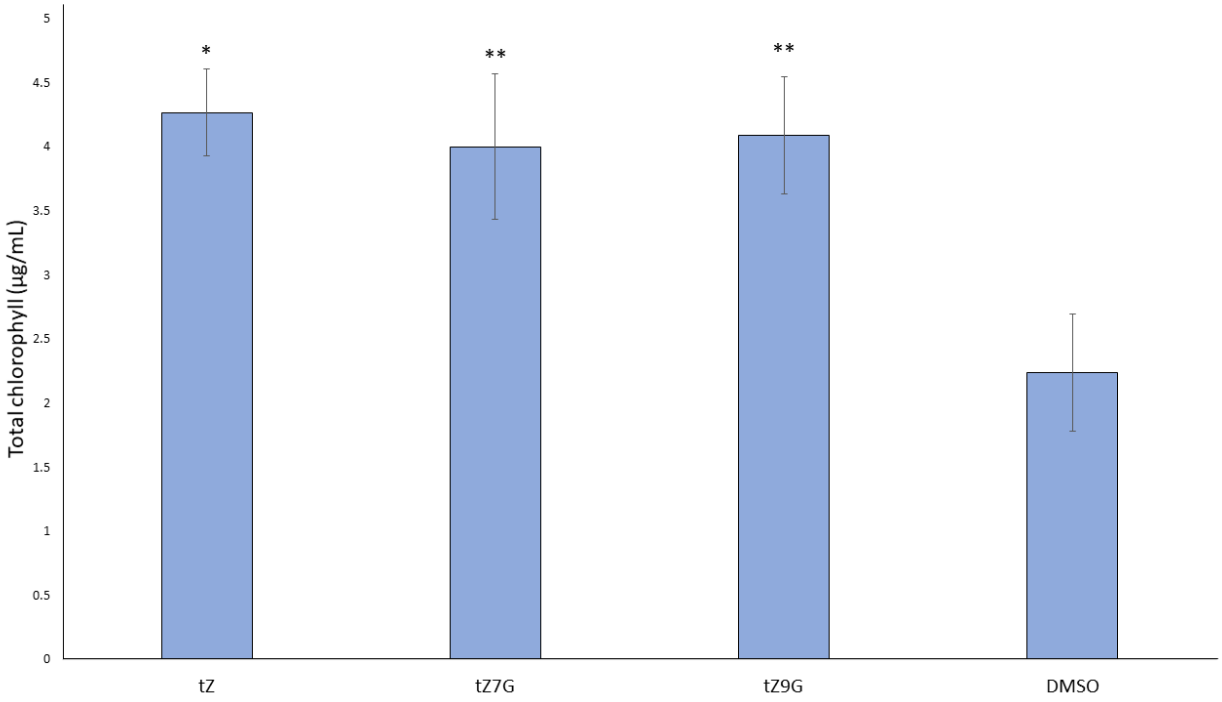


Fig 1. trans-Zeatin-N-glucosides delay chlorophyll degradation in detached cotyledons. Cotyledons from 12dag WT Arabidopsis seedlings were excised and floated abaxial side down in 3mM MES buffer (pH 5.7) supplemented with 1µM of the indicated hormone or 0.1% DMSO as a control. Solutions were placed in the dark for six days, after which chlorophyll was extracted and quantified according to Sumanta et al., 2014. Average ± SE of three biological replicates is presented. * p-value < 0.05, ** p-value < 0.01, paired two-tailed Student's t test.

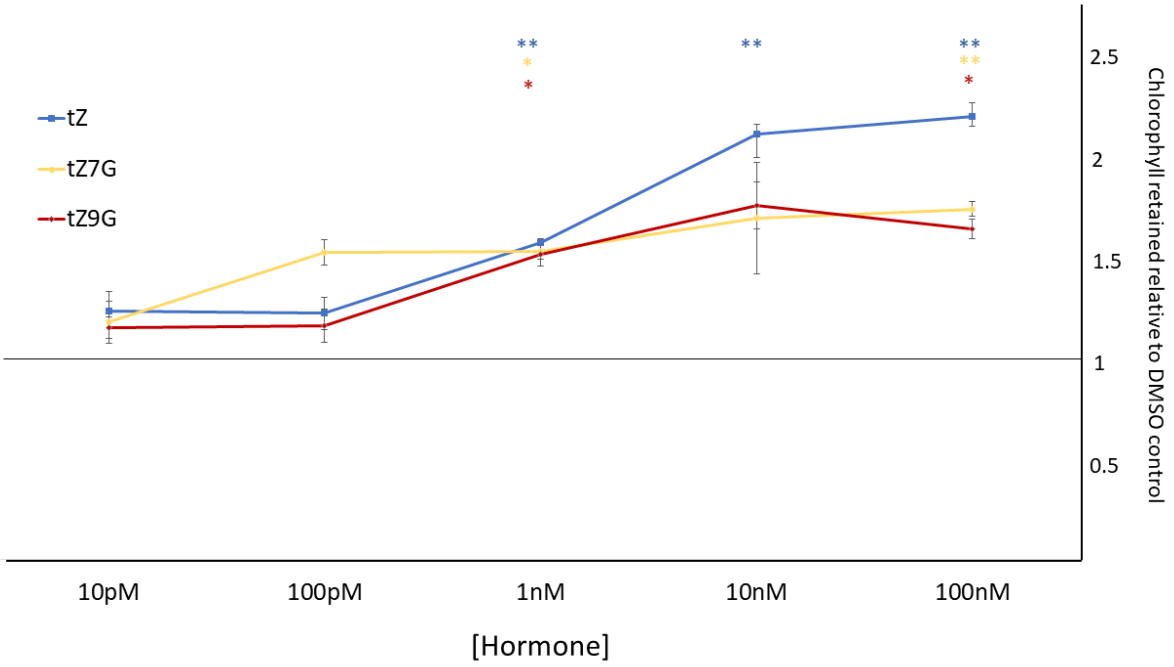


Fig 2. trans-Zeatin-N-glucosides delay chlorophyll degradation in detached cotyledons in a dose-dependent manner. Experiment performed identically to that in Figure 1 but with different concentrations of hormones. Average ± SE of three biological replicates is presented as a normalized level of chlorophyll relative to the chlorophyll content in the DMSO control cotyledons. Significance was determined by a two-way ANOVA (treatment factor, p-value = 0.0005) followed by post-hoc t-tests comparing hormone treatments to DMSO control. * p-value < 0.05, ** p-value < 0.01. Significance asterisk colors correspond to the line of the same color.

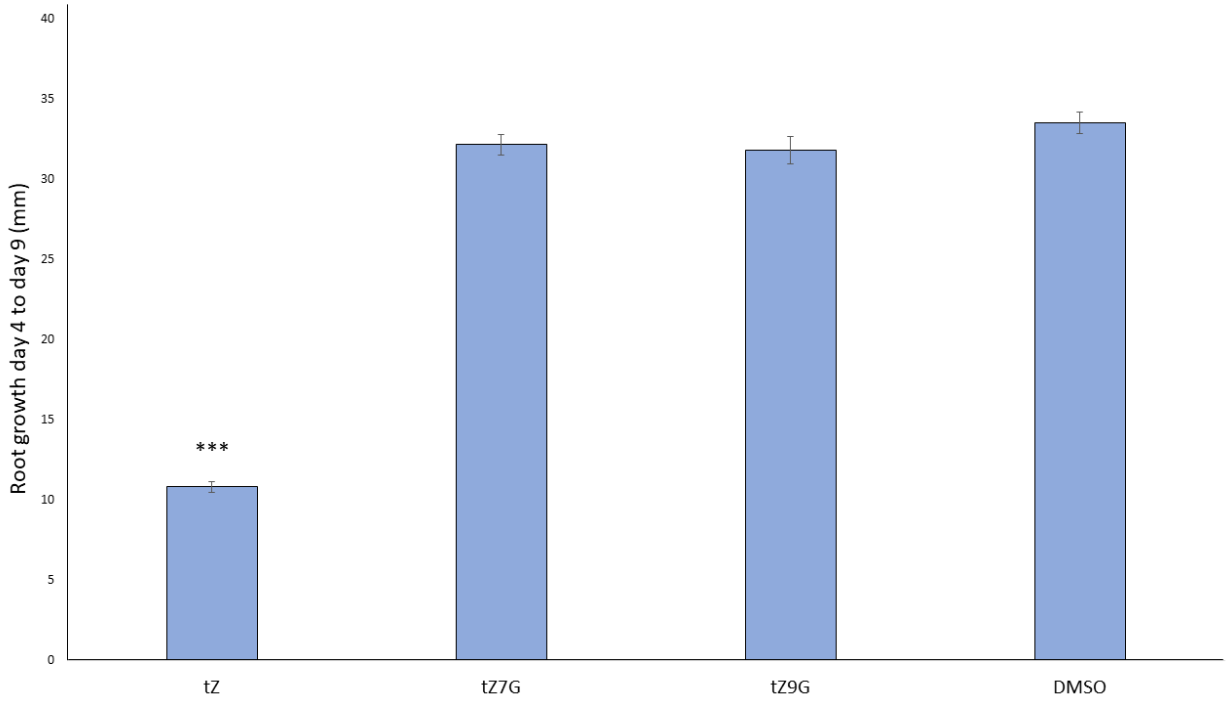


Fig 3. trans-Zeatin-N-glucosides do not inhibit root growth. Arabidopsis seedlings were transferred from standard MS + 1% sucrose at 4dag to the same media supplemented with 1µM of the indicated hormone or 0.1% DMSO as a control. Root growth since being transferred to the new media was measured on day 9. Average ± SE of three biological replicates is presented (n > 10 per treatment per replicate). *** p-value < 0.001, Student's two-tailed t test.

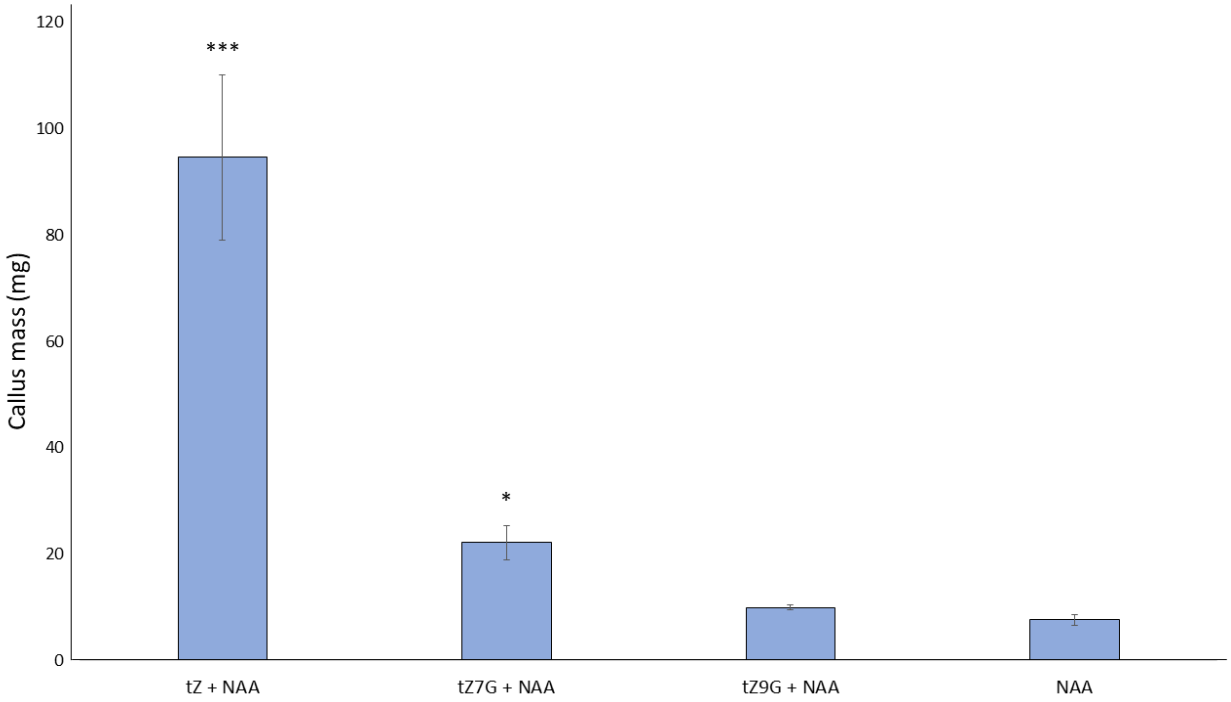
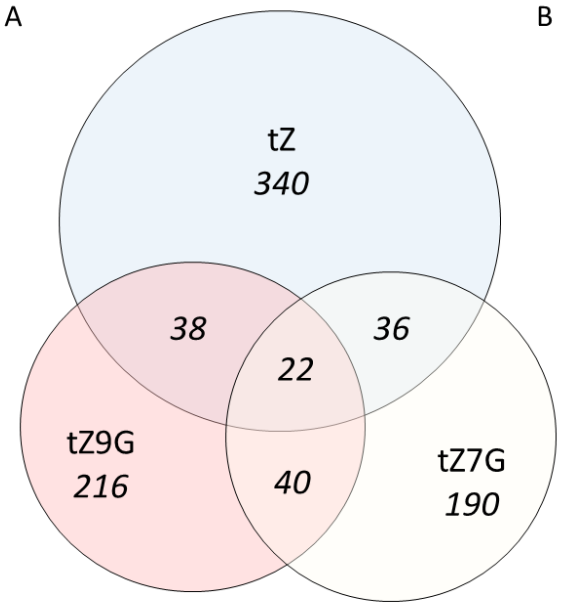


Fig 4. Trans-Zeatin-7-glucoside mildly promotes shoot initiation. Hypocotyls from etiolated Arabidopsis seedlings were transferred to MS + 1% sucrose supplemented with equimolar amounts of NAA and the indicated cytokinins. The negative controls were supplemented with an equal volume of DMSO in lieu of cytokinin. After 45 days, callus weight was measured. Average ± SE of three biological replicates is shown ($n \geq 4$ per treatment per replicate). *** p-value < 0.001, * p-value < 0.05, Student's two-tailed t test.



B

GO Term (tZ-treated)	FDR	GO Term (tZ7G-treated)	FDR	GO Term (tZ9G-treated)	FDR
Transcription factor activity	4.4e-18	Transcription factor activity	5.3e-17	Protein metabolic process	1.2e-17
Response to **cytokinin**	1.7e-13	Phosphotransfer activity	7.8e-11	Post-translational protein modification	1.1e-15
Hormone-mediated signaling pathway	6.0e-12	Nitrogen-containing metabolic process	1.2e-10	Phosphotransfer activity	2.7e-08
Cytokinin-mediated signaling pathway	1.6e-09	Kinase activity	7.9e-07	Transcription factor activity	5.5e-07
Phosphotransfer activity	3.1e-08	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	2.8e-06	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	5.1e-06
Two-component response regulator activity	4.3e-08	Adenyl nucleotide binding	0.0002	Adenyl nucleotide binding	0.0001

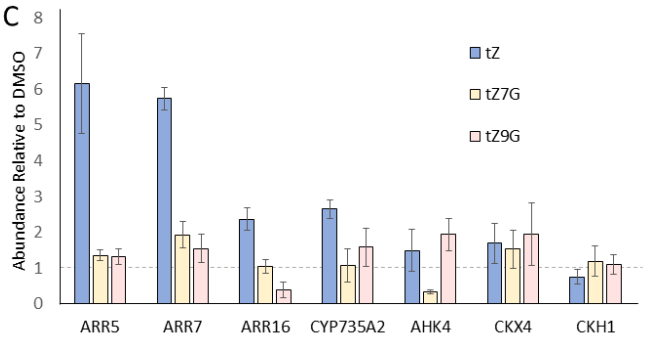


Fig 5. Trans-Zeatin-N-glucosides alter the Arabidopsis transcriptome distinctly from trans-Zeatin. A, Venn diagram showing number of differentially expressed transcripts after two-hour treatment of 10dag WT Arabidopsis seedlings with 1µM hormone. B, tZ-treated seedlings are enriched for CK-related Gene Ontology terms, unlike tZ7G- and tZ9G-treated seedlings. C, qRT-PCR confirmation of RNAseq results; data presented is average ± SE of three independent biological replicates. Treatment with tZ7G or tZ9G does not mimic treatment with tZ with regards to regulation of most tested CK-related genes.

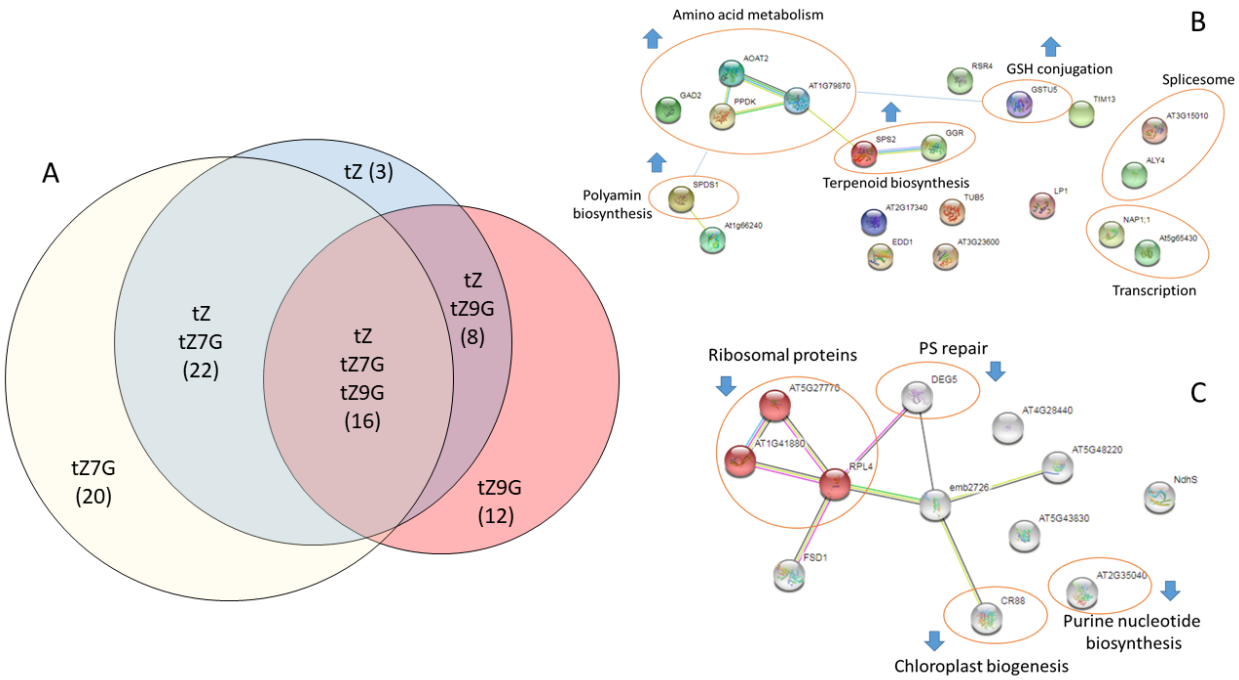


Fig 6. Trans-Zeatin-N-glucosides alter the Arabidopsis proteome in a partially overlapping manner with trans-Zeatin. A, Proportional Venn diagram showing number (in parentheses) of differentially expressed proteins after two-hour treatment of 10dag WT Arabidopsis seedlings with 1µM hormone. B, Treatment with tZ7G affects amino acid metabolism, terpenoid biosynthesis, and other pathways. C, Treatment with tZ9G affects largely plastid proteins, including those involved in chloroplast biogenesis and photosystem (PS) repair.

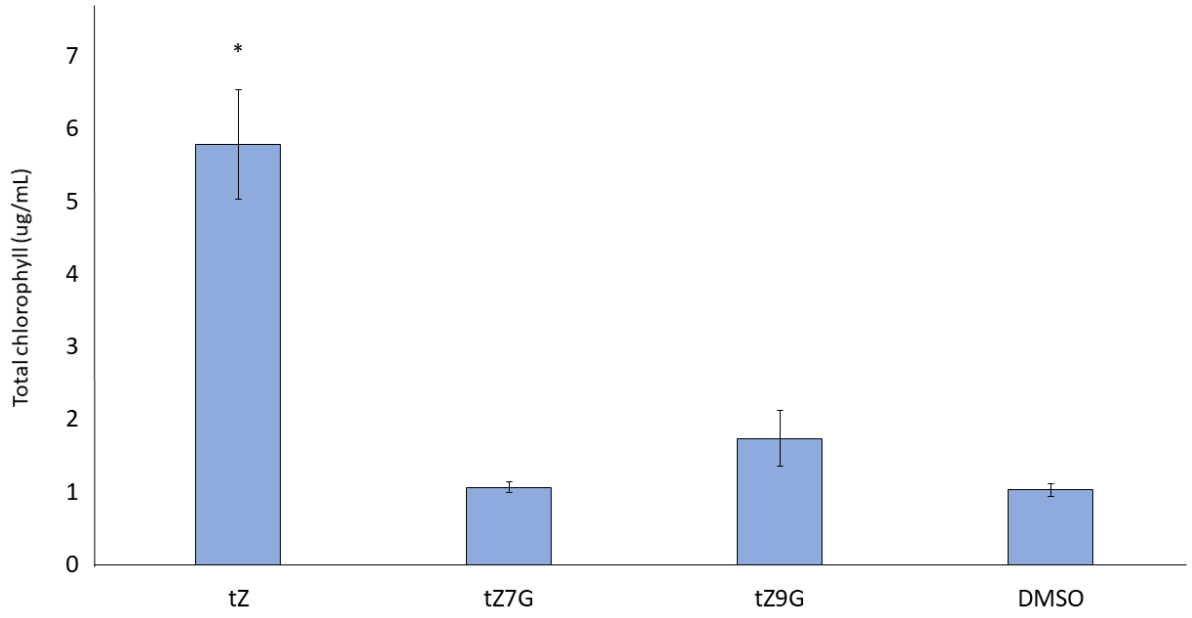


Figure 7. Trans-Zeatin-N-glucosides do not delay senescence in trans-Zeatin biosynthetic mutants. Cotyledons from 12dag *cypDM* mutants, which are deficient in tZ-type cytokinins, were subjected to a 6d-long cotyledon senescence assay. While exogenous tZ delayed senescence, tZNGs did not. Average ± SE of four independent biological replicates is presented. *$p<0.05$, Student's two-tailed t test.

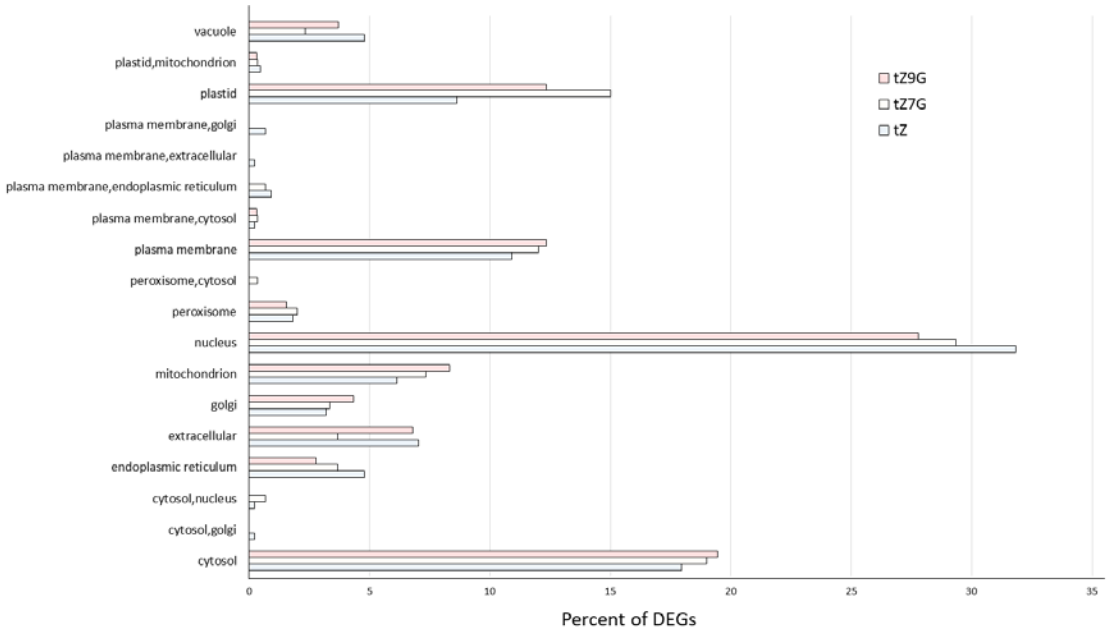


Figure S1. Subcellular distribution of DEGs is similar between tZ, tZ7G, and tZ9G treatment. Results from the transcriptome analysis were analyzed for subcellular distribution using SUBA4. Data presented is the percent of DEGs from each treatment group which localize to the noted organelle.

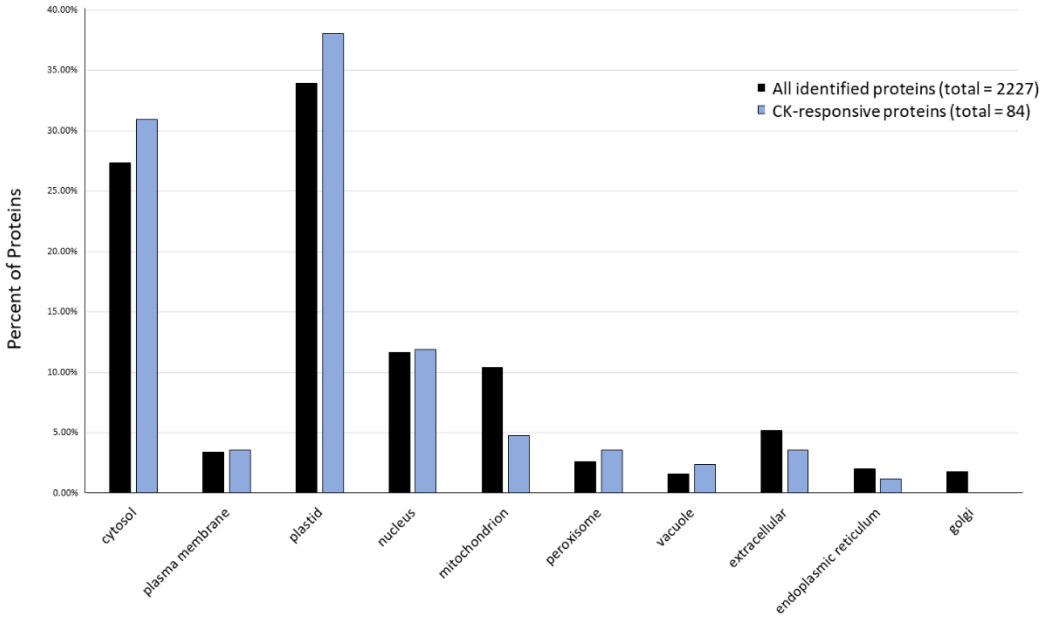


Figure S2. Cytokinin treatment results in a change in localization of detectable proteins. Results from the proteome analysis were analyzed for subcellular distribution using SUBA4. Data in blue represents the percent of differentially affected proteins showing CK responsiveness (i.e. responded to tZ, tZ7G, or tZ9G) which localize to the noted organelle. Data in black indicates the percent of all proteins identified in the analysis (i.e. both CK-responsive and non-CK-responsive) which localize to the noted organelle.

		tZ		tZ7G		tZ9G	
Accession	Protein name (UniProt)	Ratio	Adj. P-Value	Ratio	Adj. P-Value	Ratio	Adj. P-Value
AT2G45180	rRNA adenine N(6)-methyltransferase	2.9	2.9E-03	1.6	3.1E-01	0.8	7.0E-01
AT2G24940	Probable steroid-binding protein 3	0.2	2.3E-05	0.6	4.3E-01	0.7	9.6E-01
AT3G15450	*Aluminum induced protein with YGL and LRDR motifs*	0.1	3.1E-05	0.5	3.2E-01	0.3	2.2E-01
AT3G15010	UBP1-associated protein 2C	1.6	5.4E-01	3.5	4.5E-02	1.3	5.6E-01
AT1G70580	Glutamate--glyoxylate aminotransferase 2	1.6	1.3E-01	2.9	9.1E-03	1.1	5.3E-01
AT4G38460	Heterodimeric geranylgeranyl pyrophosphate synthase small subunit	1.5	2.0E-01	3.0	4.1E-03	1.0	6.2E-01
AT1G65960	Glutamate decarboxylase 2	1.5	1.3E-01	2.1	4.1E-02	1.0	6.2E-01
AT3G23600	*Alpha/beta-Hydrolases superfamily protein*	1.4	3.4E-01	3.7	4.2E-05	1.1	4.1E-01
AT4G15530	Pyruvate, phosphate dikinase 1	1.4	6.7E-01	4.1	1.4E-02	0.9	8.9E-01
AT2G29450	Glutathione S-transferase U5	1.3	3.0E-01	1.7	3.2E-02	0.9	8.8E-01
AT1G79870	Glyoxylate/hydroxypyruvate reductase A HPR2	1.3	4.3E-01	2.7	1.4E-02	0.7	9.7E-01
AT2G17340	Damage-control phosphatase	1.3	3.6E-02	2.1	8.3E-03	0.6	8.9E-01
AT5G01410	Pyridoxal 5'-phosphate synthase subunit PDX1.3	1.1	6.6E-01	1.8	1.7E-02	0.9	8.6E-01
AT1G17050	Solanesyl diphosphate synthase 2	1.0	8.3E-01	2.4	2.6E-02	1.2	3.5E-01
AT5G65430	14-3-3-like protein GF14 kappa	1.0	8.7E-01	2.8	4.5E-02	1.1	5.8E-01
AT1G23820	Spermidine synthase 1	1.0	7.2E-01	2.2	1.8E-02	1.0	5.3E-01
AT3G48110	Glycine--tRNA ligase	2.8	1.8E-01	4.5	3.8E-02	0.7	9.8E-01
AT4G26110	Nucleosome assembly protein 1	1.1	4.2E-01	0.5	2.7E-02	0.8	8.7E-01
AT1G20010	Tubulin beta-5 chain	0.7	9.8E-01	0.3	3.0E-02	0.7	9.6E-01
AT5G37720	THO complex subunit 4D	0.8	9.7E-01	0.5	2.8E-02	0.7	9.6E-01
AT1G66240	Copper transport protein ATX1	0.7	8.2E-01	0.5	1.3E-02	0.8	9.8E-01
AT1G61570	Mitochondrial import inner membrane translocase subunit TIM13	0.6	9.2E-01	0.3	4.3E-02	0.7	9.6E-01
AT2G38540	Non-specific lipid-transfer protein 1	0.6	5.6E-01	0.4	2.6E-04	0.5	4.6E-01
AT5G48220	*Aldolase-type TIM barrel family protein*	1.2	7.8E-01	1.4	7.8E-01	2.1	2.5E-02
AT4G25100	Superoxide dismutase [Fe] 1	0.8	9.8E-01	0.7	4.3E-01	1.6	1.3E-02
AT4G23890	NAD(P)H-quinone oxidoreductase subunit S	0.7	9.8E-01	1.0	9.9E-01	1.6	1.4E-02
AT1G07320	50S ribosomal protein L4	0.8	9.7E-01	0.7	5.2E-01	0.4	2.4E-02
AT2G35040	*Putative phosphoribosylaminoimidazolecarboxamide formyltransferase*	0.7	9.6E-01	1.0	9.2E-01	0.4	4.6E-02
AT2G04030	Heat shock protein 90-5	0.7	7.8E-01	0.7	5.4E-01	0.4	3.3E-02
AT4G28440	Uncharacterized protein At4g28440	0.7	2.0E-01	0.7	2.4E-01	0.4	5.9E-03
AT1G41880	60S ribosomal protein L35a-2	0.6	8.1E-01	0.8	8.2E-01	0.4	1.7E-02
AT5G27770	60S ribosomal protein L22-3	0.5	1.3E-01	0.6	1.5E-01	0.4	1.4E-02
AT4G29060	*Elongation factor Ts*	0.5	3.8E-01	0.7	3.8E-01	0.3	6.7E-05
AT4G18370	Protease Do-like 5	0.3	2.2E-01	0.4	2.1E-01	0.2	1.1E-02
AT5G43830	*Aluminum induced protein with YGL and LRDR motifs*	0.9	9.1E-01	1.1	8.8E-01	0.4	3.8E-02
AT1G56110	*Nop domain-containing protein*	4.2	3.8E-03	2.3	2.0E-01	1.0	8.1E-01
AT2G31810	Acetolactate synthase small subunit 2	4.0	2.3E-02	3.1	1.8E-01	1.4	5.6E-01
AT4G14910	Imidazoleglycerol-phosphate dehydratase 2	2.9	5.5E-02	4.1	3.5E-02	1.1	7.0E-01
AT5G62530	Delta-1-pyrroline-5-carboxylate dehydrogenase 12A1	2.8	2.8E-02	3.1	2.6E-02	0.9	9.1E-01
AT5G66090	*Cell wall integrity/stress response component*	2.7	5.7E-01	6.4	2.9E-04	2.3	1.4E-01
AT1G05385	Photosystem II D1 precursor processing protein PSB27-H2	2.7	2.2E-05	3.3	1.4E-08	1.4	3.4E-02
AT3G27360	Histone H3.2	2.3	3.4E-02	9.3	2.5E-16	1.0	6.8E-01
AT4G36810	Heterodimeric geranylgeranyl pyrophosphate synthase large subunit 1	2.1	3.8E-03	2.2	4.6E-02	1.1	3.7E-01
AT5G11670	NADP-dependent malic enzyme 2	2.1	1.3E-03	1.8	4.5E-02	1.1	5.2E-01
AT5G10920	Argininosuccinate lyase	2.0	1.4E-02	2.3	3.2E-02	1.1	3.6E-01
AT2G10940	*Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein*	2.0	1.9E-01	2.3	1.7E-05	1.4	1.1E-01
AT2G30200	*EMBRYO DEFECTIVE 3147*	1.7	2.6E-02	2.3	3.2E-03	1.3	8.5E-02
AT2G26340	*Uncharacterized protein*	1.7	5.7E-02	2.9	9.9E-03	1.1	5.5E-01
AT1G04270	40S ribosomal protein S15-1	1.7	4.4E-02	1.7	5.5E-02	1.0	6.5E-01
AT1G04690	Probable voltage-gated potassium channel subunit beta	1.6	5.5E-02	3.0	3.7E-04	1.0	5.6E-01
AT3G08940	Chlorophyll a-b binding protein CP29.2	1.8	1.9E-02	2.8	1.1E-06	1.0	6.8E-01
AT3G22960	Plastidial pyruvate kinase 1	1.9	5.6E-03	1.8	6.6E-02	1.0	6.3E-01
AT5G13450	ATP synthase subunit O	1.5	1.2E-01	2.1	2.0E-03	1.1	5.0E-01
AT1G13930	*Oleosin-B3-like protein*	0.5	1.4E-01	0.5	8.2E-03	0.6	6.7E-01
AT2G47710	*Adenine nucleotide alpha hydrolases-like superfamily protein*	0.6	5.8E-01	0.5	2.0E-02	0.7	9.4E-01
AT3G52500	*Eukaryotic aspartyl protease family protein*	0.3	2.7E-03	0.6	2.4E-01	0.7	9.5E-01
AT3G02780	Isopentenyl-diphosphate Delta-isomerase II	0.2	3.3E-02	0.5	4.6E-01	0.7	9.7E-01
AT2G38140	30S ribosomal protein S31	0.4	1.3E-01	0.7	5.4E-01	0.4	1.6E-02
AT2G41530	S-formylglutathione hydrolase	0.6	6.3E-01	0.8	8.8E-01	0.3	3.5E-03
AT3G07720	*Galactose oxidase/kelch repeat superfamily protein*	0.5	4.4E-01	0.7	5.6E-01	0.4	3.0E-02
AT3G09500	60S ribosomal protein L35-1	0.4	1.4E-02	0.7	3.7E-01	0.5	4.7E-01
AT3G48930	40S ribosomal protein S11-1	0.4	5.5E-02	0.7	4.4E-01	0.5	2.4E-01
AT2G38040	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	0.4	1.8E-01	1.0	9.8E-01	0.3	1.5E-02
AT2G40740	WRKY transcription factor 55	0.2	2.5E-04	0.8	9.2E-01	0.4	1.0E-01
AT4G29590	*S-adenosyl-L-methionine-dependent methyltransferases superfamily protein*	0.2	2.6E-04	0.8	9.0E-01	0.2	3.1E-04
AT5G66680	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit	2.9	1.9E-03	2.8	3.0E-02	1.7	2.3E-01
AT5G47030	ATP synthase subunit delta	2.4	1.0E-04	4.1	2.0E-07	1.8	3.5E-04
AT4G10450	60S ribosomal protein L9-2	2.1	1.6E-01	1.8	9.4E-01	2.1	7.9E-03

Table S1. List of all proteins differentially affected by tZ, tZ7G, and tZ9G treatments. Ratio is the relative abundance of protein in the treated sample compared to the DMSO negative control.

Transcript	Gene Symbol	Log2FC	Name/Function
AT2G36250.2	FTSZ2-1	12.71	FtsZ 2; Involved in plastid division
AT4G38160.1	pde191; MTERF6	12.46	Mitochondrial transcription termination factor
AT5G26030.2	FC1	10.87	Plastid ferrochelatase I; involved in heme biosynthesis
AT1G67740.1	PSBY	8.32	psbY precursor; mRNA imported to chloroplast to form integral membrane proteins
AT5G04330.1	CYP84A4	7.83	Cytochrome P450 84A4
AT4G29740.3	CKX4	4.16	Cytokinin Oxidase 4; degrades cytokinin
AT1G66980.1	SNC4; GDPDL2	3.95	Supressor of npr1-1; atypical receptor-like kinase
AT2G01320.1	ABCG7	3.87	Putative plastid envelope metal transporter
AT3G53920.1	SIG3	2.74	Sigma 3; plastid RNA polymerase subunit, involved in psbN transcription
AT1G17440.1	CKH1; TAF12B	1.75	Cytokinin Hypersensitive; putative negative cytokinin regulator
AT3G28970.1	AAR3	-2.47	Anti-Auxin Resistant 3; involved in response to 2,4-D
AT1G20620.1	CAT3; SEN2	-2.83	Catalase; breaks hydrogen peroxide into water and oxygen
AT5G13960.2	SUVH4; KYP	-3.36	Kryptonite; Histone-lysine N-methyltransferase; involved in histone modification
AT5G10490.2	MSL2	-3.46	MscS-like 2; Involved in plastid division
AT4G34430.2	SWI3D	-3.57	Putative chromatin remodelling protein
AT5G06530.1	ABCG22	-4.80	Involved in transpiration, mutant is more susceptible to drought
AT1G02850.4	BGLU11	-7.87	Beta glucosidase 11; putatively cleaves cytokinin-O-glucosides back into cytokinin bases
AT1G22300.2	GRF10	-8.85	General Regulatory Factor 10; 14-3-3 protein, interacts with BZR1
AT2G48120.1	PAC	-9.29	Pale Cress; mutant is deficient in chlorophyll, carotenoids, and ABA
AT4G32250.3	KOC1	-9.30	Kinase of Outer Chloroplast membrane; contributes to chloroplast biogenesis
AT4G35790.1	PLDDELTA	13.56	Phospholipase D Delta; involved in response to drought, hydrogen peroxide, cell death
AT2G32930.1	ZFN2	10.16	Zinc Finger Nuclease 2
AT1G08830.2	SOD1; CSD1	9.16	Superoxide Dismutase 1; detoxification of superoxide radicals
AT5G41315.1	GL3	9.04	Glabrous 3; bHLH, involved in trichome initiation
AT5G15410.2	DND1; CNGC2	8.43	Defense No Death 1; cAMP gated ion channel involved in pathogen response
AT4G25110.2	MCP1c	7.93	Type I Metacaspase; negatively regulates programmed cell death
AT2G36120.1	DOT1	7.91	Defective Organized Tributaries 1; involved in leaf vascular patterning
AT5G20380.1	PHT4;5	7.90	Inorganic phosphate transporter
AT1G55900.2	TIM50	6.56	Component of mitochondrial inner membrane translocase
AT4G31010.2	MCSF1	6.29	Mitchondrial CAF-like Splicing Factor 1; processes mitochondrial introns
AT1G17440.1	CKH1; TAF12B	1.53	Cytokinin Hypersensitive; putative negative cytokinin regulator
AT4G32980.1	ATH1	-2.12	Homeobox Gene 1; involved in photomorphogenesis, GA biosynthesis, flowering time
AT2G27230.1	LHW	-2.15	Lonesome Highway; transcriptional activator required for proper vascular development
AT5G67385.1	NCH1	-2.90	NRL Protein for Chloroplast Movement; plasma membrane localized
AT1G20620.1	CAT3;SEN2	-4.59	Catalase; breaks hydrogen peroxide into water and oxygen
AT4G16890.1	SNC1; BAL	-5.57	Supressor of npr1-1 constitutive; mutants constitutively express PR genes
AT5G15250.2	FTSH6	-5.76	FtsH Protease 6; chloroplast localized, involved in degradation of Lhcb3 and Lhcb1
AT2G47240.2	LACS1	-6.48	Long-chain Acyl-CoA Synthase 1; involved in cuticular wax and cutin biosynthesis
AT5G20935.1	CRR42	-6.81	Chlororespiratory Reduction 42; stromal protein involved in accumulation of NDH
AT2G48120.1	PAC	-8.91	Pale Cress; involved in chloroplast and leaf development, mutants are ABA deficient

Table S2. Trans-Zeatin-N-Glucosides regulate the transcriptome uniquely from trans-Zeatin. All transcripts listed here are significantly (padj < 0.05) regulated by tZ7G (yellow) or tZ9G (red).

Primer	Forward/Reverse	Sequence (5'→3')
AHK4	F	CCTCTCACAACTCATTACAGCTC
AHK4	R	CAACCCCGAACGCCAAAAAT
ARR5	F	CTACTCGCAGCTAAAACGC
ARR5	R	GCCGAAAGAATCAGGACA
ARR7	F	CTGGCATTGAGTAATCCGTCACTATC
ARR7	R	TGACGACTGTAGAAGGTGGAACTAGG
ARR16	F	TCAGGAGGTTCTTGTTCGTCTT
ARR16	R	AACCCAAATACTCCAATGC
CYP735A2	F	ACTTTACCCTCCTGCCACAC
CYP735A2	R	GAACTCGTTCGCATCTTCGC
CKX4	F	ACTATTGTCTCCAGGACAAGACA
CKX4	R	TTCTGAGAGACCTATACCGCT
CKH1	F	TGGAGAGTTGAAGGCACAAGT
CKH1	R	TCAAGCTTAAAATGGAGAGGACA
PDF1	F	CCATTAGATCTTGTCTCTCTGCT
PDF1	R	GACAAAACCCGTACCGAG

Table S3. Primer sequences used in qRT-PCR reactions. For analysis, all genes were normalized to PDF1.

Chapter 4: Cytokinin isopentenyladenine and its glucoside isopentenyladenine-9-glucoside delay leaf senescence through activation of cytokinin-associated genes

H. Tucker Hallmark and Aaron M. Rashotte*

101 Rouse Life Sciences, Auburn University, AL 36849

*Corresponding author

Email:

HTH – hallmark@auburn.edu

AMR – rashotte@auburn.edu

ORCID:

HTH – https://orcid.org/0000-0002-2412-1806

AMR - http://orcid.org/0000-0001-5034-4395

Abstract

It has long been reported that cytokinins (CKs), a class of phytohormones, are capable of delaying senescence in detached leaves. However, CKs are often treated as a monolithic group of compounds even though dozens of CK species are present in plants with varied degrees of reported biological activity. One specific type of CK, isopentenyladenine base (iP), has been demonstrated as having roles in delaying leaf senescence, inhibition of root growth, and promoting shoot regeneration. However, its N-glucosides isopentenyladenine-7- and -9-glucoside (iP7G, iP9G) have remained understudied and have been largely ignored for several decades, despite their relatively high concentrations in plants including the model species *Arabidopsis thaliana*. Here we show that iP and one of its glucosides, iP9G, are capable of delaying senescence in leaves, though the glucosides having little to no activity in other bioassays. Additionally, we performed the first transcriptomic study of iP-delayed cotyledon senescence which shows that iP is capable of upregulating photosynthetic genes and downregulating catabolic genes in detached cotyledons. Transcriptomic analysis also shows iP9G has mild effects on gene expression similar to that of iP during senescence, suggesting the label of “inactive” is a vast over-generalization with regards to iP-N-glucosides. These findings call into question the long-held belief that iP-N-glucosides, and possibly CK-N-glucosides as a whole, are inactive compounds. Future studies should not be so dismissive of these compounds, as they have apparent biological activity and are active in at least some biological processes similarly to “active” CKs.

Key Words: Cytokinin, Cytokinin-N-Conjugates, Isopentenyladenine, Isopentenyladenine-9-glucoside, Senescence, Transcriptome

1 Introduction

Cytokinins (CKs) are a class of phytohormones involved in a wide range of plant processes (Kieber and Schaller, 2014; Mok and Mok, 2001; Sakakibara, 2006; Skoog and Armstrong, 1970). *Arabidopsis thaliana* (hereafter referred to as Arabidopsis) has been a model species for the examination of CKs and understanding the molecular basis of cytokinin signaling and activity. Briefly, after biosynthesis and transport, a CK base binds to a CHASE-domain containing histidine kinase receptor initiating a modified two-component phosphorelay ultimately resulting in the activation of type-B Response Regulators, transcription factors (TFs) which mediate the transcriptional response to cytokinin (Hwang et al., 2012; Keshishian and Rashotte, 2015; Kieber and Schaller, 2014; Mok et al., 2000). Despite the impressively detailed understanding of the CK signaling pathway, mysteries about CK response and activity linger; in particular, what forms of CK are active, and do different types of CK lead to different effects *in planta*?

In Arabidopsis, detailed CK measurements have revealed nearly 30 different CK forms, with the two most abundant classes being isopentenyladenine-type (iP) and trans-Zeatin-type (tZ) CKs with each class composed of a several metabolites. (Kiba et al., 2013, 2019; Ko et al., 2014; Nam et al., 2012; Nishiyama et al., 2011; Sakakibara et al., 2005; Svačinová et al., 2012; Tokunaga et al., 2012; Werner et al., 2010; Zhang et al., 2014). The iP-type CKs detected in Arabidopsis include the following: iP riboside 5'monophosphates (iPRP), the first product in the CK biosynthetic pathway and a precursor to active CK; iP riboside (iPR), generally considered a transported form of iP with some bioactivity; iP (base form of CK), capable of binding CK receptors and initiating CK signaling; and iP-7- and iP-9-glucoside (iP7G, iP9G), composed of iP bases irreversibly conjugated to a glucose molecule by UGT76C2/1 and generally considered

permanently inactivated (Hou et al., 2004; Kieber and Schaller, 2014; Mok et al., 2000; Wang et al., 2011). Both iP7G and iP9G are N-glucosides, meaning the glucose molecule is attached via a nitrogen atom; these differ from O-glucosides which occur in tZ-type CKs and can be hydrolyzed back to tZ bases. Based on previous CK measurement data in Arabidopsis, iP7G is the most abundant iP-type CK, and is often the most abundant of all CKs (Kiba et al., 2019; Nishiyama et al., 2011; Sakakibara et al., 2005; Šimura et al., 2018; Svačinová et al., 2012; Tokunaga et al., 2012). Although iP9G is far less abundant than iP7G, it is often present at appreciable levels, and significantly more concentrated than other metabolites such as iP and iPR (Kiba et al., 2013, 2019; Ko et al., 2014; Nishiyama et al., 2011; Šimura et al., 2018; Tokunaga et al., 2012).

Despite the high abundance of iP7G and iP9G, collectively referred to as iPNGs, little is known about the activity of these compounds. Work from the 1980's demonstrated that Cytokinin-N-glucosides (CKNGs) are metabolically stable and do not appear to be converted into other forms of CK (Deleuze et al., 1972; Palni et al., 1984). One recent study using radiolabeled isotopes confirmed this is the case in Arabidopsis cell cultures (Hošek et al., 2019). Other classes of CK such as tZ also have N-glucoside forms, and previous work has shown that trans-Zeatin-N-glucosides have bioactivity in some assays, yet show distinct effects on the transcriptome and proteome of Arabidopsis seedlings (Hallmark et al., 2020).

Here we show that exogenous application of iP, as well as iP9G, is capable of delaying chlorophyll degradation in detached cotyledons senescence assays. To investigate the mechanism behind this phenomenon, RNA-sequencing was performed on cotyledons following a detached leaf senescence CK-bioassay. Surprisingly only three genes were differentially expressed after iP9G treatment as opposed to more than 10,000 genes from iP treatment. Genes differentially

expressed after iP treatment were enriched for roles in photosynthesis, translation, and CK response. Fv/Fm and protein content analysis validated that iP treatment leads to higher photosystem II efficiency and protein content than mock treatment. Altogether, these data suggest CK compounds should not be labelled binarily as active or inactive; modifications to CK bases, as seen in the formation iPNGs by glucosylation of iP, may simply alter or dampen the function of the molecule as opposed to fully eliminating its activity as previously believed. Additionally, this is the first published senescence transcriptome to include a naturally occurring CK base and its glucosides and reveals potential mechanisms by which iP delays senescence.

2 Results

2.1 Isopentenyladenine and one of its glucosides delays chlorophyll degradation in detached cotyledons

A hallmark of active CKs is their ability to delay senescence in leaves (Biddington and Thomas, 1978; Gan and Amasino, 1996; Singh et al., 1992a, 1992b). As the process of chlorophyll degradation is intrinsically linked with leaf senescence, measuring chlorophyll serves as a proxy for measuring how much senescence has occurred (Hörtensteiner, 2006). Here, cotyledons from 12 day after germination (12dag) seedlings were excised and floated on buffer supplemented with 1μM CK, either iP, iP7G, or iP9G, or a DMSO control. After six days in the dark, cotyledons treated with iP retain roughly three times as much chlorophyll as the control, while iP9G-treated cotyledons retain approximately twice as much as the control. The reduction in chlorophyll degradation by iP and iP9G treatment suggests both compounds are capable of delaying senescence in detached cotyledons. The difference between iP7G and the control was

not statistically significant, though iP7G retained approximately 40% more chlorophyll than the control.

2.2 Isopentenyladenine-N-glucosides do not promote shoot regeneration nor inhibit root growth

Other key features of active CKs include their abilities to promote shoot regeneration in calluses and inhibit root growth (Kieber and Schaller, 2014; Mok and Mok, 2001; Skoog and Miller, 1957). To assess whether iPNGs are functionally active as CKs in shoot regeneration, a regeneration assay was performed similarly to previously described protocols (Pernisova et al., 2018). Hypocotyls from etiolated 5dag seedlings were placed on shoot regeneration media (MS containing 1µM NAA and either 1.5µM iP, iP7G, or iP9G) and were grown under standard conditions. After three weeks on this new media, fresh weight of calluses was measured. If the CKs are active, one would expect a significant increase in callus weight, as the combination of active CK and auxin in the media promote shoot regeneration; this results in growth of the callus through reprogramming of the callus tissue to become shoot tissue (Gordon et al., 2007; Pernisova et al., 2018). Calluses on plates containing iPNGs were of similar weight to calluses grown on media containing DMSO control, while calluses on iP plates weighed significantly more, indicating only iP was promoting shoot regeneration (Fig 2).

To evaluate the effect of iPNGs on root growth, 4dag plate-grown seedlings of uniform size were transferred to media containing 1µM iP, iP7G, or iP9G, or a 0.1% DMSO control for 5 days, where root growth as length from day 4 to day 9 was measured. The concentration of 1µM was chosen as it is a common concentration for this bioassay because it is a “saturating” concentration without being immediately toxic to the plants. Only plants on iP-containing plates showed a significant reduction of root growth, approximately 74% compared to the DMSO

control (Fig 3). While iP7G and iP9G treatments led to modest reduction in root growth, 12% and 7%, respectively, neither treatment was statistically significant compared to the control (Fig 3). These data suggest iPNGs do not have roles in shoot initiation nor root growth inhibition at the concentrations tested.

2.3 Isopentenyladenine-9-glucoside minorly alters gene expression in an overlapping manner as isopentenyladenine while isopenteneyl-7-glucoside does not

To determine if iPNGs regulated gene expression similarly to iP during cotyledon senescence bioassays, tissue was collected at the end of cotyledon senescence bioassays and snap frozen. RNA was extracted and sequenced, and RNA-sequencing (RNAseq) was performed on cotyledons after 6d dark treatment with 1µM iP, iP7G, iP9G, and 0.1% DMSO. Three replicates of each treatment were performed, and at least twenty million paired-end reads per replicate were generated; reads were mapped back to the Arabidopsis reference genome with a mapping rate >97% for every sample (Anders et al., 2015; Cheng et al., 2017; Pertea et al., 2016). Differential Expression Analysis was performed using DESeq2 (Love et al., 2014). More than 10,000 differentially expressed genes (DEGs) were identified between iP and DMSO (Fig 4A). Surprisingly, only three DEGs were identified between iP9G and DMSO, and no DEGs between iP7G vs DMSO (Fig 4A). It is worth noting that treatment with iP9G and iP7G led to a non-statistically significant two-fold change difference in expression of approximately 1800 and 1400 genes, respectively. What is of particular interest is that the three statistically significant iP9G DEGs were key CK response genes: type-A response regulators ARR6, ARR7, and CK degrading enzyme CKX4. Each of these genes appears on the “Golden List” of transcripts routinely upregulated by CK treatment (Bhargava et al., 2013), so a heatmap was generated to

determine if other genes from this list were transcriptionally affected by iPNGs, regardless of statistical significance (Fig 4C). Based on a previous meta-analysis, a list of 38 genes was identified which show nearly universal upregulation following CK treatment (Bhargava et al., 2013). This subset of "Golden List" genes were upregulated by CK treatment in at least six microarray experiments and were also validated by a method other than microarray such as RNA-seq or qRT-PCR (Bhargava et al., 2013). Many of the 38 genes show upregulation by iP9G compared to DMSO, though mostly these changes are not as dramatic as what is seen with iP treatment (Fig 4C). Additionally, qRT-PCR confirmation of RNAseq results was performed on common CK-responsive genes as well as markers of leaf senescence including SAG12, SEN4, and NAC17 (Fig 4D). As expected, CK-responsive genes were upregulated in iP while senescence genes were downregulated (Fig 4D). Though the effects were mild, iPNGs also appeared to induce expression of CK-responsive genes and had mild, variable effects on the subset of selected senescence markers (Fig 4D).

2.4 Isopentenyladenine treatment significantly alters expression of senescence-associated transcription factors

Leaf senescence is a tightly regulated process which relies on highly coordinated gene regulation (Lim et al., 2007). As such, proper expression of TFs, and as a result their target genes, is necessary for senescence to occur. To determine if any previously identified senescence-responsive or senescence-mediating TFs were altered by iP treatment, a list of 48 TFs was generated and the effect of iP was evaluated (Chen et al., 2002; Kim et al., 2018, 2013; Miao et al., 2004; Ülker et al., 2007; Yang et al., 2011; Zwack et al., 2013) (Table 1). All TFs in the list have shown upregulation during leaf senescence, with the exception of CRF6 whose expression

decreases during senescence (Zwack et al., 2013). Just over half (25/48) of the TFs were significantly regulated by iP treatment as compared to the DMSO control, and a majority of those (21/25) displayed expression patterns opposite to what is typically seen during senescence (Table 1).

Of particular interest are CRF6, NAC017, WRKY6. CRF6 is a CK-responsive TF which negatively regulates senescence and is downregulated during senescence (Zwack et al., 2012, 2013). Interestingly CRF6 is also induced by oxidative stress and promotes stress tolerance by modulating the expression of CK-associated genes (Zwack et al., 2016). Treatment with iP led to significant upregulation of CRF6 (Table 1). Notably, CRF6 is a direct target of NAC017 (Ng et al., 2013). NAC017 is one member of the "NAC troika" involved in senescence response and is upregulated during leaf senescence (Kim et al., 2018). Further study of NAC017 has identified it as a key player in organellar stress response and retrograde signaling, and it is an activator of many genes involved in senescence, cell death, and autophagy (Meng et al., 2019). WRKY6 was identified in a screen for transcriptionally upregulated TFs during leaf senescence, and deeper analysis has characterized it as being a positive regulator of leaf senescence by directly activating expression of senescence genes; overexpression of WRKY6 leads to an early senescent phenotype (Chen et al., 2002; Robatzek and Somssich, 2002; Zhang et al., 2018). Both NAC017 and WRKY6 are significantly downregulated by iP treatment (Table 1).

2.5 Isopentenyladenine upregulates genes involved in photosynthesis and ribosome biogenesis while downregulating senescence and catabolism associated genes

After identifying key senescence TFs possibly responsible for the high number of DEGs seen between iP and DMSO treatment, further analysis of the 10,163 DEGs was performed to identify

potential mechanisms by which iP may be delaying senescence in detached cotyledons. Gene Ontology (GO) enrichment analysis was performed on both upregulated and downregulated gene lists. GO terms significantly enriched in the upregulated gene lists include cell components such as “chloroplast,” “ribosomal subunit,” and “chloroplast photosystem II”; enriched molecular functions include “structural constituent of ribosome” and “chlorophyll binding”; and enriched biological processes include “photosynthesis,” “ribosome biogenesis,” “response to cytokinin,” and “photosystem II assembly” (Fig 5A). GO terms significantly enriched in the downregulated gene lists include cell parts such as “authophagosome” and “proteasome complex”; enriched molecular functions include “endopeptidase activity,” “hydrolase activity,” and “ubiquitin-protein transferase activity”; enriched biological processes include “proteolysis,” “catabolic process,” and “leaf senescence” (Fig 5B). These data indicate significant roles for iP in delaying leaf senescence by upregulating photosynthetic and translation machinery during CK response, while downregulating catabolic processes like proteolysis and autophagy often associated with senescence and cell death (Bassham, 2007; Hildebrandt et al., 2015).

To evaluate the degree of co-expression and potential physical interaction between some of the most significantly regulated DEGs, gene networks were generated with the top 250 up- and downregulated genes from each list using geneMANIA in Cytoscape (Mostafavi et al., 2008; Shannon et al., 2003). As expected, multiple nodes associated with “photosynthetic membrane” and “response to cytokinin” were found clustered in the upregulated network (Fig 5C, E) while nodes associated with “organ senescence” and “amino acid catabolic processes” were found in the downregulated network (Fig 5D, F).

To evaluate the physiological impacts of changes seen from transcriptomic analysis of cotyledons from 6d dark senescence assays, three examinations were performed. First,

cotyledons from this assay were measured for chlorophyll fluorescence as a proxy for photosynthetic response and photosystem II integrity, as genes associated with photosynthesis were significantly upregulated by iP treatment. As expected, cotyledons treated with iP have significantly higher photosystem II efficiency as determined by Fv/Fm compared to that of DMSO control (Fig 6A). Second, iP-treated cotyledons were examined for overall protein levels, as genes associated with translation and ribosome biogenesis were significantly upregulated by iP treatment. The iP-treated cotyledons were found to have higher protein content than their DMSO counterparts (Fig 6B). Finally, amounts of cell death was examined in post-senescence assay cotyledons with Trypan Blue vital staining, as genes associated with senescence and cell death were significantly downregulated by iP treatment. iP-treated cotyledons appear to have the least staining of any treatment while DMSO-treated cotyledons stained the most heavily (Fig 6C). Interestingly, both iP7G and iP9G appear to have an intermediate amount of staining (Fig 6C).

3 Discussion

3.1 Isopentenyladenine-N-glucosides do not mimic isopentenyladenine in most bioassays

Active CKs have canonical activities such as inhibiting root growth, promoting shoot regeneration, and delaying senescence (Kieber and Schaller, 2014; Mok and Mok, 2001; Skoog and Miller, 1957). In each of these CK-bioassays conducted in this study, the CK base iP has strong and standard CK functional activity, similar to other CK examined in similar assays (Figs 1-3). In direct contrast, the iP N-glucoside iP7G did not have significant effects in any of these bioassays examined here at the tested concentration (Figs 1-3). While this cannot fully rule out potential CK activity or functions for iP7G in any of these processes, as higher concentrations of

iP7G could be further examined. The concentrations used in this study were chosen because they are on the higher end of what is commonly used in CK assays, but considering the fact that iP7G is routinely measured at orders of magnitude more highly concentrated than iP (Kiba et al., 2019; Nishiyama et al., 2011; Sakakibara et al., 2005; Šimura et al., 2018; Svačinová et al., 2012; Tokunaga et al., 2012), analysis of higher concentrations of exogenously applied iP7G might be more fruitful in identifying potential functions. It is worth noting that trans-Zeatin-N-glucosides both showed high activity at 1µM in a detached leaf senescence assay, and these compounds have comparable endogenous concentration relative to iPNGs (Hallmark et al., 2020), potentially indicating iP7G truly does not have significant anti-senescent capability. With regards to iP9G, there was a significant increase in chlorophyll retention during the detached leaf senescence assay (Fig 1), but no significant activity was detected in root growth inhibition or shoot initiation (Figs 2,3). This may point to a role for iP9G in delaying leaf senescence, and it is noteworthy that though iP9G is often measured at much higher concentrations than iP, iP9G levels are typically significantly less than iP7G levels (Kiba et al., 2013, 2019; Ko et al., 2014; Nishiyama et al., 2011; Šimura et al., 2018; Tokunaga et al., 2012). These data suggest iP9G is an active CK, albeit not as robustly active as its respective base; iP7G, however, does not appear to be an active CK as determined by these bioassays.

3.2 Isopentenyladenine-9-glucoside has modest effects on the senescing leaf transcriptome

Following a 6d detached leaf senescence experiment, iP9G treatment surprisingly only led to the differential expression of three genes (Fig 4A). Perhaps more surprisingly, however, is that these three genes are ARR6, ARR7, and CKX4, all of which are commonly upregulated by active CK treatments and play roles in CK signaling and degradation (Fig 4B) (Bhargava et al., 2013;

Galuszka et al., 2007; Rashotte et al., 2003). While these were the only three genes to be found significantly differentially expressed, several other key CK-responsive genes showed upregulation following iP9G treatment as compared to mock treatment, including ARR4, ARR3, ARR15, CRF6, ASL9, and GRXS13 (Fig 4C). The modest upregulation of these genes by iP9G as compared with their significant upregulation by iP furthers the notion that iP9G has iP-like activity, but this activity is decreased relative to the base.

3.3 Isopentenyladenine treatment significantly alters the transcriptome of senescing leaves

It has long been known that active CKs delay senescence, and key regulators of CK-delayed senescence have been previously identified. Phosphorylation of ARR2 as a result of CK binding to AHK3 has been shown to be necessary for CK to delay senescence (Kim et al., 2006). Additionally, the CK-responsive TF CRF6 is required for CK treatment to delay senescence (Zwack et al., 2013, 2016). There has been extensive characterization of the senescing leaf transcriptome in Arabidopsis (Breeze et al., 2011; Guo et al., 2004; Kim et al., 2018; Woo et al., 2016), yet, to the authors knowledge, no transcriptome of detached leaves treated with natural CKs has been performed. A previous CK senescence transcriptome used the synthetic CK benzyladenine riboside and its halogenated forms over a 48h treatment (Vylíčilová et al., 2016). This novel experiment allows one to see potential mechanisms of the anti-senescence activity of naturally-occurring CKs and how this classic CK bioassay functions.

Many TFs typically upregulated during senescence showed significant downregulation with iP treatment as compared to the DMSO control (Table 1). Several of these transcription factors including WRKY6 and NAC017 are known to positively regulate genes involved in senescence associated processes such as autophagy and cell death (Kim et al., 2018; Meng et al., 2019;

Robatzek and Somssich, 2002; Zhang et al., 2018). Therefore, the repression of these TFs by iP treatment is likely one of the mechanisms by which iP treatment led to downregulation of senescence-associated genes and inhibited leaf senescence.

Because such a large number of genes (10,163) were affected by iP treatment, gene ontology (GO) analysis was performed to gain a big-picture view of what types of genes were affected. Because a similar number of genes were up- and downregulated by iP relative to DMSO (5106 and 5057, respectively), GO analysis was performed on both lists independently of one another (Fig 5A,B).

It has previously been noted that chloroplast and photosystem genes plays a key role in leaf senescence (Woo et al., 2016), so it is unsurprising that there is enrichment for chloroplasts and photosystems in the Cell Component GO terms (Fig 5A). Enriched Biological Process GO terms reveal some intriguing trends: upregulation of genes involved in photosynthesis, ribosome biogenesis, chlorophyll biosynthesis, response to CK, and photosystem II assembly (Fig 5A). Genes associated with the GO terms “photosynthetic membrane” and “response to cytokinin” were present in gene networks generated using the top 250 iP-upregulated genes (Fig 5C, E). GO analysis of the downregulated gene list led to several interesting enriched GO terms that were distinct from the upregulated list, suggesting that CK may both induce and repress a number of processes to delay senescence. GO terms for both the Cellular Component “autophagasome” and the Biological Process “autophagy” are significantly enriched in the downregulated gene lists (Fig 5B). Autophagy is known to play an important role in leaf senescence as it is a catabolic process which aids in recycling nutrients and macromolecules (Bassham, 2007; Xiong et al., 2005). Degradation of macromolecules including proteins by endopeptidases and the proteasome are common hallmarks of senescence (Hildebrandt et al.,

2015; Lim et al., 2007; Lohman et al., 1994), so unsurprisingly GO terms for the Biological Processes “catabolic process” and “proteolysis,” the Molecular Functions “endopeptidase activity” and “ubiquitin-protein transferase activity,” and the Cellular Component “proteasome complex” and “lytic vacuole” are enriched in the downregulated gene list. Important to note is the GO term enrichment for “aging” and “leaf senescence” in the downregulated gene list as one would expect. Genes associated with both “organ senescence” and “amino acid catabolic processes” are enriched in gene networks generated using the top 250 iP-downregulated genes (Fig 5D, F). Catabolism of proteins and ultimately amino acids is a consequence of senescence as cells use the breakdown of these molecules for energy (Avin-Wittenberg et al., 2015; Hildebrandt et al., 2015).

3.4 Examination of cotyledons after senescence bioassays supports transcriptome findings

As many biological processes were affected by iP treatment of iP as a CK according to transcriptome findings, we decided to assay some of these processes *in planta* following the detached leaf senescence assays. One of the most significantly upregulated biological processes in this assay by iP was photosynthesis, specifically photosystem II assembly (Fig 5A). It had already been determined that iP treatment led to higher chlorophyll retention than DMSO treatment in these assays (Fig 1), however measurement of chlorophyll fluorescence can determine the efficiency of photosystem II. Based on transcriptome results, one would expect higher photosystem II efficiency in iP treatment relative to DMSO, and this result was borne out in Fv/Fm measurements revealing iP-treated cotyledons had approximately double the Fv/Fm of that of DMSO-treated cotyledons (Fig 6A). This supports previous findings that CK treatment increases photosynthetic capability in detached leaves, though the previous work was performed

with synthetic CK ribosides and halogenated derivatives (Vylíčilová et al., 2016) as opposed to a natural CK base and its glucosides as performed here. While not statistically significant, it is interesting to note that the median Fv/Fm of the iP9G treatment group is 15% higher and shows less variability than the DMSO treatment group (Fig 6A), which may possibly relate to the higher chlorophyll retention seen with iP9G in earlier assays (Fig 1).

Genes involved in ribosome biogenesis were found to be significantly upregulated while genes involved in various protein degradation processes including proteasome-mediated degradation and autophagy were downregulated. To determine if these changes in gene expression were mirrored in protein level, cotyledons were collected following senescence assays and subjected to protein extraction and quantification using methods previously established by Li et al., 2017. As visualized by Coomassie Brilliant Blue staining, iP-treated cotyledons appear to have higher overall protein content relative to the other treatments (Fig 6B). While not as drastic, iP7G and iP9G have slightly more protein than DMSO treatment (Fig 6B). These data suggest the upregulation of translation-associated genes and downregulation of proteolysis-related genes leads to increased protein production, or decreased protein degradation, in the detached cotyledons. This finding is consistent with previously reported decreases in leaf protein during senescence (Lim et al., 2007; Lohman et al., 1994; Noodén et al., 1997).

Treatment of cotyledons with iP led to a significant downregulation of genes associated with leaf senescence and cell death. To determine if these transcriptional changes led to a change in cell death, Trypan blue staining was performed and revealed limited cell death as compared to the DMSO control (Fig 6C). Treatment with iPNGs appeared to also lead to less cell death than DMSO, though to a lesser degree than iP. Again, these findings support both the transcriptome

findings (Fig 5) and support the role for iP in delaying senescence and cell death in detached cotyledons.

3.5 iPNGs are not identical in their activity to tZ-N-glucosides

Previous work with tZ-N-glucosides (tZNGs) revealed the compounds are capable of delaying senescence in senescence assays similarly to iP9G, but, like iPNGs, tZNGs had minimal to no effect on root growth or shoot regeneration (Hallmark et al., 2020). A stark difference between tZNGs and iPNGs, however, is their effects on gene expression; a two-hour treatment with tZNGs led to hundreds of DEGs (Hallmark et al., 2020), while only three DEGs were identified with iP9G treatment and zero with iP7G (Fig 4A). While the conditions of the transcriptome experiment differ greatly, one may hypothesize that tZNGs play their own more unique roles in gene expression, whereas iPNGs have minimal but overlapping effects with iP (Fig 4A). The ability of iP9G and tZNGs to delay senescence in detached leaves may indicate a role for CKNGs collectively in delaying senescence or regulating levels of CK bases.

3.6 Mechanism of iP9G activity remains unclear

While showing some activity in the detached leaf chlorophyll retention assay (Fig 1) along with upregulation of CK-related genes in the senescence transcriptome (Fig 4), the exact mechanism by which iP9G displays activity is unclear. As previously reported, conversion of CK-N-glucosides to CK bases is an unlikely culprit (Hallmark et al., 2020; Hošek et al., 2019; Palni et al., 1984). As iP9G appears to act as a less active form of iP at the transcriptional level (Fig 4), it stands to reason that iP9G is somehow activating the cytokinin response pathway, at least during the senescence assay. Two non-mutually exclusive mechanisms may be in play. First, exogenous

treatment with iP9G may make it unfavorable for iP base to be converted to iP9G, leading to an increase in iP levels and concomitantly activation of the cytokinin signaling pathway. Second, iP9G may directly bind CK receptors albeit with much lower affinity than iP. Previous tests of Arabidopsis CK receptor binding activity have not included iP-N-glucosides (Romanov et al., 2006; Spíchal et al., 2004; Yamada et al., 2001). There is also the issue of subcellular localization. CKNGs have been previously shown to be located largely in the apoplast and vacuole in leaves (Jiskrová et al., 2016), while CK receptors are primarily localized to the endoplasmic reticulum (Caesar et al., 2011; Wulfetange et al., 2011). Recent work, though, suggests CK receptors also appear to be present at the plasma membrane under certain conditions (Kubiasová et al., 2019), possibly placing them in a better position to interact with CKNGs. Whatever the mechanism, it is clear iP9G has CK activity which has gone previously unrecognized. While glycosylation of iP certainly dampens its effects, data presented here suggest this glycosylation is not fully inactivating. Moving forward, cytokinin biologists should reassess the generalization that CK-N-glucosides are biologically inert and consider that CK activities previously thought to be performed by CK bases alone might also be performed by their respective glucosides.

4 Materials and Methods

4.1 Plants Material and Growth Conditions

Seeds were sterilized with 70% ethanol and 20% bleach with 0.05% Tween before being plated on full strength MS agar plates. Seeds were then stratified 4 days at 4°C in the dark before being moved to a growth chamber running a 16h/8h light cycle (100µE) with diurnal temperature of 22°C/18°C. All plants were of the wildtype Col-0 ecotype of Arabidopsis.

4.2 Cotyledon Senescence Assay

The cotyledon senescence assays were carried out as described in Hallmark, et al. 2020. Briefly, cotyledons of 12 day after germination Arabidopsis seedlings were excised at the petiole and floated on 3mM MES buffer, pH 5.7. Buffer was supplemented with either 1µM CK (iP, iP7G, iP9G) or an equivalent volume of DMSO as a solvent control. CK were obtained as analytical standards from OlChemIm (Olomouc, Czech Republic). Previous work with CKNGs obtained from OlChemIm revealed no contamination with CK bases including no conversion of CKNGs to CK base during chlorophyll retention assays (Gajdošová, 2011). Once in solution, cotyledons were placed in the dark at 20°C for six days, after which chlorophyll was extracted and quantified as previously described (Sumanta et al., 2014). Three independent biological replicates were performed with 15 cotyledons per replicate.

4.3 Root Growth Assay

Seedlings were germinated on full strength MS agar. Four days after germination, seedlings of uniform size were transferred to MS plates supplemented with 1µM CK (iP, iP7G, iP9G) or an equivalent volume DMSO (0.1%). Transferred seedlings continued to grow until day 9, at which point root growth since day 4 was measured using ImageJ (NIH). Three independent biological replicates were performed with at least 8 seedlings per replicate.

4.4 Shoot Regeneration Assay

Shoot regeneration was completed similarly to a previously described assay (Pernisova et al., 2018) with modifications. The assay was carried out under standard growth conditions described

above 1µM NAA and 1.5µM CK (iP, iP7G, iP9G) or an equivalent volume of DMSO (0.15%). Fresh weight of calluses were measured 21 days after hypocotyls were transferred to hormone containing media. Three independent biological replicates were performed with at least 5 calluses per replicate.

4.5 Transcriptome and Gene Expression Analysis

Cotyledons (n = 20 per replicate) were collected from three biologically independent cotyledon senescence assays performed as described above. RNA was extracted using a Qiagen RNeasy Plant Mini Kit according to manufacturer's instructions. RNA was sent to Novogene, Inc. for quality check, library preparation, and sequencing on an Illumina HiSeq X. Read quality was evaluated using FastQC and trimming was performed using Trimmomatic (Andrews, 2010; Bolger et al., 2014). Read mapping, quantification, and differential expression analysis were carried out using HISAT2, HTSeq, and DESeq2, respectively (Anders et al., 2015; Love et al., 2014; Pertea et al., 2016). At least 20 million paired-end reads were generated per sample, and these reads mapped back to the TAIR10 Arabidopsis reference genome with a mapping rate of >97% for every sample (Cheng et al., 2017). The significance threshold for differential expression was set at adjusted p-value < 0.05. GO analysis was performed using AgriGO 2.0 (Tian et al., 2017). Raw sequencing data is available for download at NCBI Sequence Read Archive under BioProject ID PRJNA616296. qRT-PCR confirmation of RNAseq was completed using Sybr-green and sequence-specific primers on an Eppendorf Realplex2 as previously described (Zwack et al., 2013). Gene networks were visualized in Cytoscape using the geneMANIA plugin (Mostafavi et al., 2008; Shannon et al., 2003). Heatmap was generated using Heatmapper (Babicki et al., 2016).

4.6 Chlorophyll Fluorescence Analysis

Following three biologically independent cotyledon senescence assays performed as described above, cotyledons had Fv/Fm measured using standard settings from FluroCam7 software on a Handy FluorCam (Photon Systems Instruments) as previously described (Zwack et al., 2016). Three independent replicates consisting of ten cotyledons per treatment were performed.

4.7 Protein Content Determination

To measure protein content following cotyledon senescence assays, described above, cotyledons were collected from three independent replicates (n = 10 per treatment per replicate). Protein content was determined using a previously described protocol with minor modifications (Li et al., 2017). Cotyledons were ground in protein sample buffer (62.5mM Tris-HCl, 2% [w/v] SDS, 10% [v/v] glycerol, 5% beta-mercaptoethanol, 0.005% [w/v] bromophenol blue). Solutions were then boiled for five minutes and centrifuged for 10 minutes at 10,000g. Supernatant was collected and 25µL per sample was loaded into an Invitrogen 12% NuPAGE Bis-Tris gel. Electrophoresis was carried out at 115 V for 100 minutes. Proteins were visualized with Coomassie Brilliant Blue staining, and ImageJ (NIH) was used to quantify staining.

4.8 Histochemical Staining

For visualization of cell death in tissue following cotyledon senescence assays, described above, cotyledon tissue was stained with Trypan Blue as previously described (Fernández-Bautista et al., 2016). Briefly, cotyledons were submerged in Trypan Blue stain for 25 minutes and gently

shaken before stain was removed and cotyledons were de-stained. Three independent biological replicates were performed using ten cotyledons per treatment.

4.9 Data and statistical analyses

Unless otherwise stated, physiological data presented is the mean ± SE of at least three independent biological replicates, and statistical significance was determined by Student's two-tailed t-tests.

5 References

Anders, S., Pyl, P.T., and Huber, W. (2015). HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics *31*, 166–169.

Andrews, S. (2010). FastQC: A quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

Avin-Wittenberg, T., Bajdzienko, K., Wittenberg, G., Alseekh, S., Tohge, T., Bock, R., Giavalisco, P., and Fernie, A.R. (2015). Global Analysis of the Role of Autophagy in Cellular Metabolism and Energy Homeostasis in Arabidopsis Seedlings under Carbon Starvation. Plant Cell *27*, 306–322.

Babicki, S., Arndt, D., Marcu, A., Liang, Y., Grant, J.R., Maciejewski, A., and Wishart, D.S. (2016). Heatmapper: web-enabled heat mapping for all. Nucleic Acids Res. *44*, W147–W153.

Bassham, D.C. (2007). Plant autophagy—more than a starvation response. Curr. Opin. Plant Biol. *10*, 587–593.

Bhargava, A., Clabaugh, I., To, J.P., Maxwell, B.B., Chiang, Y.-H., Schaller, E.G., Loraine, A., and Kieber, J.J. (2013). Identification of Cytokinin Responsive Genes Using Microarray Meta-analysis and RNA-seq in Arabidopsis thaliana. Plant Physiol. pp.113.217026.

Biddington, N.L., and Thomas, T.H. (1978). Influence of Different Cytokinins on the Transpiration and Senescence of Excised Oat Leaves. Physiol. Plant. *42*, 369–374.

Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics *30*, 2114–2120.

Breeze, E., Harrison, E., McHattie, S., Hughes, L., Hickman, R., Hill, C., Kiddle, S., Kim, Y., Penfold, C.A., Jenkins, D., et al. (2011). High-Resolution Temporal Profiling of Transcripts during Arabidopsis Leaf Senescence Reveals a Distinct Chronology of Processes and Regulation. Plant Cell *23*, 873–894.

Caesar, K., Thamm, A.M.K., Witthöft, J., Elgass, K., Huppenberger, P., Grefen, C., Horak, J., and Harter, K. (2011). Evidence for the localization of the Arabidopsis cytokinin receptors AHK3 and AHK4 in the endoplasmic reticulum. J. Exp. Bot. *62*, 5571–5580.

Chen, W., Provart, N.J., Glazebrook, J., Katagiri, F., Chang, H.-S., Eulgem, T., Mauch, F., Luan, S., Zou, G., Whitham, S.A., et al. (2002). Expression Profile Matrix of Arabidopsis Transcription Factor Genes Suggests Their Putative Functions in Response to Environmental Stresses. Plant Cell *14*, 559–574.

Cheng, C., Krishnakumar, V., Chan, A., Thibaud-Nissen, F., Schobel, S., and Town, C. (2017). Araport11: a complete reannotation of the Arabidopsis thaliana reference genome. Plant J. *89*, 789–804.

Deleuze, G.G., McChesney, J.D., and Fox, J.E. (1972). Identification of a stable cytokinin metabolite. Biochem. Biophys. Res. Commun. *48*, 1426–1432.

Fernández-Bautista, N., Domínguez-Núñez, J., Moreno, M.M., and Berrocal-Lobo, M. (2016). Plant Tissue Trypan Blue Staining During Phytopathogen Infection. BIO-Protoc. *6*.

Gajdošová, S. (2011). Biological effects and metabolism of cis-Zeatin-type cytokinins in plants. Charles University.

Galuszka, P., Popelková, H., Werner, T., Frébortová, J., Pospíšilová, H., Mik, V., Köllmer, I., Schmülling, T., and Frébort, I. (2007). Biochemical Characterization of Cytokinin Oxidases/Dehydrogenases from Arabidopsis thaliana Expressed in Nicotiana tabacum L. J. Plant Growth Regul. *26*, 255–267.

Gan, S., and Amasino, R.M. (1996). Cytokinins in plant senescence: From spray and pray to clone and play. BioEssays *18*, 557–565.

Guo, Y., Cai, Z., and Gan, S. (2004). Transcriptome of Arabidopsis leaf senescence. Plant Cell Environ. *27*, 521–549.

Hallmark, H.T., Černý, M., Brzobohatý, B., and Rashotte, A.M. (2020). trans-Zeatin-N-glucosides have biological activity in Arabidopsis thaliana. PLOS ONE *15*, e0232762.

Hildebrandt, T.M., Nunes Nesi, A., Araújo, W.L., and Braun, H.-P. (2015). Amino Acid Catabolism in Plants. Mol. Plant *8*, 1563–1579.

Hörtensteiner, S. (2006). Chlorophyll Degradation During Senescence. Annu. Rev. Plant Biol. *57*, 55–77.

Hošek, P., Hoyerová, K., Kiran, N.S., Dobrev, P.I., Zahajská, L., Filepová, R., Motyka, V., Müller, K., and Kamínek, M. (2019). Distinct metabolism of N-glucosides of isopentenyladenine and trans-zeatin determines cytokinin metabolic spectrum in Arabidopsis. New Phytol.

Hou, B., Lim, E.-K., Higgins, G.S., and Bowles, D.J. (2004). N-Glucosylation of Cytokinins by Glycosyltransferases of Arabidopsis thaliana. J. Biol. Chem. *279*, 47822–47832.

Hwang, I., Sheen, J., and Müller, B. (2012). Cytokinin Signaling Networks. Annu. Rev. Plant Biol. *63*, 353–380.

Jiskrová, E., Novák, O., Pospíšilová, H., Holubová, K., Karády, M., Galuszka, P., Robert, S., and Frébort, I. (2016). Extra- and intracellular distribution of cytokinins in the leaves of monocots and dicots. New Biotechnol. *33*, 735–742.

Keshishian, E.A., and Rashotte, A.M. (2015). Plant cytokinin signalling. Essays Biochem. *58*, 13–27.

Kiba, T., Takei, K., Kojima, M., and Sakakibara, H. (2013). Side-Chain Modification of Cytokinins Controls Shoot Growth in Arabidopsis. Dev. Cell *27*, 452–461.

Kiba, T., Takebayashi, Y., Kojima, M., and Sakakibara, H. (2019). Sugar-induced de novo cytokinin biosynthesis contributes to Arabidopsis growth under elevated CO 2. Sci. Rep. *9*, 1–15.

Kieber, J.J., and Schaller, G.E. (2014). Cytokinins. Arab. Book Am. Soc. Plant Biol. *12*.

Kim, H.J., Ryu, H., Hong, S.H., Woo, H.R., Lim, P.O., Lee, I.C., Sheen, J., Nam, H.G., and Hwang, I. (2006). Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in Arabidopsis. Proc. Natl. Acad. Sci. *103*, 814–819.

Kim, H.J., Park, J.-H., Kim, J., Kim, J.J., Hong, S., Kim, J., Kim, J.H., Woo, H.R., Hyeon, C., Lim, P.O., et al. (2018). Time-evolving genetic networks reveal a NAC troika that negatively regulates leaf senescence in Arabidopsis. Proc. Natl. Acad. Sci. *115*, E4930–E4939.

Kim, Y.-S., Sakuraba, Y., Han, S.-H., Yoo, S.-C., and Paek, N.-C. (2013). Mutation of the Arabidopsis NAC016 Transcription Factor Delays Leaf Senescence. Plant Cell Physiol. *54*, 1660–1672.

Ko, D., Kang, J., Kiba, T., Park, J., Kojima, M., Do, J., Kim, K.Y., Kwon, M., Endler, A., Song, W.-Y., et al. (2014). Arabidopsis ABCG14 is essential for the root-to-shoot translocation of cytokinin. Proc. Natl. Acad. Sci. *111*, 7150–7155.

Kubiasová, K., Montesinos, J.C., Šamajová, O., Nisler, J., Mik, V., Plíhalová, L., Novák, O., Marhavý, P., Zalabák, D., Berka, K., et al. (2019). Cytokinin fluoroprobe and receptor CRE1/AHK4 localize to both plasma membrane and endoplasmic reticulum. BioRxiv 744177.

Li, L., Nelson, C.J., Trösch, J., Castleden, I., Huang, S., and Millar, A.H. (2017). Protein Degradation Rate in Arabidopsis thaliana Leaf Growth and Development. Plant Cell *29*, 207–228.

Lim, P.O., Kim, H.J., and Gil Nam, H. (2007). Leaf Senescence. Annu. Rev. Plant Biol. *58*, 115–136.

Lohman, K.N., Gan, S., John, M.C., and Amasino, R.M. (1994). Molecular analysis of natural leaf senescence in Arabidopsis thaliana. Physiol. Plant. *92*, 322–328.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. *15*, 550.

Meng, X., Li, L., Clercq, I.D., Narsai, R., Xu, Y., Hartmann, A., Claros, D.L., Custovic, E., Lewsey, M.G., Whelan, J., et al. (2019). ANAC017 Coordinates Organellar Functions and Stress Responses by Reprogramming Retrograde Signaling. Plant Physiol. *180*, 634–653.

Miao, Y., Laun, T., Zimmermann, P., and Zentgraf, U. (2004). Targets of the WRKY53 transcription factor and its role during leaf senescence in Arabidopsis. Plant Mol. Biol. *55*, 853–867.

Mok, D.W., and Mok, and M.C. (2001). Cytokinin Metabolism and Action. Annu. Rev. Plant Physiol. Plant Mol. Biol. *52*, 89–118.

Mok, M.C., Martin, R.C., and Mok, D.W.S. (2000). Cytokinins: Biosynthesis, Metabolism and Perception. Vitro Cell. Dev. Biol. Plant *36*, 102–107.

Mostafavi, S., Ray, D., Warde-Farley, D., Grouios, C., and Morris, Q. (2008). GeneMANIA: a real-time multiple association network integration algorithm for predicting gene function. Genome Biol. *9*, S4.

Nam, Y.-J., Tran, L.-S.P., Kojima, M., Sakakibara, H., Nishiyama, R., and Shin, R. (2012). Regulatory Roles of Cytokinins and Cytokinin Signaling in Response to Potassium Deficiency in Arabidopsis. PLOS ONE *7*, e47797.

Ng, S., Ivanova, A., Duncan, O., Law, S.R., Aken, O.V., Clercq, I.D., Wang, Y., Carrie, C., Xu, L., Kmiec, B., et al. (2013). A Membrane-Bound NAC Transcription Factor, ANAC017, Mediates Mitochondrial Retrograde Signaling in Arabidopsis. Plant Cell tpc.113.113985.

Nishiyama, R., Watanabe, Y., Fujita, Y., Le, D.T., Kojima, M., Werner, T., Vankova, R., Yamaguchi-Shinozaki, K., Shinozaki, K., Kakimoto, T., et al. (2011). Analysis of Cytokinin Mutants and Regulation of Cytokinin Metabolic Genes Reveals Important Regulatory Roles of Cytokinins in Drought, Salt and Abscisic Acid Responses, and Abscisic Acid Biosynthesis. Plant Cell *23*, 2169–2183.

Noodén, L.D., Guiamét, J.J., and John, I. (1997). Senescence mechanisms. Physiol. Plant. *101*, 746–753.

Palni, L.M.S., Palmer, M.V., and Letham, D.S. (1984). The stability and biological activity of cytokinin metabolites in soybean callus tissue. Planta *160*, 242–249.

Pernisova, M., Grochova, M., Konecny, T., Plackova, L., Harustiakova, D., Kakimoto, T., Heisler, M.G., Novak, O., and Hejatko, J. (2018). Cytokinin signalling regulates organ identity via the AHK4 receptor in Arabidopsis. Development *145*.

Pertea, M., Kim, D., Pertea, G.M., Leek, J.T., and Salzberg, S.L. (2016). Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. Nat. Protoc. *11*, 1650–1667.

Rashotte, A.M., Carson, S.D.B., To, J.P.C., and Kieber, J.J. (2003). Expression Profiling of Cytokinin Action in Arabidopsis. Plant Physiol. *132*, 1998–2011.

Robatzek, S., and Somssich, I.E. (2002). Targets of AtWRKY6 regulation during plant senescence and pathogen defense. Genes Dev. *16*, 1139–1149.

Romanov, G.A., Lomin, S.N., and Schmülling, T. (2006). Biochemical characteristics and ligand-binding properties of Arabidopsis cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. J. Exp. Bot. *57*, 4051–4058.

Sakakibara, H. (2006). CYTOKININS: Activity, Biosynthesis, and Translocation. Annu. Rev. Plant Biol. *57*, 431–449.

Sakakibara, H., Kasahara, H., Ueda, N., Kojima, M., Takei, K., Hishiyama, S., Asami, T., Okada, K., Kamiya, Y., Yamaya, T., et al. (2005). Agrobacterium tumefaciens increases cytokinin production in plastids by modifying the biosynthetic pathway in the host plant. Proc. Natl. Acad. Sci. *102*, 9972–9977.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., and Ideker, T. (2003). Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. Genome Res. *13*, 2498–2504.

Šimura, J., Antoniadi, I., Široká, J., Tarkowská, D., Strnad, M., Ljung, K., and Novák, O. (2018). Plant Hormonomics: Multiple Phytohormone Profiling by Targeted Metabolomics. Plant Physiol. *177*, 476–489.

Singh, S., Palni, L.M.S., and Letham, D.S. (1992a). Cytokinin Biochemistry in Relation to Leaf Senescence V. Endogenous Cytokinin Levels and Metabolism of Zeatin Riboside in Leaf Discs from Green and Senescent Tobacco (Nicotiana rustica) Leaves. J. Plant Physiol. *139*, 279–283.

Singh, S., Letham, D.S., and Palni, L.M.S. (1992b). Cytokinin biochemistry in relation to leaf senescence. VII. Endogenous cytokinin levels and exogenous applications of cytokinins in relation to sequential leaf senescence of tobacco. Physiol. Plant. *86*, 388–397.

Skoog, F., and Armstrong, D. (1970). Cytokinins. Annu. Rev. Plant Physiol. *21*, 359–384.

Skoog, F., and Miller, C.O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp. Soc. Exp. Biol. *54*, 118–130.

Spíchal, L., Rakova, N.Y., Riefler, M., Mizuno, T., Romanov, G.A., Strnad, M., and Schmülling, T. (2004). Two Cytokinin Receptors of Arabidopsis thaliana, CRE1/AHK4 and AHK3, Differ in their Ligand Specificity in a Bacterial Assay. Plant Cell Physiol. *45*, 1299–1305.

Sumanta, N., Haque, C.I., Nishika, J., and Suprakash, R. (2014). Spectrophotometric Analysis of Chlorophylls and Carotenoids from Commonly Grown Fern Species by Using Various Extracting Solvents. Res. J. Chem. Sci. *9*.

Svačinová, J., Novák, O., Plačková, L., Lenobel, R., Holík, J., Strnad, M., and Doležal, K. (2012). A new approach for cytokinin isolation from Arabidopsis tissues using miniaturized purification: pipette tip solid-phase extraction. Plant Methods *8*, 17.

Tian, T., Liu, Y., Yan, H., You, Q., Yi, X., Du, Z., Xu, W., and Su, Z. (2017). agriGO v2.0: a GO analysis toolkit for the agricultural community, 2017 update. Nucleic Acids Res. *45*, W122–W129.

Tokunaga, H., Kojima, M., Kuroha, T., Ishida, T., Sugimoto, K., Kiba, T., and Sakakibara, H. (2012). Arabidopsis lonely guy (LOG) multiple mutants reveal a central role of the LOG-dependent pathway in cytokinin activation. Plant J. *69*, 355–365.

Ülker, B., Shahid Mukhtar, M., and Somssich, I.E. (2007). The WRKY70 transcription factor of Arabidopsis influences both the plant senescence and defense signaling pathways. Planta *226*, 125–137.

Vylíčilová, H., Husičková, A., Spíchal, L., Srovnal, J., Doležal, K., Plíhal, O., and Plíhalová, L. (2016). C2-substituted aromatic cytokinin sugar conjugates delay the onset of senescence by maintaining the activity of the photosynthetic apparatus. Phytochemistry *122*, 22–33.

Wang, J., Ma, X.-M., Kojima, M., Sakakibara, H., and Hou, B.-K. (2011). N-Glucosyltransferase UGT76C2 is Involved in Cytokinin Homeostasis and Cytokinin Response in Arabidopsis thaliana. Plant Cell Physiol. *52*, 2200–2213.

Werner, T., Nehnevajova, E., Köllmer, I., Novák, O., Strnad, M., Krämer, U., and Schmülling, T. (2010). Root-Specific Reduction of Cytokinin Causes Enhanced Root Growth, Drought Tolerance, and Leaf Mineral Enrichment in Arabidopsis and Tobacco. Plant Cell *22*, 3905–3920.

Woo, H.R., Koo, H.J., Kim, J., Jeong, H., Yang, J.O., Lee, I.H., Jun, J.H., Choi, S.H., Park, S.J., Kang, B., et al. (2016). Programming of Plant Leaf Senescence with Temporal and Inter-Organellar Coordination of Transcriptome in Arabidopsis. Plant Physiol. *171*, 452–467.

Wulfetange, K., Lomin, S.N., Romanov, G.A., Stolz, A., Heyl, A., and Schmulling, T. (2011). The Cytokinin Receptors of Arabidopsis Are Located Mainly to the Endoplasmic Reticulum. PLANT Physiol. *156*, 1808–1818.

Xiong, Y., Contento, A.L., and Bassham, D.C. (2005). AtATG18a is required for the formation of autophagosomes during nutrient stress and senescence in Arabidopsis thaliana. Plant J. *42*, 535–546.

Yamada, H., Suzuki, T., Terada, K., Takei, K., Ishikawa, K., Miwa, K., Yamashino, T., and Mizuno, T. (2001). The Arabidopsis AHK4 Histidine Kinase is a Cytokinin-Binding Receptor that Transduces Cytokinin Signals Across the Membrane. Plant Cell Physiol. *42*, 1017–1023.

Yang, S.-D., Seo, P.J., Yoon, H.-K., and Park, C.-M. (2011). The Arabidopsis NAC Transcription Factor VNI2 Integrates Abscisic Acid Signals into Leaf Senescence via the COR/RD Genes. Plant Cell *23*, 2155–2168.

Zhang, K., Novak, O., Wei, Z., Gou, M., Zhang, X., Yu, Y., Yang, H., Cai, Y., Strnad, M., and Liu, C.-J. (2014). Arabidopsis ABCG14 protein controls the acropetal translocation of root-synthesized cytokinins. Nat. Commun. *5*.

Zhang, Y., Liu, Z., Wang, X., Wang, J., Fan, K., Li, Z., and Lin, W. (2018). DELLA proteins negatively regulate dark-induced senescence and chlorophyll degradation in Arabidopsis through interaction with the transcription factor WRKY6. Plant Cell Rep. *37*, 981–992.

Zwack, P.J., Shi, X., Robinson, B.R., Gupta, S., Compton, M.A., Gerken, D.M., Goertzen, L.R., and Rashotte, A.M. (2012). Vascular Expression and C-Terminal Sequence Divergence of Cytokinin Response Factors in Flowering Plants. Plant Cell Physiol. *53*, 1683–1695.

Zwack, P.J., Robinson, B.R., Risley, M.G., and Rashotte, A.M. (2013). Cytokinin Response Factor 6 Negatively Regulates Leaf Senescence and is Induced in Response to Cytokinin and Numerous Abiotic Stresses. Plant Cell Physiol. *54*, 971–981.

Zwack, P.J., De Clercq, I., Howton, T.C., Hallmark, H.T., Hurny, A., Keshishian, E.A., Parish, A.M., Benkova, E., Mukhtar, M.S., Van Breusegem, F., et al. (2016). Cytokinin Response Factor 6 Represses Cytokinin-Associated Genes during Oxidative Stress. Plant Physiol. *172*, 1249–1258.

AGI	Gene Name	log2FC iP	log2FC iP7G	log2FC iP9G	Previous identifying study
AT3G28210	SAP12	**-4.31**	-0.18	-0.09	Chen et al., 2002
AT2G28710	AT2G28710	**-3.15**	-0.08	-0.24	Chen et al., 2002
AT2G46680	HB-7	**-3.07**	0.03	-0.15	Chen et al., 2002
AT2G47190	MYB2	**-3.06**	0.08	-0.14	Chen et al., 2002
AT1G62300	WRKY6	**-2.74**	-0.09	-0.16	Chen et al., 2002
AT1G56650	PAP1	-2.41	-0.42	0.36	Chen et al., 2002
AT3G23250	ATMYB15	**-2.21**	0.15	0.06	Chen et al., 2002
AT1G06180	MYB13	**-2.10**	-0.13	0.00	Chen et al., 2002
AT4G39780	AT4G39780	**-2.05**	0.14	-0.04	Chen et al., 2002
AT5G47230	ERF5	**-1.64**	0.06	-0.02	Chen et al., 2002
AT1G46768	RAP2.1	**-1.53**	-0.10	-0.24	Chen et al., 2002
AT3G51960	BZIP24	**-1.25**	-0.06	-0.33	Chen et al., 2002
AT2G27580	SAP3	**-1.22**	0.01	-0.16	Chen et al., 2002
AT5G65210	TGA1	**-1.19**	-0.04	-0.14	Chen et al., 2002
AT1G30490	PHV	**-1.17**	-0.02	-0.05	Chen et al., 2002
AT5G06960	OBF5	**-0.79**	-0.08	-0.08	Chen et al., 2002
AT3G14230	RAP2.2	**-0.79**	-0.02	-0.06	Chen et al., 2002
AT4G34990	MYB32	-0.75	0.05	-0.01	Chen et al., 2002
AT5G06100	MYB33	**-0.71**	0.01	-0.06	Chen et al., 2002
AT2G40950	BZIP17	**-0.67**	0.02	0.11	Chen et al., 2002
AT4G01910	AT4G01910	**-0.65**	0.03	-0.05	Chen et al., 2002
AT1G34190	NAC017	**-0.58**	0.05	-0.01	Kim et al., 2018
AT3G01470	HB-1	-0.46	-0.03	-0.07	Chen et al., 2002
AT4G11680	AT4G11680	-0.41	0.11	0.07	Chen et al., 2002
AT5G10030	TGA4	-0.36	-0.04	-0.17	Chen et al., 2002
AT2G23340	DEAR3	-0.35	0.08	0.09	Chen et al., 2002
AT2G25000	WRKY60	-0.35	-0.03	0.15	Chen et al., 2002
AT1G13960	WRKY4	-0.34	-0.04	0.05	Chen et al., 2002
AT5G13180	VNI2	-0.22	0.03	0.04	Yang et al., 2011
AT1G78080	RAP2.4	0.15	0.20	0.24	Chen et al., 2002
AT4G01250	WRKY22	0.16	-0.01	0.04	Zhou et al., 2011
AT2G46270	GBF3	0.19	-0.16	0.00	Chen et al., 2002
AT3G23240	ERF1	0.22	0.06	0.18	Chen et al., 2002
AT1G13260	RAV1	0.26	-0.19	-0.05	Woo et al., 2010
AT5G09330	NAC082	0.30	0.01	0.01	Kim et al., 2018
AT4G23810	WRKY53	0.38	-0.10	0.26	Miao et al., 2004
AT4G24240	WRKY7	0.40	-0.30	-0.03	Chen et al., 2002
AT3G56400	WRKY70	0.49	-0.06	-0.10	Ülker et al., 2007
AT2G31180	MYB14	0.57	0.56	0.02	Chen et al., 2002
AT5G22380	NAC090	0.57	0.77	1.91	Kim et al., 2018
AT1G34180	NAC016	**0.61**	0.02	0.14	Kim et al., 2013
AT4G01720	WRKY47	0.64	-0.18	0.18	Chen et al., 2002
AT2G33310	IAA13	**0.72**	0.02	-0.01	Chen et al., 2002
AT2G02820	MYB88	**0.82**	0.02	-0.16	Chen et al., 2002
AT4G38620	MYB4	1.19	-0.04	-0.08	Chen et al., 2002
AT3G46130	MYB48	1.61	-0.81	0.50	Chen et al., 2002
AT3G61630	CRF6	**2.23**	0.03	0.56	Zwack et al., 2013 (CRF6 is downregulated during senescence)
AT2G01940	SGR5	**2.31**	0.23	-0.16	Chen et al., 2002

Table 1. Treatment with iP leads to downregulation of many TFs typically upregulated during senescence. All TFs listed in this table, except CRF6, have been previously reported to be upregulated during leaf senescence and/or play roles in leaf senescence. Approximately half of the list show downregulation as noted by blue colored cells. Darker blue and darker yellow indicate more severe down- and upregulation, respectively. The log2FC is log-2-based fold change relative to DMSO control. Bold-faced values indicate the differential expression is statistically significant (padj < 0.05).

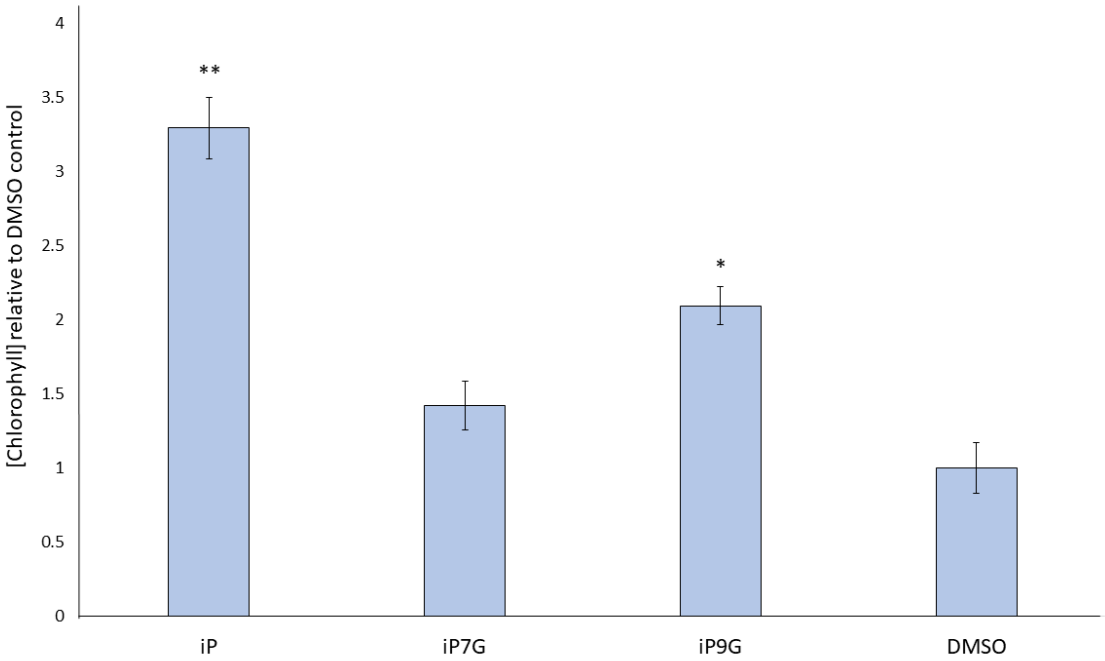


Figure 1. Isopentenyladenine and its 9-glucoside delays senescence in detached cotyledons. Cotyledons from 12dag seedlings were floated on 3mM MES buffer supplemented with 1µM of indicated hormone or 0.1% DMSO as a solvent control. Average ± SE of three independent replicates. Student's t test, *$p<0.05$ **$p<0.01$.

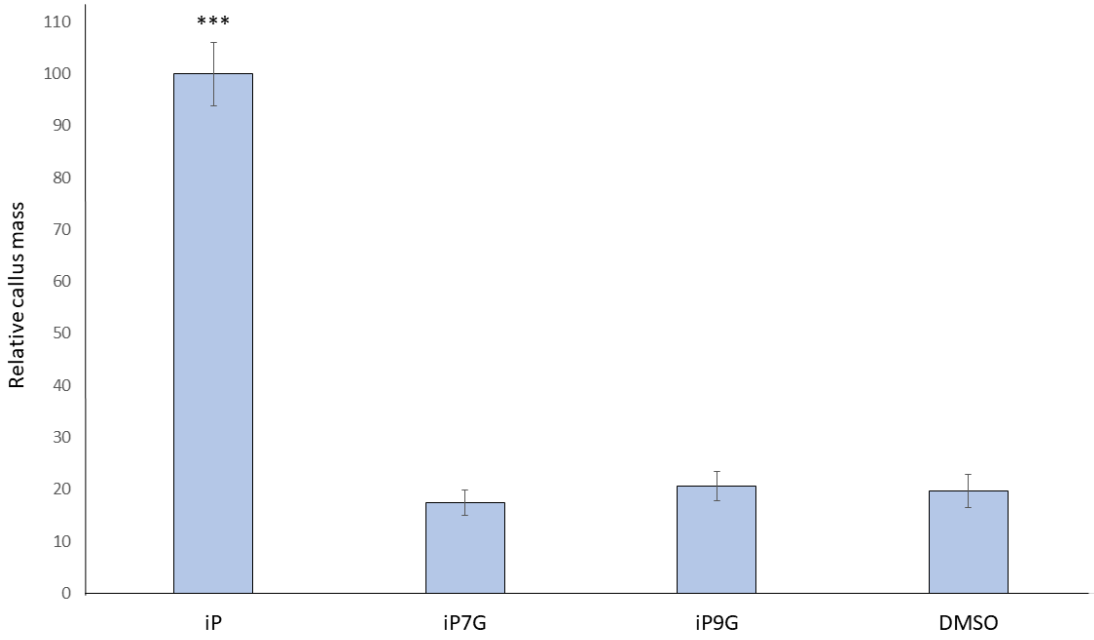


Figure 2. Isopentenyladenine-N-glucosides do not promote shoot regeneration. Hypocotyls from 5dag etiolated seedlings were placed on MS+1% sucrose media supplemented with 1µM NAA and 1.5µM of the indicated CK, or an equivalent volume of DMSO as a negative control. Plates were incubated at standard growth conditions for 21d, after which point callus mass was measured. Average ± SE is presented from three biological replicates of at least five calli per replicate. Student's t test, ***$p<0.001$.

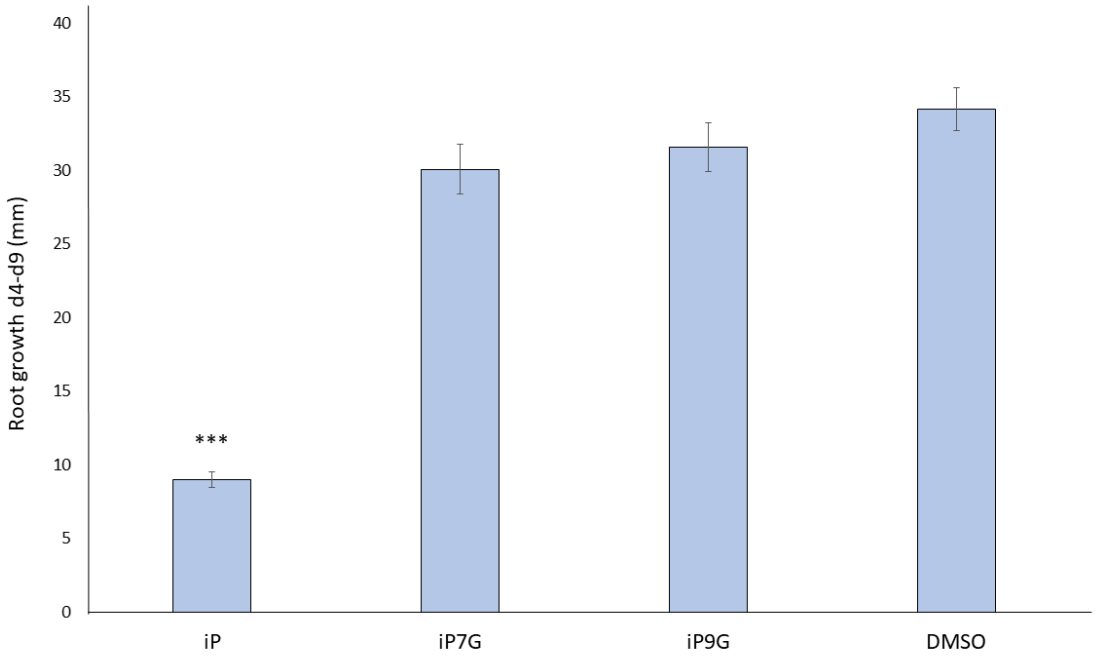


Figure 3. Isopentenyladenine-N-glucosides do not inhibit root growth. Plants were germinated vertically on standard MS + 1% sucrose and transferred to the same media supplemented with 1µM of the indicated CK or 0.1% DMSO as a negative control on day 4. Root growth from day 4 to day 9 was measured. Data presented are averages ± SE of 4 independent biological replicates, with at least 8 plants per replicate. Student's t test, ***$p<0.001$.

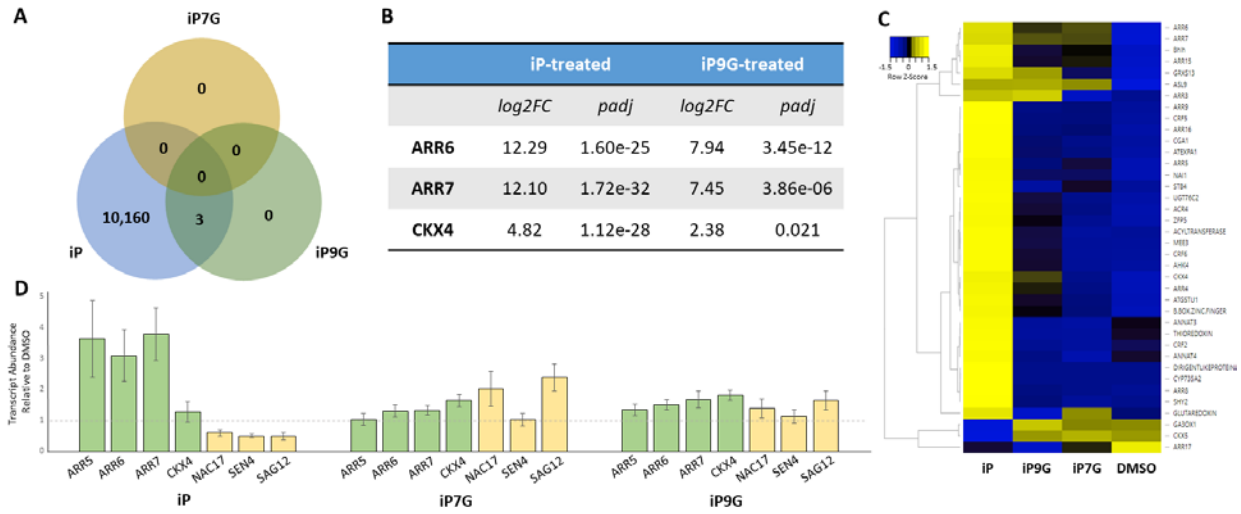


Figure 4. iP9G has minimal effects on the transcriptome of senescing cotyledons. Cotyledons from 12dag seedlings were floated on 3mM MES buffer supplemented with 1µM of indicated hormone or 0.1% DMSO as a solvent control for six days, after which RNA was extracted and sequenced. A, Venn diagram showing the number of significantly differentially expressed genes between treatment groups as compared to the DMSO control. B, The three DEGs altered by both iP and iP9G with log2-based fold change and adjusted p-value. C, heatmap showing expression of canonically CK-upregulated genes. D, qPCR confirmation of RNAseq results. Green bars represent genes typically upregulated by CK treatment, yellow bars represent genes upregulated during leaf senescence.

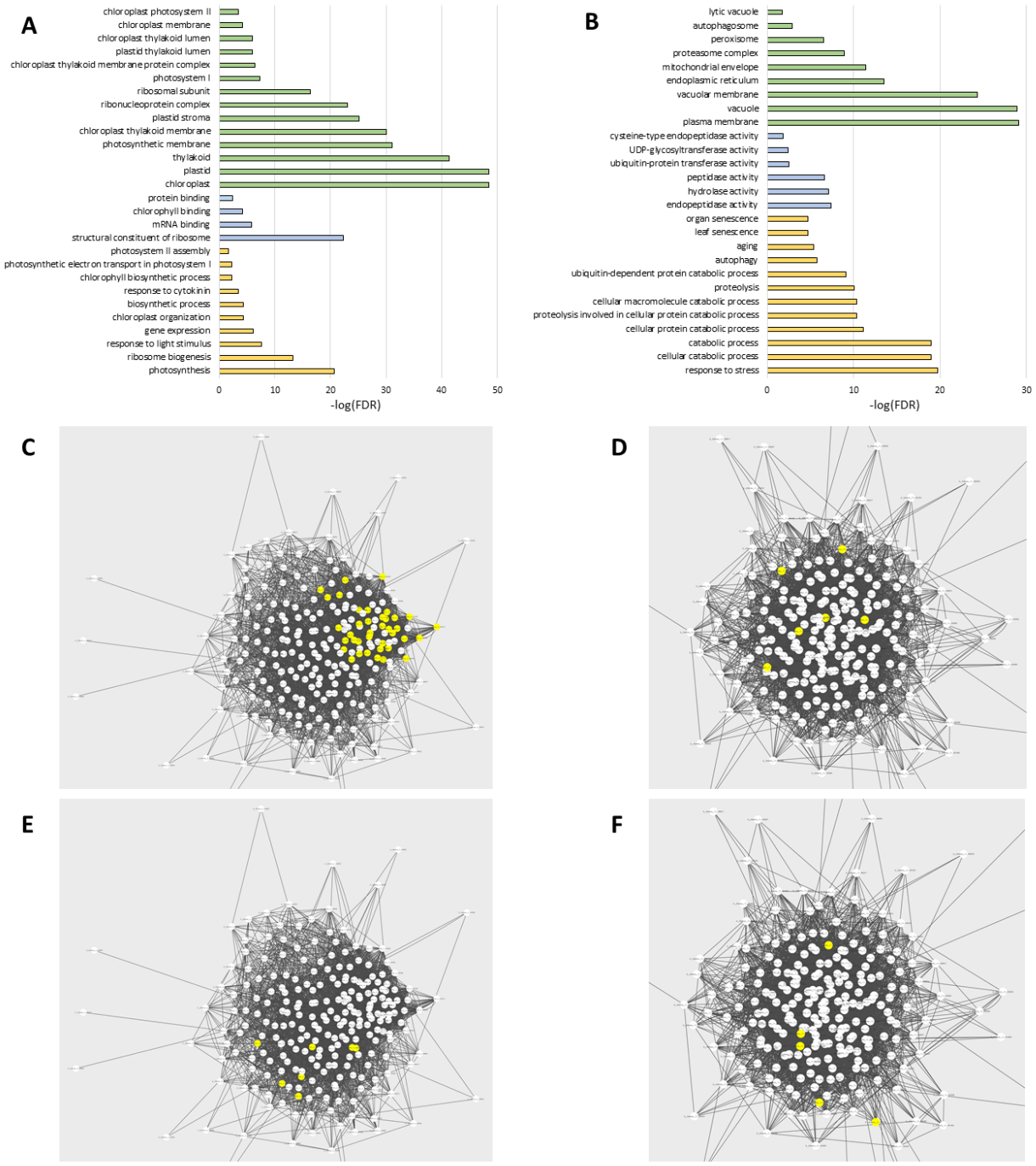


Figure 5. iP treatment of senescing cotyledons has significant effects on transcriptional regulation. Select GO terms significantly enriched in (A) iP-upregulated and (B) iP-downregulated list. Green bars associated with Cell Part GO terms, Blue with Molecular Function GO terms, Gold with Biological Process GO terms. C-F are predicted Gene Networks

generated using geneMANIA in Cytoscape. C and E are based on top 250 iP-upregulated genes; D and F are based on top 250 iP-downregulated genes. Nodes in yellow represent genes associated with GO terms “photosynthetic membrane” (C), “organ senescence” (D), “response to cytokinin” (E), and “cellular amino acid catabolic processes” (F).

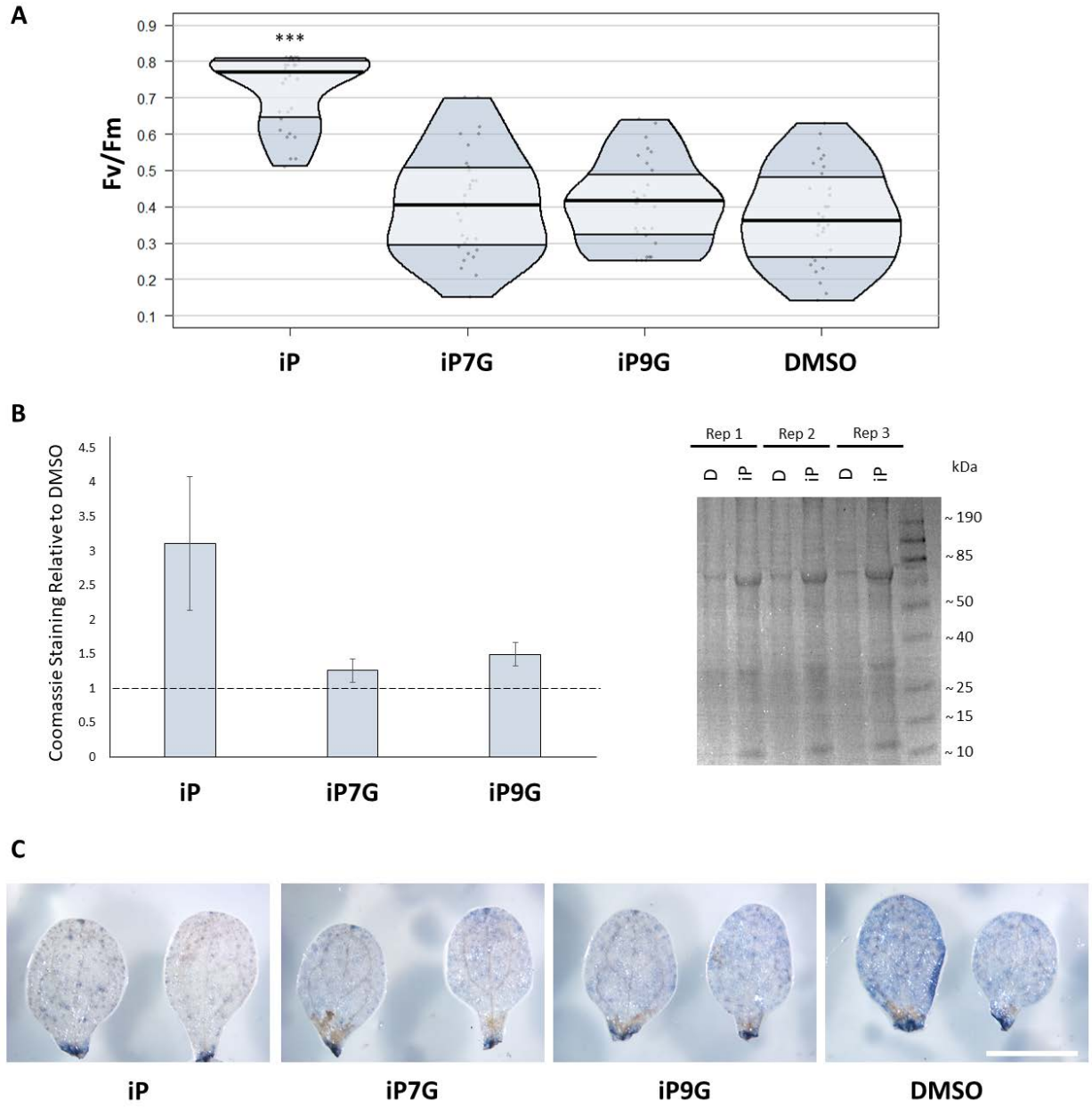


Figure 6. Senescing cotyledons treated with iP have higher photosystem II efficiency, higher protein content, and less cell death than mock-treated cotyledons. A, Cotyledons were treated identically to figure 1; at the end of six days, cotyledons were subjected to Fv/Fm measurement to determine photosystem II efficiency. Black bars represent median, grey dots indicate raw data points, opaque boxes represent inter-quartile range. Results are from three independent replicates with 10 cotyledons per treatment per replicate. Student's t test, ***p-value<0.001. B, Protein

content from cotyledons in A was determined according to Li et al, 2017. Protein was extracted from 10 cotyledons per treatment group from three independent replicates. 25µL protein extract was run on NuPAGE Bis-Tris gel, stained with Coomassie Blue, and protein was quantified in ImageJ. C, Cotyledons were stained with Trypan blue to visualize cell death. Images are representative of three independent replicates of ten cotyledons per replicate. All images taken at the same magnification, scale bar = 3mm.

Acknowledgements

This study was supported through the Auburn University Internal Grant Program. The authors thank Lauren Collins and Lesley Turner for their technical assistance throughout the project. The authors also thank Dr. Sang-Wook Park and his lab for assistance with the protein work.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

HTH designed the study, conducted the experiments, analyzed data, and wrote the manuscript. AMR secured funding, designed the study, analyzed data, and wrote the manuscript.

Chapter 5: Short communication – trans-Zeatin-N-glucoside-delayed senescence involves regulation of trans-Zeatin levels

H. Tucker Hallmark, Aaron M. Rashotte*

Department of Biological Sciences, 101 Rouse Life Sciences, Auburn University, AL 36849

*Corresponding author

HTH: hallmark@auburn.edu, AMR: rashotte@auburn.edu

Keywords: cytokinin, trans-Zeatin, senescence, cytokinin receptors

Abstract

A key feature of active cytokinins is their ability to delay senescence in detached leaves. Recently it was shown that trans-Zeatin-N-glucosides (tZNGs), previously believed to be biologically inactive, are capable of delaying senescence when exogenously applied to cotyledons. In this short communication, we further dissect this finding through the use of senescence assays in different mutant backgrounds as well as cytokinin (CK) measurements. Evidence suggests tZNGs delay senescence by increasing levels of trans-Zeatin base (tZ), thought to be a main active form of cytokinin. The increase in tZ does not appear to be a result of conversion of tZNGs back to tZ, but rather through the stimulation of new CK production, leading to an overaccumulation of all tZ-type CKs. These findings suggest a potential role for tZNGs in finetuning levels of other CKs, possibly through regulation of enzymatic activity.

Introduction

Cytokinins (CKs) are phytohormones involved in many plant processes, including leaf senescence (Mok et al., 2000; Sakakibara, 2006; Kieber and Schaller, 2014). CKs are a family of hormones composed of dozens of forms of adenine-derived molecules, though perhaps the most commonly studied forms of CKs are the base forms, in particular trans-Zeatin (tZ). Base forms of CK, including tZ, have long been shown to delay senescence in detached leaves (Biddington and Thomas, 1978; Smart et al., 1991; Gan and Amasino, 1995; Zwack et al., 2013). However, tZ can be irreversibly conjugated to a glucose molecule via a nitrogen atom, producing a trans-Zeatin-N-glucoside (tZNG), of which there are two main species: trans-Zeatin-7- and -9-glucoside (tZ7G, tZ9G) (Mok et al., 2000; Hou et al., 2004; Sakakibara, 2006; Kieber and Schaller, 2014; Li et al., 2015). tZNGs are often considered inactive forms of CK, but

interestingly they are some of the most abundant CKs in Arabidopsis and other Angiosperms (Auer, 1997; Sakakibara et al., 2005; Nishiyama et al., 2011; Svačinová et al., 2012; Kiba et al., 2013). Recent work from our lab has demonstrated roles for tZNGs in delaying senescence in cotyledons (Hallmark et al., 2020), yet the mechanism behind this process was not immediately apparent.

Here, we use Arabidopsis mutants in the CK biosynthetic and signaling pathways to dissect the mechanism of how CKNGs are delaying senescence. Results suggest tZ7G and tZ9G delay senescence in a manner dependent on endogenous tZ base and the CK receptors AHK2 and AHK3. UGT76C2 and UGT76C1, the enzymes responsible for the conversion of tZ to tZNGs, may also play a role in tZNG-delayed senescence.

Results

Endogenous tZ is required for tZNGs to delay senescence

Based on current knowledge of tZNG-delayed senescence, the mechanism of this process likely falls into one of two mutually exclusive categories: tZNGs are directly capable of delaying senescence (i.e. they bind a receptor and trigger the delayed senescence response) or tZNGs are indirectly capable of delaying senescence. In the latter case, the most probable way in which tZNGs would indirectly delay senescence is through conversion to tZ base. Though it has been widely reported that tZNGs are in fact not convertible, a recent study has claimed that within minutes of exogenous application, tZNGs are converted to tZ base (Hošek et al., 2019); these data are refuted by previously presented results (Hallmark et al., 2020).

To test if reconversion of tZNGs to tZ is responsible for tZNG-delayed senescence, a cotyledon senescence assay was performed using the previously described Arabidopsis *cypDM* mutant line.

The *cypDM* mutant (*dm* denotes double mutant) lacks functional copies of CYP735A1 and CYP735A2, the enzymes responsible for converting iP-type CKs to tZ-type CKs, causing the plant to endogenously lack tZ (Kiba et al., 2013). If senescence is still delayed in *cypDM* cotyledons after treatment with tZNGs, this would indicate one of two primary possibilities: either tZNGs are capable of delaying senescence directly, or tZNGs are converted to tZ, which in turn delays senescence. If senescence is not delayed, this is strong evidence that tZNGs do not directly delay senescence and that they are not converted to tZ.

As seen in Figure 1, treatment with tZ, but not tZNGs, caused chlorophyll retention relative to the DMSO control in *cypDM* cotyledons.

Cytokinin receptors are required for tZNGs to delay senescence

In Arabidopsis, three CK receptors have been identified: AHK2, AHK3, and AHK4 (Inoue et al., 2001; Nishimura et al., 2004). Analysis indicated each receptor differed in their ligand specificity, and AHK2 and AHK3 appear to be involved more in response to tZ than AHK4 (Spíchal et al., 2004; Romanov et al., 2006; Kiba et al., 2013).

To evaluate the role of CK receptors in response to tZNG treatment, a cotyledon senescence assay was performed using double receptor mutants, *ahk2ahk3*, *ahk2ahk4*, *ahk3ahk4*. This way, each double mutant only has one functional receptor, allowing us to determine if one specific receptor is more or less involved in response to these hormones. As previously reported (Hallmark et al., 2020), treatment with tZ, tZ7G, and tZ9G significantly delayed senescence in wildtype; each of these treatments led to approximately twice as much chlorophyll retention as the DMSO control (Figure 2). For *ahk2ahk3*, treatment with tZ nor tZNGs significantly delayed senescence relative to DMSO, though there was roughly twice as much chlorophyll retention in

tZ as compared to DMSO (Figure 2). Treatment of *ahk2ahk3* with tZ7G and tZ9G led to non-significant increases of 14% and 7% in chlorophyll retention, respectively (Figure 2). These results support previous findings that *ahk2ahk3* is unresponsive to tZ (Nishimura et al., 2004). Treatment of *ahk2ahk4* with tZ led to a significant 321% increase in chlorophyll retention, while treatment with tZ7G and tZ9G led to non-significant increases of 64% and 121%, respectively (Figure 2). Finally, Treatment of *ahk3ahk4* with tZ led to a significant 364% increase in chlorophyll retention, while treatment with tZ7G and tZ9G led to non-significant increases of 14% and 57%, respectively (Figure 2). The tZ treatment data suggest AHK2 and AHK3 are stronger positive regulators of tZ signaling than AHK4, as the *ahk2ahk3* double mutant showed the least responsiveness to tZ out of all genotypes; this supports previous reports of AHK2 and AHK3 as the primary tZ receptors (Nishimura et al., 2004; Spíchal et al., 2004; Romanov et al., 2006; Stolz et al., 2011). The trend is similar with the tZNGs, with AHK3 showing the most responsiveness to tZ7G and tZ9G in the *ahk2ahk4* mutant background, though the response was still not statistically significant.

The responsiveness, or lack thereof, of receptor double mutants to tZNGs suggests a combination of receptors must be present for tZNG response. This could indicate tZNG response requires heterodimers of AHKs. A simpler and possibly more likely scenario, however, is that tZNG response requires tZ response; as the data shows, when both AHK2 and AHK3 are absent, there is not a significant response to tZ (Figure 2).

It should be noted that the four genotypes differed in their chlorophyll level following DMSO treatment. This could be due to differences in chlorophyll levels in the seedlings prior to the assay, or it could be due to differences in senescence response. Further examination is required to determine the cause of this difference.

Levels of all tZ-type cytokinins increase following tZ and tZNG treatment

To determine if treatment with tZNGs does in fact lead to an increase in tZ content, cotyledons from a senescence assay were subjected to CK measurement via LC-MS. Treatment with tZ, tZ7G, and tZ9G led to an increase in concentration of all tZ-type CKs compared to the DMSO control (Figure 3). Following tZ treatment, levels of tZ, tZ7G, and tZ9G rose 755%, 4700%, and 14,160%, respectively, as compared to DMSO (Figure 3A). Following tZ7G treatment, levels of tZ, tZ7G, and tZ9G rose 418%, 6483%, and 11,493%, respectively, as compared to DMSO (Figure 3B). Following tZ9G treatment, levels of tZ, tZ7G, and tZ9G rose 761%, 2511%, and 30,129%, respectively, as compared to DMSO (Figure 3C). It is again worth noting tZNGs do not appear to be converted to tZ (Deleuze et al., 1972; Palni et al., 1984; Hallmark et al., 2020), though much of the rest of the tZ biosynthetic pathway is made up of reversible steps (Mok et al., 2000; Sakakibara, 2006). A potential mechanism by which tZNG treatment leads to an increase in tZ is presented in Figure 3D, showing that elevated levels of tZNGs, the products of tZ glucosylation, may inhibit the glucosylation of tZ, over time leading to an accumulation of tZ. This could be a form of product inhibition of enzyme kinetics (Walter and Frieden, 2006). There are changes in other CK metabolite levels as well, particularly DZR, though tZ-type CK are the only CKs which show universal increases across all treatments relative to DMSO. Importantly, additional replicates of this experiment are required to confirm these findings, but initial results suggest treatment with any of the tested tZ-type CKs leads to an increase in tZ, potentially leading to the CK effects seen in the senescence assays.

UGT76C2 and UGT76C1 are not required for tZNGs to delay senescence

One hypothesis of how tZNGs delay senescence includes a process requiring cytokinin-glucosylating enzymes, UGT76C2 and UGT76C1. In the CK-glucosylating reaction, tZ (substrate) is converted by these UGT enzymes into tZNGs (product). One hypothesis is that exogenous application of product causes the reaction to become metabolically unfavorable, so substrate builds up (Walter and Frieden, 2006); in turn, the built-up tZ may lead to delayed senescence. To test this hypothesis, cotyledon senescence assays were carried out in two separate alleles of the *ugtDM* mutant which lacks functional copies of both UGT76C2 and UGT76C1. A transgenic line overexpressing UGT76C2 (UGT76C2OX50) was also included. As shown in Figure 4, tZNG treatment delays senescence in *ugtDM* mutants, though not as severely as tZ treatment. In *ugtDM-A*, tZ treatment leads to a significant increase in chlorophyll retention of 425%, while treatment with tZ7G and tZ9G lead to non-significant increases of 150% and 217%, respectively (Figure 4). In *ugtDM-B*, tZ treatment leads to a non-significant increase in chlorophyll retention of 250%, while treatment with tZ7G and tZ9G lead to non-significant increases of 100% and 119%, respectively (Figure 4). In UGT76C2OX, tZ treatment leads to a significant increase in chlorophyll retention of 290%, while treatment with tZ7G and tZ9G lead to non-significant increases of 36% and 45%, respectively (Figure 4).

While much of these data are not statistically significant, the trend suggests that tZNGs still have some ability to delay senescence regardless of the presence of UGT76C1 and UGT76C2, though lacking these enzymes clearly leads to a less dramatic response to tZNGs. The lack of tZNG-responsiveness seen in UGT76C2-overexpressing plants may be due to the endogenously elevated levels of tZNGs caused by overexpressing the enzyme, though cytokinin measurement analysis would be required to confirm this. Overall these data suggest potential roles for the UGT enzymes in mediating response to tZNGs.

Discussion

Previous studies cast doubt on the ability of tZNGs to be converted to tZ. Treatment with a variety of glucosidase enzymes has been shown to not convert CKNGs back to CK bases; radiolabelled CKNGs have not shown conversion to other CK metabolites; and tZNGs have shown distinct biological and gene expression activity relative to tZ (Deleuze et al., 1972; Palni et al., 1984; Brzobohaty et al., 1993; Hallmark et al., 2020). Further evidence to support the hypothesis that tZNGs are not converted to tZ is presented here in that exogenous application of tZNGs does not lead to tZ-like effects in plants lacking endogenous tZ (Figure 1). However, it must be noted all data presented in this short communication are from cotyledons, so the possibility that tZNGs are converted to tZ in other organs cannot be fully ruled out.

Work on CK receptors has indicated AHK2 and AHK3 are key tZ responders which are primarily localized to shoot tissue while AHK4 is more involved in iP response and is primarily localized to root tissue (Spíchal et al., 2004; Romanov et al., 2006; Stolz et al., 2011; Kiba et al., 2013). Furthermore, AHK3 has been identified as being required for proper CK-delayed senescence (Kim et al., 2006). We tested double receptor mutants to determine if any receptor in particular was responsible for response to tZNGs during senescence assays; while none of the receptor mutants responded significantly to tZNGs, *ahk2ahk3* had the most dampened response (Figure 2). This suggested AHK2 and AHK3 were more important in response to tZNGs than AHK4, which falls in line with previous findings of the tZ- and senescence-specificity of AHK2 and AHK3 (Spíchal et al., 2004; Romanov et al., 2006; Stolz et al., 2011; Kiba et al., 2013). These results also furthered the notion that tZNGs may be delaying senescence via a tZ-dependent mechanism.

To determine if tZ levels are affected by tZNG treatment, cotyledons were collected following a senescence assay and subjected to cytokinin measurement. Interestingly, levels of all tZ-type CKs were increased by tZ and tZNG treatment (Figure 3A-C). This may reflect an attempt by the cotyledons to "equilibrate" their tZ levels. One potential mechanism for this is that treatment with tZNGs makes it unfavorable for UGT76C2 and UGT76C1, the enzymes responsible for cytokinin-N-glucosylation, to convert tZ to tZNGs due to inhibition of the enzymes due to overaccumulation of product (Walter and Frieden, 2006) (Figure 3D). In turn, this may lead to a buildup of tZ which then leads to the senescence delay; this type of product inhibition of enzyme activity is seen in many organisms. It is important to note that this is only the result of two technical replicates from one single biological replicate, and further CK measurement should be performed to validate this finding.

To determine if UGT76C2 and UGT76C1 are involved in modulating tZNG-delayed senescence, senescence assays were performed using two independently transformed *ugtDM* lines which lack functional copies of both enzymes. If tZNG treatment does lead to an accumulation of tZ via inhibition of the UGT enzymes, it would be expected that tZNG treatment would not delay senescence in *ugtDM* cotyledons. While variability was high, tZNGs did not significantly delay senescence in most cases (Figure 4). One exception is tZ9G delaying senescence in *ugtDM-B.* A line overexpressing UGT76C2 was also subjected to the assay and appeared to have no response to exogenous tZNG application, as well as a decreased sensitivity to tZ (Figure 4).

These data suggest anti-senescent activity seen during exogenous application of tZNGs is likely a result of increased tZ levels. The accumulation of tZ following tZNG treatment possibly involves the UGT enzymes responsible for glucosylating tZ, but other mechanisms certainly cannot be ruled out. These findings may implicate tZNGs, highly abundant forms of CK, act as a

regulator of other CK levels, potentially finetuning the levels of receptor-binding CK such as tZ. Further study is warranted to test this hypothetical role for tZNGs, and CKNGs as a whole.

Materials and Methods

Plants Material and Growth Conditions

Seeds were surface sterilized with 70% ethanol followed by 20% bleach with 0.05% Tween for ten minutes prior to plating on full strength MS agar plates containing 1% sucrose. Seeds were stratified in the dark at 4°C for four days before being moved to a growth chamber with a diurnal light and temperature cycle of 16h/8h 22°C/18°C. Plants used were Col-0 background *Arabidopsis thaliana*, and *ugtDM* and UGT76C2-overexpressing lines were a kind gift from Dr. Tomàs Werner.

Senescence assay

Detached cotyledon senescence assays were carried out as previously described (Hallmark et al., 2020). Briefly, cotyledons of 12 day after germination (12dag) seedlings were excised at the petiole and floated on 3mM MES buffer, pH 5.7. Buffer was supplemented with 1µM tZ, tZ7G, or tZ9G, or an equivalent volume (0.1%) DMSO as a solvent control. Solutions were placed in the dark at 20°C for 6 days before being removed. For chlorophyll extraction and determination, an established methanol-based protocol was used (Sumanta et al., 2014).

Cytokinin measurements

Cotyledons were collected following a senescence assay and lyophilized before being sent to Institute of Experimental Botany, Czech Republic, for CK determination by UPLC-MS/MS as previously described (Dobrev et al., 2017).

Acknowledgements

We thank Dr. Eva Pokorna and the Motyka Lab at Institute of Experimental Botany in Czech Republic for performing the CK measurements. We also thank the Werner lab for providing *ugtDM* seeds.

References

Auer CA (1997) Cytokinin conjugation: recent advances and patterns in plant evolution. Plant Growth Regul **23**: 17–32

Biddington NL, Thomas TH (1978) Influence of Different Cytokinins on the Transpiration and Senescence of Excised Oat Leaves. Physiol Plant **42**: 369–374

Brzobohaty B, Moore I, Kristoffersen P, Bako L, Campos N, Schell J, Palme K (1993) Release of active cytokinin by a beta-glucosidase localized to the maize root meristem. Science **262**: 1051–1054

Deleuze GG, McChesney JD, Fox JE (1972) Identification of a stable cytokinin metabolite. Biochem Biophys Res Commun **48**: 1426–1432

Dobrev PI, Hoyerová K, Petrášek J (2017) Analytical Determination of Auxins and Cytokinins. *In* T Dandekar, M Naseem, eds, Auxins Cytokinins Plant Biol. Methods Protoc. Springer, New York, NY, pp 31–39

Gan S, Amasino RM (1995) Inhibition of Leaf Senescence by Autoregulated Production of Cytokinin. Science **270**: 1986–1988

Hallmark HT, Černý M, Brzobohatý B, Rashotte AM (2020) trans-Zeatin-N-glucosides have biological activity in Arabidopsis thaliana. PLOS ONE **15**: e0232762

Hošek P, Hoyerová K, Kiran NS, Dobrev PI, Zahajská L, Filepová R, Motyka V, Müller K, Kamínek M (2019) Distinct metabolism of N-glucosides of isopentenyladenine and trans-zeatin determines cytokinin metabolic spectrum in Arabidopsis. New Phytol. doi: 10.1111/nph.16310

Hou B, Lim E-K, Higgins GS, Bowles DJ (2004) N-Glucosylation of Cytokinins by Glycosyltransferases of Arabidopsis thaliana. J Biol Chem **279**: 47822–47832

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

Kiba T, Takei K, Kojima M, Sakakibara H (2013) Side-Chain Modification of Cytokinins Controls Shoot Growth in Arabidopsis. Dev Cell **27**: 452–461

Kieber JJ, Schaller GE (2014) Cytokinins. Arab Book Am Soc Plant Biol. doi: 10.1199/tab.0168

Kim HJ, Ryu H, Hong SH, Woo HR, Lim PO, Lee IC, Sheen J, Nam HG, Hwang I (2006) Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in Arabidopsis. Proc Natl Acad Sci **103**: 814–819

Li Y, Wang B, Dong R, Hou B (2015) AtUGT76C2, an Arabidopsis cytokinin glycosyltransferase is involved in drought stress adaptation. Plant Sci **236**: 157–167

Mok MC, Martin RC, Mok DWS (2000) Cytokinins: Biosynthesis, Metabolism and Perception. Vitro Cell Dev Biol Plant **36**: 102–107

Nishimura C, Ohashi Y, Sato S, Kato T, Tabata S, Ueguchi C (2004) Histidine Kinase Homologs That Act as Cytokinin Receptors Possess Overlapping Functions in the Regulation of Shoot and Root Growth in Arabidopsis. Plant Cell **16**: 1365–1377

Nishiyama R, Watanabe Y, Fujita Y, Le DT, Kojima M, Werner T, Vankova R, Yamaguchi-Shinozaki K, Shinozaki K, Kakimoto T, et al (2011) Analysis of Cytokinin Mutants and Regulation of Cytokinin Metabolic Genes Reveals Important Regulatory Roles of Cytokinins in Drought, Salt and Abscisic Acid Responses, and Abscisic Acid Biosynthesis. Plant Cell **23**: 2169–2183

Palni LMS, Palmer MV, Letham DS (1984) The stability and biological activity of cytokinin metabolites in soybean callus tissue. Planta **160**: 242–249

Reed MC, Lieb A, Nijhout HF (2010) The biological significance of substrate inhibition: A mechanism with diverse functions. BioEssays **32**: 422–429

Romanov GA, Lomin SN, Schmülling T (2006) Biochemical characteristics and ligand-binding properties of Arabidopsis cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. J Exp Bot **57**: 4051–4058

Sakakibara H (2006) CYTOKININS: Activity, Biosynthesis, and Translocation. Annu Rev Plant Biol **57**: 431–449

Sakakibara H, Kasahara H, Ueda N, Kojima M, Takei K, Hishiyama S, Asami T, Okada K, Kamiya Y, Yamaya T, et al (2005) Agrobacterium tumefaciens increases cytokinin production in plastids by modifying the biosynthetic pathway in the host plant. Proc Natl Acad Sci **102**: 9972–9977

Smart CM, Scofield SR, Bevan MW, Dyer TA (1991) Delayed Leaf Senescence in Tobacco Plants Transformed with tmr, a Gene for Cytokinin Production in Agrobacterium. Plant Cell **3**: 647–656

Spíchal L, Rakova NY, Riefler M, Mizuno T, Romanov GA, Strnad M, Schmülling T (2004) Two Cytokinin Receptors of Arabidopsis thaliana, CRE1/AHK4 and AHK3, Differ in their Ligand Specificity in a Bacterial Assay. Plant Cell Physiol **45**: 1299–1305

Stolz A, Riefler M, Lomin SN, Achazi K, Romanov GA, Schmülling T (2011) The specificity of cytokinin signalling in Arabidopsis thaliana is mediated by differing ligand affinities and expression profiles of the receptors. Plant J **67**: 157–168

Sumanta N, Haque CI, Nishika J, Suprakash R (2014) Spectrophotometric Analysis of Chlorophylls and Carotenoids from Commonly Grown Fern Species by Using Various Extracting Solvents. Res. J. Chem. Sci. 9:

Svačinová J, Novák O, Plačková L, Lenobel R, Holík J, Strnad M, Doležal K (2012) A new approach for cytokinin isolation from Arabidopsis tissues using miniaturized purification: pipette tip solid-phase extraction. Plant Methods **8**: 17

Walter C, Frieden E (2006) The Prevalence and Significance of the Product Inhibition of Enzymes. Adv. Enzymol. Relat. Areas Mol. Biol. John Wiley & Sons, Ltd, pp 167–274

Zwack PJ, Robinson BR, Risley MG, Rashotte AM (2013) Cytokinin Response Factor 6 Negatively Regulates Leaf Senescence and is Induced in Response to Cytokinin and Numerous Abiotic Stresses. Plant Cell Physiol **54**: 971–981

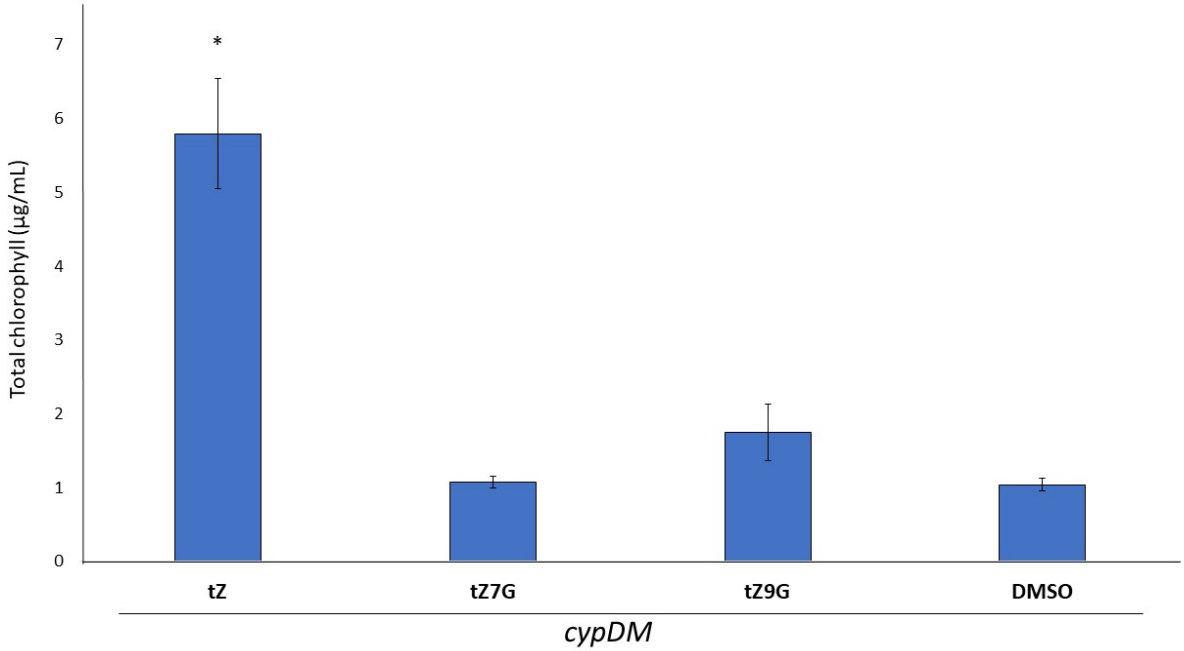


Figure 1. trans-Zeatin-N-glucosides do not delay senescence in trans-Zeatin biosynthetic mutants. Cotyledons from 12dag *cypDM* mutants, which are deficient in tZ-type cytokinins, were subjected to a 6d senescence assay with either 1µM CK or 0.1% DMSO. After six days in the dark, chlorophyll was extracted and quantified according to Sumanta et al., 2014. While exogenous tZ delayed senescence, tZNGs did not. Mean ± SE of four independent biological replicates is presented, *$p<0.05$, Student's t test.

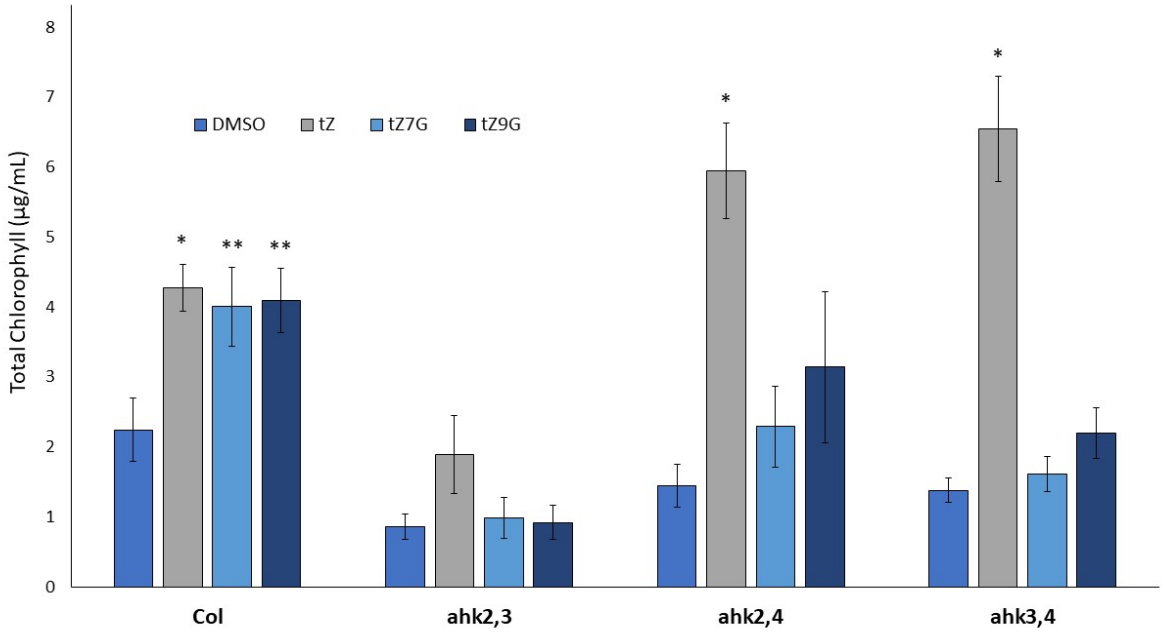


Figure 2. Cytokinin receptors are required for tZNGs to delay senescence. Cotyledons from 12dag seedlings of the noted genotypes were subjected to a 6d senescence assay with either 1µM CK or 0.1% DMSO. After six days in the dark, chlorophyll was extracted and quantified according to Sumanta et al., 2014. Mean ± SD of three independent replicates, *p<0.05, **p<0.01, Student's paired t-test.

A	iP	tZ	DZ	cZ
RMP	23%	D	ND	28%
R	152%	833%	415%	187%
base	64%	755%	17%	52%
7G	52%	4700%	31%	282%
9G	144%	14160%	131%	ND
OG		281%	ND	ND
ROG		NDD	NDD	225%

B	iP	tZ	DZ	cZ
RMP	0%	D	ND	45%
R	60%	2967%	756%	134%
base	64%	418%	28%	113%
7G	63%	6483%	38%	400%
9G	29%	11493%	221%	ND
OG		439%	ND	ND
ROG		NDD	NDD	334%

C	iP	tZ	DZ	cZ
RMP	115%	D	ND	61%
R	132%	1294%	439%	120%
base	41%	761%	27%	117%
7G	46%	2511%	56%	451%
9G	11%	30129%	349%	ND
OG		269%	ND	ND
ROG		NDD	ND	186%

D

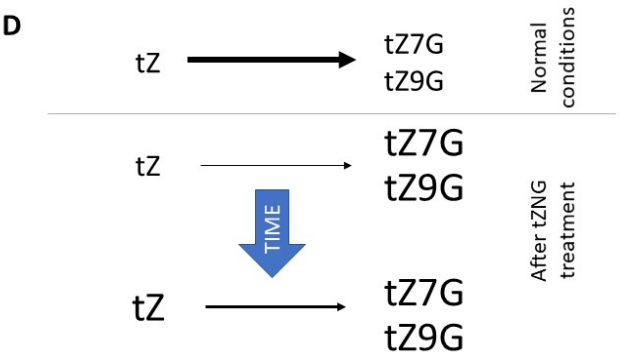


Figure 3. Treatment with tZ and tZNGs leads to increased levels of all tZ-type cytokinins. Cotyledons treated with tZ (A), tZ7G (B), and tZ9G (C) were subjected to cytokinin measurement via LC/MS. Levels of tZ base, tZ7G, and tZ9G are highlighted in green, blue, and yellow, respectively. Values shown are percentage of each CK species relative to the DMSO negative control. Each column represents a specific class of CKs, and each row represents a specific form of CK present in that class, i.e. cells in green are representative of tZ base. Values are from a single biological replicate. tZ base, tZ7G, and tZ9G are highlighted in green, blue, and yellow, respectively. ND, not detected in any sample, NDD, not detected in negative control treatment but detected after treatment. D, possible mechanism by which tZNG treatment leads to increase in tZ. Weight of the black arrow indicates favorability of UGT76C1/2-mediated glucosylation of tZ. Size of the text indicates relative abundance of tZ, tZ7G, and tZ9G. Blue arrow indicates progression of time.

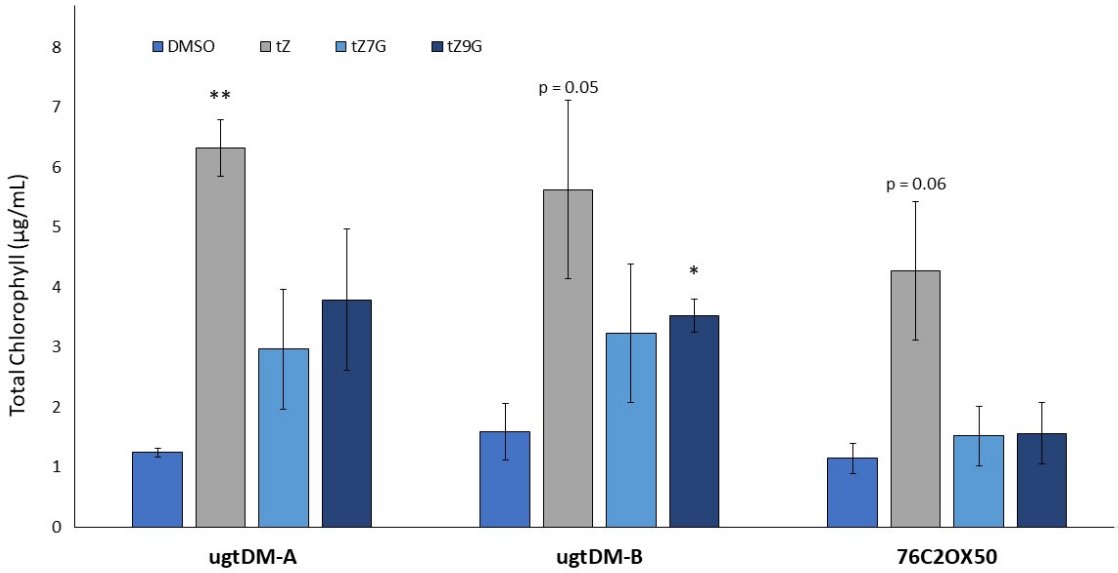


Figure 4. Cytokinin-N-glucosylating enzymes UGT76C2 and UGT76C1 may be involved in response to tZNGs. Cotyledons from 12dag seedlings were subjected to a 6d senescence assay with either 1µM CK or 0.1% DMSO. Genotypes *ugtDM-A* and *ugtDM-B* are separate alleles of double mutants lacking UGT76C1 and UGT76C2. Genotype 76C2OX50 is a transgenic line overexpressing UGT76C2. After six days in the dark, chlorophyll was extracted and quantified according to Sumanta et al., 2014. Mean ± SD of three independent replicates, *p<0.05, **p<0.01, Student's paired t-test.

Chapter 6: Meta-analysis of cytokinin measurements in *Arabidopsis thaliana*

H. Tucker Hallmark and Aaron M. Rashotte*

*Corresponding author

Author information

Email:

HTH – hallmark@auburn.edu

AMR – rashotte@auburn.edu

ORCID:

HTH – https://orcid.org/0000-0002-2412-1806

AMR – http://orcid.org/0000-0001-5034-4395

Abstract

The term cytokinin (CK) refers to a class of adenine-derived phytohormones which plays key roles in plant development, including regulation of shoot growth, root elongation, and senescence. CK compounds consist of an adenine molecule with an N^6-substituted sidechain; however many modifications can be made to this simple structure which alter the functionality or activity of the metabolite. Though CKs were discovered more than half a century ago, highly accurate and quantitative measurements of these molecules have only been possible for less than two decades. At least two dozen CKs are known to occur in *Arabidopsis thaliana*, the species in which much of the CK biosynthetic and signaling pathway has been studied. Many published studies of Arabidopsis physiology have included detailed CK measurement data, though typically only a few CK forms are discussed due to the complexity of CK metabolism. Here, we present a brief meta-analysis of CK concentrations in *Arabidopsis* through comparing twelve distinct CK measurement datasets. Plants used in these studies varied in age and growth conditions, and as such, this is a heuristic analysis with the goal of roughly determining the relative abundance of different CKs. We find that CK-N-glucosides (CKNGs), a class of CKs recently shown to have roles in regulation of leaf senescence, are typically the most concentrated form of CKs. CK bases, the most active form of the hormone, are the least abundant form.

Keywords: cytokinin, cytokinin measurement, phytohormone, meta-analysis, Arabidopsis

Introduction

The first cytokinin (CK) was first identified more than half a century ago (Miller et al., 1955). In the more than sixty years since, CKs have been identified as having key roles in physiological processes such as shoot growth, root growth, and delaying leaf senescence (Mok and Mok, 2001; Sakakibara, 2006; Kieber and Schaller, 2014). As key regulators of such important biological activity, it was important to establish protocols by which to accurately measure these CKs to fully understand their role in these processes. CKs are a class of dozens of endogenous adenine-derived hormones which all have the same general structure of an adenine base with an N^6-substituted side chain. The four main groups of CKs (the isoprenoid classes) differ in the composition of their sidechains, and additional modifications can be made to those sidechains or the adenine backbone to alter the activity or functionality of the CK (Yoshida and Oritani, 1972; Fox et al., 1973; Letham et al., 1983; Sakakibara, 2006; Kiba et al., 2013). The four isoprenoid classes of CKs are trans-Zeatins (tZ), cis-Zeatins (cZ), dihydrozeatins (DZ), and isopentenyladenines (iP); each form has a precursor form (CK-ribomonophosphate), a transported form (CK-riboside), a base form (CK base, typically considered the most active form), a storage form (CK-O-glucoside, not present in iP), and CK-N-glucosides (CKNGs), which were previously believed to be inactive though recent work suggests this is not entirely correct (Sakakibara, 2006; Hallmark et al., 2020; Vylíčilová et al., 2020). Table 1 shows the different CK forms included in this meta-analysis adapted from Novák et al., 2003.

In 2003, a protocol for the extraction and quantification of CKs by high performance liquid-chromatography followed by single-quadrupole mass spectrometry (HPLC-MS) was established (Novák et al., 2003). This was a major advance in CK determination because of the high sensitivity of the assay combined with the relative simplicity of being able to test for many CK

forms in a single analysis (Novák et al., 2003). This method was later improved upon by incorporating ultra-performance liquid chromatography followed by tandem mass spectrometry (UPLC-MS/MS), leading to increased sensitivity and higher throughput analysis (Novák et al., 2008). Just a year later, an independently developed method very similar to the UPLC-MS/MS protocol was established which allowed similar quantification of CKs in addition to other hormones in the same analysis (Kojima et al., 2009). These have protocols have been utilized to generate a large amount of CK measurement data in a variety of species. Here, we selected twelve examinations, detailed below, to compare findings from studies in perhaps the most well-examined species with regards to CK measurement, Arabidopsis.

Methodology

In determining which CK datasets to include in this meta-analysis, certain criteria had to be applied to remove potential confounding variables. First, all measurements had to be performed on whole wildtype *Arabidopsis thaliana* plants, excluding studies which measured only leaves or other specific organs. Second, plants that were examined must be between ten days and three weeks post-germination. At this age, true leaves should be present as well as lateral roots, but bolting will not have yet occurred. Third, plants had to be grown under typical conditions, excluding studies of plants grown under continuous light or dark regimes. Fourth, all isoprenoid type CKs must be measured and provided either in the main text or in supplemental data. This resulted in twelve studies being identified for use in the meta-analysis, with three CK examinations performed labs in the Czech Republic and nine in Japan. Studies which met these qualifications and are included in the analysis are listed in Table 2.

Mean concentrations of each CK metabolite were collected from figures and tables, then total isoprenoid CK content was summed and each CK concentration was normalized as a percentage of total measured isoprenoid CKs. CKs that were not detected were assigned a value of 0. Each study had at least 3 replicates, and the value included in the meta-analysis was the mean of those replicates.

In the case that roots and shoots were measured separately, these means were combined. We acknowledge that shoot tissue and root tissue are not of equal masses, so adding these concentrations will not lead to the precise concentration. However, because the data are normalized by percentage, we assume general trends will remain unaffected. Because multiple replicates were performed for each experiment, there is a degree of variability noted in each publication; only the means from each study were considered in this meta-analysis.

Results and Discussion

Dihydrozeatins are the least abundant CK bases and ribosides

Among the twelve datasets included in the analysis, the four base CK forms are similar in concentration with the exception of DZ base (Figure 1). Median levels of tZ, iP, and cZ bases are 0.69%, 0.42%, and 0.35%, respectively. DZ base, however, had a median level of 0% (tZ>iP>cZ>>DZ). Only three of the twelve studies detected DZ base, and when detected, DZ was present at only 0.01-0.06%. This trend continued with the riboside forms, generally considered to be transportable forms of CK with some CK activity (Spíchal et al., 2004; Romanov et al., 2006; Hirose et al., 2008; Kudo et al., 2010). The median levels of tZR, iPR, and cZR are 1.53%, 0.61%, and 0.98%, respectively. Yet the median level of DZR is less than 0.01% (tZR>cZR>iPR>>DZR).

It is interesting to find cZ-type and tZ-type CKs present at similar levels, as cZ-type CKs are generally considered inactive in Arabidopsis due to low binding affinity in receptor-binding assays and low activity in gene expression analyses (Spíchal et al., 2004; Romanov et al., 2006).

Precursor and glucoside forms make up the majority of measured CKs

One of the most abundant classes of CKs are the precursor forms consisting of tZRP, iPRP, cZRP, and DZRP (Figure 1). These forms have median levels of 4.58%, 10.04%, 4.73%, and 0.03%, respectively (iPRP>cZRP>tZRP>>DZRP). This continues the trend of lowly abundant DZ-type CKs, but the finding also shows a higher abundance of iP precursors to tZ- and cZ-type precursors. This may be unsurprising, as the main commitment step of the CK biosynthetic pathway is the formation of iPRP via isopentenyltransferase enzymes (Kakimoto, 2001; Takei et al., 2001; Miyawaki et al., 2006). From this point on in the pathway, iP-type CKs can be hydroxylated to form tZ-type CKs (Takei et al., 2004). It is worth noting that cZ-type CKs are synthesized via a distinct pathway in *Arabidopsis* involving tRNA (Kasahara et al., 2004). Therefore, it is interesting that cZ- and tZ-type precursors are present at similar concentrations. By far the most abundant class of CKs is CKNGs, which make up over half of the CKs measured. The two most abundant forms of CKNGs are iP7G and tZ7G, which combined make up roughly half of all CK in the plants tested (Figure 1). Median levels of these CKs are 25.05% and 17.54%, respectively. The next most abundant CKNGs are tZ9G and iP9G, which have median levels of 7.28% and 1.61%, respectively (iP7G>tZ7G>tZ9G>iP9G). CKs have been shown to be N-glucosylated via two enzymes, UGT76C2 and UGT76C1 (Hou et al., 2004; Wang et al., 2011; Wang et al., 2013; Li et al., 2015). The results of this meta-analysis support the in vitro finding that these enzymes prefer N^7 glucosylation over N^9 glucosylation, though the

precise mechanism for this preference is unknown (Hou et al., 2004). This preference appears to be even more apparent with regards to iP-type CKs, where iP7G is over ten-fold more abundant than iP9G, versus tZ-type cytokinins where tZ7G is just over twice as abundant as tZ9G. These findings may suggest a role of the side chain in deciding enzyme preference.

Though historically labelled as inactive, recent published work and much of this dissertation (Hallmark et al., 2020, Chapter 4, Chapter 5) suggest that CKNGs are not inactive forms, and likely play roles in modulating levels of other CKs. Perhaps the most intriguing finding of this meta-analysis is that the most active forms of CKs, CK bases, are the least abundant class of CK. CK ribosides, a transported form of CK which also show some ability to bind CK receptors and initiate CK signaling (Spíchal et al., 2004; Romanov et al., 2006), are the second least abundant class. The most abundant classes appear to be the CKNGs, which are irreversible products of the glucosylation of CK bases, and CK ribomonophosphates, which are precursor forms of CK ribosides and bases. These data may suggest that both precursor and "dead-end" products of CK metabolism are important regulators of the metabolites most involved in CK signaling. We posit that these "inactive" CK forms, including glucosides and precursors, are acting to buffer the levels of active CK compounds. As illustrated in Figure 2, the "Active Zone" of CK metabolites in the CK biosynthesis pathway is composed of CK bases and ribosides which have been demonstrated to have receptor binding capabilities. Metabolites in the "Active Zone" are much less abundant than those in the "Buffer Zone," which is composed of canonically "inactive" CK forms. Perhaps the ability of plants to convert active CK bases to CKNGs or back to precursors allows for finer control of active CK levels as opposed to simply degrading CK bases when they are in excess. Finer tuning of CK levels via these mechanisms could mean better programmed development and stress response without the energy and resource requirements involved in

activating expression of cytokinin biosynthetic or degrading enzymes. More research in this area is required to determine if this hypothesis is accurate. What is certain, though, is that CKNGs are highly abundant in *Arabidopsis thaliana*; in eleven of the twelve datasets analyzed in this study, the most concentrated CK was either tZ7G or iP7G. This points toward something interesting, which is that the twelve datasets were relatively homogenous. Less than half of the metabolites have statistical outliers, despite the variations in age and growth conditions of the plants (Table 2).

Future examinations of CK levels would ideally focus on plants across their developmental lifespan, as well as organ and even cell-type specific measurements. While some of this is already being done in Arabidopsis (Rybel et al., 2014; Antoniadi et al., 2015; Cucinotta et al., 2018), expanding these types of targeted CK measurements across taxa would likely be illuminating with regards to the function and evolution of different CK compounds.

References

Antoniadi I, Plačková L, Simonovik B, Doležal K, Turnbull C, Ljung K, Novák O (2015) Cell-Type-Specific Cytokinin Distribution within the Arabidopsis Primary Root Apex. Plant Cell **27**: 1955–1967

Cucinotta M, Manrique S, Cuesta C, Benkova E, Novak O, Colombo L (2018) CUP-SHAPED COTYLEDON1 (CUC1) and CUC2 regulate cytokinin homeostasis to determine ovule number in Arabidopsis. J Exp Bot **69**: 5169–5176

Fox JE, Cornette J, Deleuze G, Dyson W, Giersak C, Niu P, Zapata J, McChesney J (1973) The Formation, Isolation, and Biological Activity of a Cytokinin 7-Glucoside. Plant Physiol **52**: 627–632

Hallmark HT, Černý M, Brzobohatý B, Rashotte AM (2020) trans-Zeatin-N-glucosides have biological activity in Arabidopsis thaliana. PLOS ONE **15**: e0232762

Hirose N, Takei K, Kuroha T, Kamada-Nobusada T, Hayashi H, Sakakibara H (2008) Regulation of cytokinin biosynthesis, compartmentalization and translocation. J Exp Bot **59**: 75–83

Hou B, Lim E-K, Higgins GS, Bowles DJ (2004) N-Glucosylation of Cytokinins by Glycosyltransferases of Arabidopsis thaliana. J Biol Chem **279**: 47822–47832

Kakimoto T (2001) Identification of Plant Cytokinin Biosynthetic Enzymes as Dimethylallyl Diphosphate:ATP/ADP Isopentenyltransferases. Plant Cell Physiol **42**: 677–685

Kasahara H, Takei K, Ueda N, Hishiyama S, Yamaya T, Kamiya Y, Yamaguchi S, Sakakibara H (2004) Distinct Isoprenoid Origins of cis- and trans-Zeatin Biosyntheses in Arabidopsis. J Biol Chem **279**: 14049–14054

Kiba T, Takebayashi Y, Kojima M, Sakakibara H (2019) Sugar-induced de novo cytokinin biosynthesis contributes to Arabidopsis growth under elevated CO 2. Sci Rep **9**: 1–15

Kiba T, Takei K, Kojima M, Sakakibara H (2013) Side-Chain Modification of Cytokinins Controls Shoot Growth in Arabidopsis. Dev Cell **27**: 452–461

Kieber JJ, Schaller GE (2014) Cytokinins. Arab Book Am Soc Plant Biol. doi: 10.1199/tab.0168

Ko D, Kang J, Kiba T, Park J, Kojima M, Do J, Kim KY, Kwon M, Endler A, Song W-Y, et al (2014) Arabidopsis ABCG14 is essential for the root-to-shoot translocation of cytokinin. Proc Natl Acad Sci **111**: 7150–7155

Kudo T, Kiba T, Sakakibara H (2010) Metabolism and Long-distance Translocation of Cytokinins. J Integr Plant Biol **52**: 53–60

Letham DS, Palni LMS, Tao G-Q, Gollnow BI, Bates CM (1983) Regulators of cell division in plant tissues XXIX. The activities of cytokinin glucosides and alanine conjugates in cytokinin bioassays. J Plant Growth Regul **2**: 103–115

Li Y, Wang B, Dong R, Hou B (2015) AtUGT76C2, an Arabidopsis cytokinin glycosyltransferase is involved in drought stress adaptation. Plant Sci **236**: 157–167

Miller CO, Skoog F, Von Saltza MH, Strong FM (1955) KINETIN, A CELL DIVISION FACTOR FROM DEOXYRIBONUCLEIC ACID1. J Am Chem Soc **77**: 1392–1392

Miyawaki K, Tarkowski P, Matsumoto-Kitano M, Kato T, Sato S, Tarkowska D, Tabata S, Sandberg G, Kakimoto T (2006) Roles of Arabidopsis ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. Proc Natl Acad Sci **103**: 16598–16603

Mok DW, Mok and MC (2001) Cytokinin Metabolism and Action. Annu Rev Plant Physiol Plant Mol Biol **52**: 89–118

Nam Y-J, Tran L-SP, Kojima M, Sakakibara H, Nishiyama R, Shin R (2012) Regulatory Roles of Cytokinins and Cytokinin Signaling in Response to Potassium Deficiency in Arabidopsis. PLOS ONE **7**: e47797

Nishiyama R, Watanabe Y, Fujita Y, Le DT, Kojima M, Werner T, Vankova R, Yamaguchi-Shinozaki K, Shinozaki K, Kakimoto T, et al (2011) Analysis of Cytokinin Mutants and Regulation of Cytokinin Metabolic Genes Reveals Important Regulatory Roles of Cytokinins in Drought, Salt and Abscisic Acid Responses, and Abscisic Acid Biosynthesis. Plant Cell **23**: 2169–2183

Novák O, Hauserová E, Amakorová P, Doležal K, Strnad M (2008) Cytokinin profiling in plant tissues using ultra-performance liquid chromatography–electrospray tandem mass spectrometry. Phytochemistry **69**: 2214–2224

Novák O, Tarkowski P, Tarkowská D, Doležal K, Lenobel R, Strnad M (2003) Quantitative analysis of cytokinins in plants by liquid chromatography–single-quadrupole mass spectrometry. Anal Chim Acta **480**: 207–218

Romanov GA, Lomin SN, Schmülling T (2006) Biochemical characteristics and ligand-binding properties of Arabidopsis cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. J Exp Bot **57**: 4051–4058

Rybel BD, Adibi M, Breda AS, Wendrich JR, Smit ME, Novák O, Yamaguchi N, Yoshida S, Isterdael GV, Palovaara J, et al (2014) Integration of growth and patterning during vascular tissue formation in Arabidopsis. Science. doi: 10.1126/science.1255215

Sakakibara H (2006) CYTOKININS: Activity, Biosynthesis, and Translocation. Annu Rev Plant Biol **57**: 431–449

Sakakibara H, Kasahara H, Ueda N, Kojima M, Takei K, Hishiyama S, Asami T, Okada K, Kamiya Y, Yamaya T, et al (2005) Agrobacterium tumefaciens increases cytokinin production in plastids by modifying the biosynthetic pathway in the host plant. Proc Natl Acad Sci **102**: 9972–9977

Šimura J, Antoniadi I, Široká J, Tarkowská D, Strnad M, Ljung K, Novák O (2018) Plant Hormonomics: Multiple Phytohormone Profiling by Targeted Metabolomics. Plant Physiol **177**: 476–489

Spíchal L, Rakova NY, Riefler M, Mizuno T, Romanov GA, Strnad M, Schmülling T (2004) Two Cytokinin Receptors of Arabidopsis thaliana, CRE1/AHK4 and AHK3, Differ in their Ligand Specificity in a Bacterial Assay. Plant Cell Physiol **45**: 1299–1305

Svačinová J, Novák O, Plačková L, Lenobel R, Holík J, Strnad M, Doležal K (2012) A new approach for cytokinin isolation from Arabidopsis tissues using miniaturized purification: pipette tip solid-phase extraction. Plant Methods **8**: 17

Takei K, Sakakibara H, Sugiyama T (2001) Identification of Genes Encoding Adenylate Isopentenyltransferase, a Cytokinin Biosynthesis Enzyme, in Arabidopsis thaliana. J Biol Chem **276**: 26405–26410

Takei K, Yamaya T, Sakakibara H (2004) Arabidopsis CYP735A1 and CYP735A2 Encode Cytokinin Hydroxylases That Catalyze the Biosynthesis of trans-Zeatin. J Biol Chem **279**: 41866–41872

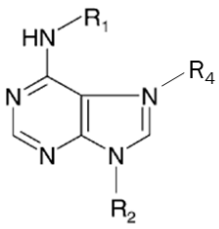
Vylíčilová H, Bryksová M, Matušková V, Doležal K, Plíhalová L, Strnad M (2020) Naturally Occurring and Artificial N9-Cytokinin Conjugates: From Synthesis to Biological Activity and Back. Biomolecules **10**: 832

Wang J, Ma X-M, Kojima M, Sakakibara H, Hou B-K (2011) N-Glucosyltransferase UGT76C2 is Involved in Cytokinin Homeostasis and Cytokinin Response in Arabidopsis thaliana. Plant Cell Physiol **52**: 2200–2213

Wang J, Ma X-M, Kojima M, Sakakibara H, Hou B-K (2013) Glucosyltransferase UGT76C1 finely modulates cytokinin responses via cytokinin N-glucosylation in Arabidopsis thaliana. Plant Physiol Biochem **65**: 9–16

Yoshida R, Oritani T (1972) Cytokinin glucoside in roots of the rice plant. Plant Cell Physiol **13**: 337–343

Zhang K, Novak O, Wei Z, Gou M, Zhang X, Yu Y, Yang H, Cai Y, Strnad M, Liu C-J (2014) Arabidopsis ABCG14 protein controls the acropetal translocation of root-synthesized cytokinins. Nat Commun. doi: 10.1038/ncomms4274



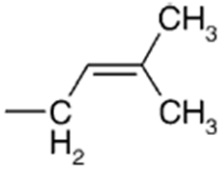
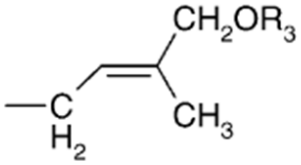
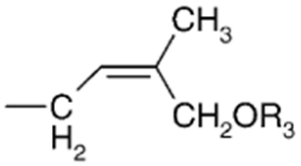
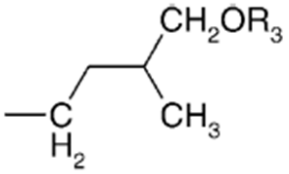
R_1	R_2	R_3	R_4	Compound	Abbreviation
	H	-	-	*N*^{6}-isopentenyladenine	iP
	R	-	-	*N*^{6}-isopentenyladenosine	iPR
	H	-	G	*N*^{6}-isopentenyladenine-7-glucoside	iP7G
	G	-	-	*N*^{6}-isopentenyladenine-9-glucoside	iP9G
	RP	-	-	*N*^{6}-isopentenyladenine-5′-monophosphate	iPRP
	H	H	-	*trans*-Zeatin	tZ
	R	H	-	*trans*-Zeatin riboside	tZR
	H	G	-	*trans*-Zeatin-*O*-glucoside	tZOG
	H	H	G	*trans*-Zeatin-7-glucoside	tZ7G
	G	H	-	*trans*-Zeatin-9-glucoside	tZ9G
	RP	H	-	*trans*-Zeatin-5′-monophosphate	tZRP
	H	H	-	*cis*-Zeatin	cZ
	R	H	-	*cis*-Zeatin riboside	cZR
	H	G	-	*cis*-Zeatin-*O*-glucoside	cZOG
	G	H	-	*cis*-Zeatin-9-glucoside	cZ9G
	RP	H	-	*cis*-Zeatin-5′-monophosphate	cZRP
	H	H	-	Dihydrozeatin	DZ
	R	H	-	Dihydrozeatin riboside	DZR
	H	G	-	Dihydrozeatin-*O*-glucoside	DZOG
	H	H	G	Dihydrozeatin-7-glucoside	DZ7G
	G	H	-	Dihydrozeatin-9-glucoside	DZ9G
	RP	H	-	Dihydrozeatin-5′-monophosphate	DZRP

Table 1. Adapted table from Novak, et al., 2003, showing structures, names, and abbreviations of CKs included in the meta-analysis. Shown above the table is a skeleton cytokinin structure. R_1 is the *N*6 substituted side chain. R_2 represents a group attached to *N*9. R_3 represents a group attached to the hydroxyl group of the side chain. R_4 represents a group attached to *N*7. H:

Hydrogen, R: β-D-ribofuranosyl, G: β-D-glucopyranosyl, RP: β-D-ribofuranosyl-5’-monophosphate.

CK Measurement Datasets Used in Meta-analysis		
Reference	*Plant age*	*Plant Growth Conditions*
(Šimura et al., 2018)	14 days	MS agar plates, 16h-light/8h-dark
(Zhang et al., 2014)	10 days	Half-strength MS agar plates, 16h-light/8h-dark
(Svačinová et al., 2012)	10 days	MS agar plates, 16h-light/8h-dark
(Sakakibara et al., 2005)	18 days	MS agar plates, 16h-light/8h-dark
(Nishiyama et al., 2011)	10 days	GM agar plates, 16h-light/8h-dark
(Wang et al., 2011)	14 days	MS agar plates, 16h-light/8h-dark
(Nam et al., 2012)	11 days	LSM agarose plates, 16h-light/8h-dark
(Wang et al., 2013)	14 days	MS agar plates, 16h-light/8h-dark
(Kiba et al., 2013)	18 days	MGRL agar plates, 16h-light/8h-dark
(Ko et al., 2014)	14 days	MGRL agar plates, 16h-light/8h-dark
(Kiba et al., 2019)	12 days	Half-strength MS agar plates, 12h-light/12h-dark
(Keshishian et al., in revision)	10 days	Soil grown, 16h-light/8h-dark

Table 2. List of studies used in the meta-analysis. All plants were *Arabidopsis thaliana* Col-0 ecotype. Age and growth conditions are indicated.

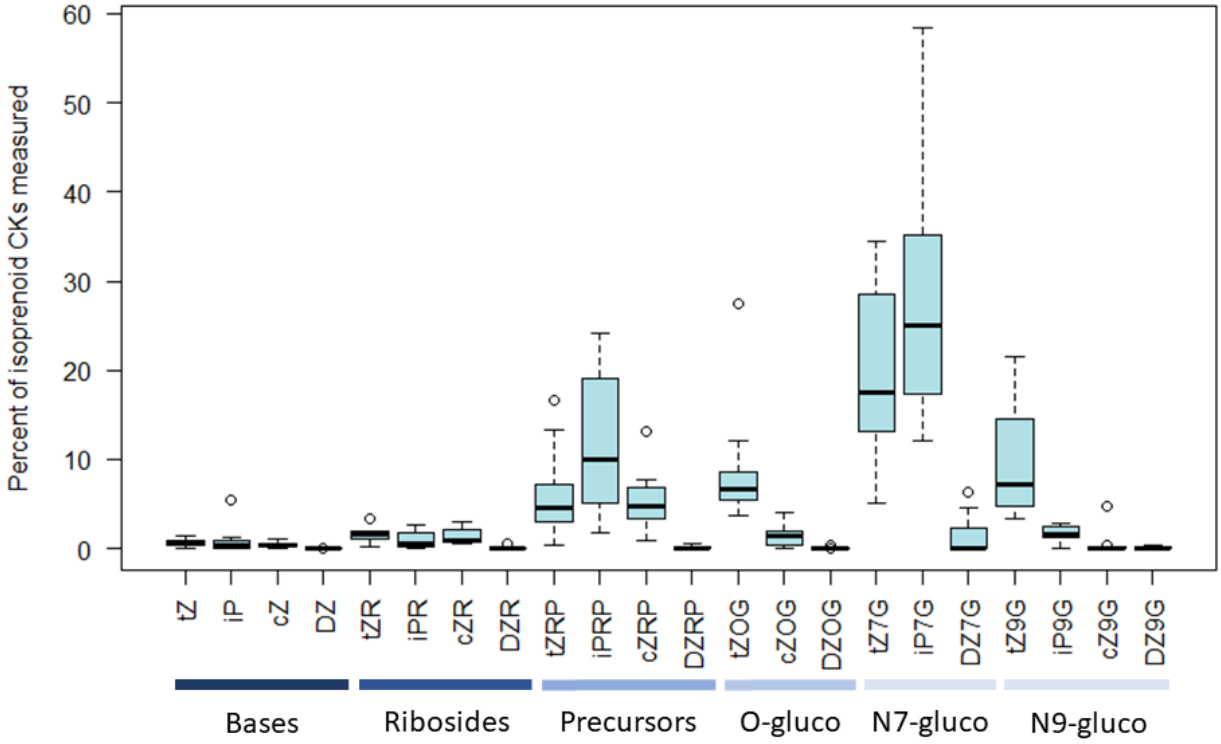


Figure 1. Analysis of twelve CK measurement datasets reveals CKNGs are the dominant forms of CK in *Arabidopsis thaliana* plants between ten and 18 days old. Least abundant group are the CK bases, followed by ribosides. The most abundant group are the CKNGs, with N^7-glucosides being more concentrated than N^9-glucosides. Dark bar represents median, blue rectangle represents interquartile range, dashed lines represent minimum and maximum, open circles represent outliers.

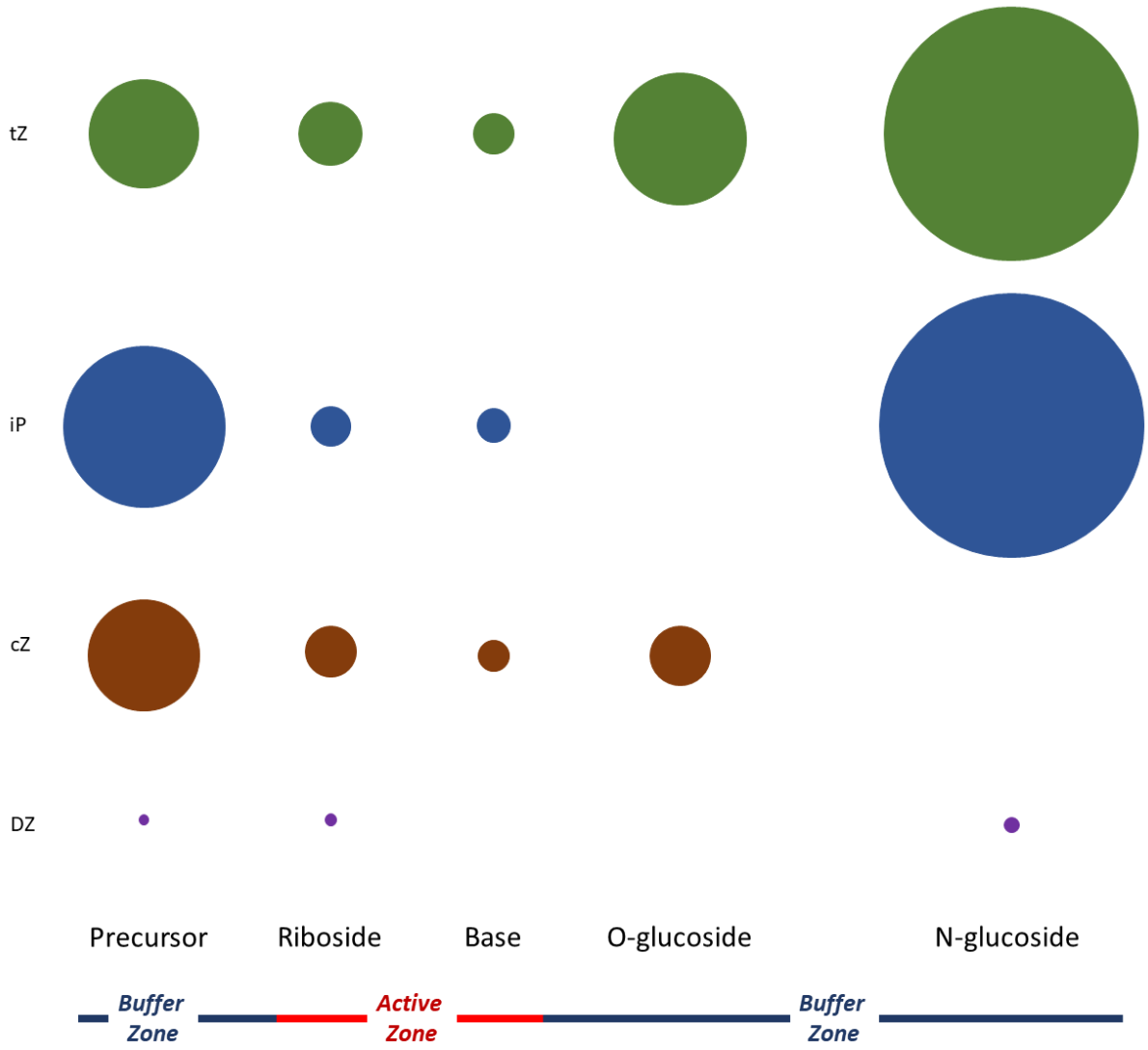


Figure 2. Proposed model of potential roles of precursors and CKNGs in regulating active CK levels. The area of each circle is proportional to its median abundance across the twelve studies analyzed. Both bases and ribosides are included in the "Active Zone" while precursors and glucosides make up the "Buffer Zone." Green, blue, brown, and purple circles represent tZ-, iP-, cZ-, and DZ-type CKs, respectively.