Cytokinin levels and signaling respond to wounding and the perception of herbivore elicitors in *Nicotiana attenuata*

Martin Schäfer1*, Ivan D. Meza-Canales1, Aura Navarro-Quezada1, Christoph Brütting1, Radomira Vanková2, Ian T. Baldwin1 and Stefan Meldau1,3*

1Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, 07745, Jena, Germany, 2Laboratory of Hormonal Regulations in Plants, Institute of Experimental Botany AS CR, 165 02 Prague 6-Lysolaje, Czech Republic, 3German Centre for Integrative Biodiversity Research (iDiv), 04107, Leipzig, Germany. *Correspondences: stefan.meldau@kws.com, mschaefer@ice.mpg.de

Abstract  Nearly half a century ago insect herbivores were found to induce the formation of green islands by manipulating cytokinin (CK) levels. However, the response of the CK pathway to attack by chewing insect herbivores remains unclear. Here, we characterize the CK pathway of *Nicotiana attenuata* (Torr. ex S. Wats.) and its response to wounding and perception of herbivore-associated molecular patterns (HAMPs). We identified 44 genes involved in CK biosynthesis, inactivation, degradation, and signaling. Leaf wounding rapidly induced transcriptional changes in multiple genes throughout the pathway, as well as in the levels of CKs, including isopentenyladenosine and cis-zearin riboside; perception of HAMPs present in the oral secretions (OS) of the specialist herbivore *Manduca sexta* amplified these responses. The jasmonate pathway, which triggers many herbivore-induced processes, was not required for these HAMP-triggered changes, but rather suppressed the CK responses. Interestingly CK pathway changes were observed also in systemic leaves in response to wounding and OS application indicating a role of CKs in mediating long distance systemic processes in response to herbivory. Since wounding and grasshopper OS elicited similar accumulations of CKs in *Arabidopsis thaliana* L., we propose that CKs are integral components of wounding and HAMP-triggered responses in many plant species.

Keywords: *Arabidopsis thaliana*; cytokinins; herbivore-associated molecular patterns; herbivory; insect; jasmonic acid; *Manduca sexta*; *Nicotiana attenuata*; wounding

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INTRODUCTION

Plant survival in nature strongly relies on the ability of plants to adjust their physiology to maximize their fitness in environments that frequently change. The detection of specific herbivore-derived cues, the so-called herbivory-associated molecular patterns (HAMPs), allows plants to distinguish herbivore attack from wounding and often leads to the activation of herbivore-specific responses (Erb et al. 2012). HAMP perception is known to occur in many plants, including maize (*Zea mays*), thale cress (*Arabidopsis thaliana*), soybean (*Glycine max*), and wild tobacco (*Nicotiana attenuata*) (Erb et al. 2012).

*Nicotiana attenuata* is an ecological model organism for analyzing plant responses to herbivory. The interaction with the lepidopteran herbivore *Manduca sexta*, a main defoliator in the plant’s natural environment, has been intensively studied. *N. attenuata* specifically responds to fatty acid-amino acid conjugates (FACs), which are major HAMPs present in *M. sexta* oral secretions (OS; Bonaventure et al. 2011). Analysis of FAC-triggered responses has provided important insights into HAMP recognition and signaling, as well as into the diverse defense and tolerance strategies that plants use against herbivore attack (Bonaventure et al. 2011). FAC perception in *N. attenuata* triggers the biosynthesis of oxylipins, including jasmonic acid (JA) and the JA-isoleucine conjugate (JA-Ile; Kallenbach et al. 2010). Oxylipins play a central role in the regulation of most anti-herbivore defenses in plants (De Geyter et al. 2012). JA-Ile, the active jasmonate, is perceived by the ubiquitin-E3 ligase complex protein CORONATINE INSENSITIVE 1 (COI1), leading to the degradation of JASMONATE-ZIM-DOMAIN (JAZ) proteins, which are negative transcriptional regulators of JA-responsive genes (Chini et al. 2009).

However, the oxylipin sector is not the only hormonal pathway that is involved in the regulation of herbivory-specific responses. Other phytohormones, which respond to wounding and HAMP perception, are ethylene, abscisic acid, or salicylic acid (Erb et al. 2012). In addition to these well-studied defense hormones, the roles of growth-related hormones, such as auxins, brassinosteroids, cytokinins (CKs), and gibberellins are much less understood (Erb et al. 2012). Our lack of knowledge of the role of these hormones in biotic interactions can...
mainly be attributed to difficulties in measuring these low abundant compounds and their common characterization as “growth-related hormones” putting them out of the scope of traditional defense pathway-oriented plant-herbivore interaction research.

It has long been suspected that CKs function in plant-insect interactions. Some insects like leaf miners have been shown to use CKs to modify the tissue surrounding their mines, resulting in the well-described phenomenon of “green islands” (Engelbrecht 1968) or certain sawflies that can induce “leaf galls” (Elzen 1983). In addition to the manipulation of CKs by insect herbivores, an increasing number of studies have provided evidence for an active role of CKs in regulating plant defense responses against herbivores (Giron et al. 2013). Active CKs, such as isopentenyladenine (IP), transisopentenyltransferases (IPTs), whereas the CK nucleoside 5-phosphate or tRNA. This rate limitation of CKs for the release of the free CK bases from the CK nucleotides can be directly converted into free bases by the activity of cytokinin nucleoside 5'-monophosphate phosphoribohydrolases (LOGs) are responsible for the release of the free CK bases from the CK nucleotides (Kurakawa et al. 2007; Kuroha et al. 2009). Active CKs such as isopentenyladenine (IP), trans-zeatin (tZ), cis-zeatin (cZ), and dihydrozeatin (DHZ) and their respective ribosides (~R) can be inactivated by CK oxidase/dehydrogenases (CKX) or by the formation of sugar conjugates like O-glucosides (~OG), riboside O-glucosides (~ROG), N7-glucosides (~7G), and N9-glucosides (~9G) through the activity of glucosyltransferases, like zeatin O-glucosyltransferase or N-glucosyltransferases (ZOG, UGT). CK are perceived by histidine kinases (CHK), which phosphorylate histidine phosphotransfer proteins (HPT). HPTs relay the signal to type-B response regulators (RR), which stimulate transcription of CK-response genes and type-A RRs that in turn mediate the feedback inhibition of the signal. The color code (light gray: CK biosynthesis, medium gray: CK degradation and inactivation, dark gray: CK signaling) is used consistently in all figures.

Figure 1. Cytokinin metabolism and signaling

Cytokinins (CK) biosynthesis is initiated by the activity of isopentenyltransferases (IPT). Monophosphorylated CK nucleotides can be directly converted into free bases by the activity of cytokinin nucleoside 5'-monophosphate phosphoribohydrolases (LOG). Active CKs such as isopentenyladenine (IP), trans-zeatin (tZ), cis-zeatin (cZ), and dihydrozeatin (DHZ) and their respective ribosides (~R) can be inactivated by CK oxidase/dehydrogenases (CKX) or by the formation of sugar conjugates like O-glucosides (~OG), riboside O-glucosides (~ROG), N7-glucosides (~7G), and N9-glucosides (~9G) through the activity of glucosyltransferases, like zeatin O-glucosyltransferase or N-glucosyltransferases (ZOG, UGT). CK are perceived by histidine kinases (CHK), which phosphorylate histidine phosphotransfer proteins (HPT). HPTs relay the signal to type-B response regulators (RR), which stimulate transcription of CK-response genes and type-A RRs that in turn mediate the feedback inhibition of the signal. The color code (light gray: CK biosynthesis, medium gray: CK degradation and inactivation, dark gray: CK signaling) is used consistently in all figures.

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including CKX, CK-glucosyltransferase, CK-receptor, HPT, and RR genes (Brenner et al. 2012; Bhargava et al. 2013).

Here, we used the model plant N. attenuata, from which we identified homologues of known CK-related genes, from 454 transcripts to test: (i) if the CK pathway responds to wounding and HAMPs; (ii) if JAs influence CK levels and signaling during herbivory; and (iii) if the CK pathways is activated in non-treated systemic tissues. Our results demonstrate that CKs function as an integral component of the herbivory-induced signaling cascade.

RESULTS

CK metabolism and signaling genes in N. attenuata

Genes involved in CK biosynthesis and signaling have previously been described in several plant species (Pils and Heyl 2009; Frébort et al. 2011), with in-depth studies in A. thaliana (e.g., Frébort et al. 2011), rice (Oryza sativa; e.g., Tsai et al. 2012), and a moss (Physcomitrella patens; Ishida et al. 2010). Here, we present a comprehensive description and phylogenetic analysis of these genes in a Solanaceous plant. We used 454 transcriptome sequencing data obtained from RNA extractions of various plant tissues to identify the gene homologs in N. attenuata. We used cloned cDNA sequences, as well as homology search to protein sequences in public databases and identified 44 genes involved in CK metabolism and signaling. Given that many annotated genomes are available now in easy to access databases, such as NCBI (www.ncbi.nlm.nih.gov/) or Phytozome (http://www.phytozome.org/), we mined 15 proteomes, including green algae, lower land plants, monocots, and dicots and compared the results about gene number expansion and reduction to previous studies (e.g., Pils and Heyl 2009; Tsai et al. 2012). Our analysis also includes the CK biosynthesis genes, which were often excluded from previous phylogenetic studies of the CK pathway. An overview of the investigated gene families is shown in Figure 1; their phylogenetic relationships are shown in Figures S1B–S8B.

Comparisons of gene numbers among different plants revealed that genes involved in CK biosynthesis (IPT, LOC), inactivation (ZOG), and signaling output (RR) were more susceptible to rapid intra-species gene gains than were the CK perception (CHK) and signal transfer proteins (HPT), which represent the core elements of the CK phosphorylase. The genes involved in CK degradation (CKX) also belong to genes with more restrained intra-species duplication (Figures S1B–S8B). Our data-mining effort in green plants also shows that the genes encoding for the CK inactivation and degradation enzymes ZOG and CKX appear for the first time in land plants.

Regulation of CK-related genes by wounding and simulated herbivory

To investigate if these genes are transcriptionally regulated by wounding and perception of M. sexta-derived cues, we mined our recently established herbivory-regulated microarray dataset (Onkokesung et al. 2012). In this microarray experiment, time kinetics of labeled copy RNA probes from leaves 1, 5, and 17 h after treatment with wounding and water (W + W) or 0.5, 1, 5, 9, 13, 17, and 21 h after treatment with wounding and the immediate application of M. sexta OS to the puncture wounds (W + OS), as well as from untreated control leaves were hybridized to a N. attenuata-specific Agilent microarray platform (GEO microarray repository, GPL13527). Since W + OS treatments mimic plant responses to actual M. sexta attack (Hallatschke et al. 2001), these treatments allow for the rigorous discrimination of wound-induced responses (W + W) from those elicited by herbivore perception (W + OS). Figure 2 provides an overview of highly regulated transcripts, whereas the detailed results from all analyzed genes are shown in Figures S1–S8. We independently determined transcript expression of selected genes by quantitative PCR (qPCR) to confirm the microarray results (Figures S9, S10).

Figure 2 shows the transcript levels of genes with high homologies to CK biosynthesis enzymes, such as NaIPT5 and several LOGs (NaLOG1, NaLOG4, and NaLOG5), and also the transcripts of signaling components including the phosphotransfer protein, NaHPT2, and the RR, NaRRA5, to be differentially regulated by W + W and W + OS, when compared to untreated leaf tissues. The putative inactivation and degradation enzymes, NaCKX5, NaZOG1, NaZOG2, and NaZOG3, were particularly induced by W + W and W + OS treatments. While the transcript levels of some genes were quickly regulated within the first hours after single treatments (e.g., NaLOG1, NaLOG4, NaRRA5, NaCKX5, NaZOG1, NaZOG2, and NaZOG3), long-term effects, lasting frequently more than 20 h, were also observed (e.g., NaIPT5, NaLOG5, and NaHPT2). Treatment-dependent up- as well as downregulations of transcripts were observed. In addition to genes such as NaIPT5, whose transcripts responded to wounding irrespective of the presence of OS, other transcripts (e.g., NaRRA5) were highly responsive to OS-derived cues, which highly amplified wound-induced accumulations. Interestingly downregulation of NaIPT5 transcripts and upregulation of NaRRA5 and NaCKX5 transcripts could also be achieved by tZ and cZR application to N. attenuata leaves (Figure S11). Given the rapid and strong regulation of the CK biosynthesis and signaling pathway at the transcriptional level, these data suggest that wounding and OS perception strongly affect the accumulation of CK metabolites.

CK levels are regulated by wounding and simulated herbivory

Another kinetic experiment was performed (lasting 4 h after treatments) to analyze rapid changes in CK metabolites to the W + W and W + OS treatments (Figures 3, S12). The levels of the active CKs, namely IP, IPR, and cZR, as well as the CK inactivation forms, tZROG, cZROG, and tZ7G responded particularly strongly to wounding and OS application (Figure 3). While IP and IPR levels peaked already at 30 min after wounding and OS application and declined afterwards, cZR, tZROG, cZROG, and tZ7G levels had accumulated at 1 h and remained at elevated levels at least for the 4 h duration of the analysis. IP, tZROG, cZROG, and tZ7G levels attained maximum increases of 25%–50%. IPR levels doubled and cZR levels increased fourfold. Similar to the observed transcript changes, OS application to the puncture wounds further elevated some of the wound-induced CK levels compared to wounding alone. OS application increased the wound-induced IPR, tZROG, and cZROG levels by approximately 25% and cZR levels were elevated by 75%, when compared to wounding alone.
These results were consistent with the analysis of the transcripts demonstrating wound- and HAMP perception-specific regulation of CK levels.

Fatty acid-amino acid conjugates are the main HAMPs in the OS of M. sexta (Halitschke et al. 2001). We used synthetic FACs to evaluate if they could account for the inductions of the CK pathway. Figure 4 shows that FACs are sufficient to elevate wound-induced CK levels, as well as NaRRA5 transcript accumulation. Transcript levels of NaRRA5 homologues have been used as marker genes for analyzing CK-dependent responses in several plant species (e.g., Kurakawa et al. 2007; Stolz et al. 2011). Since NaRRA5 was also highly responsive to CK application in N. attenuata (Figure S11), it was used as CK-responsive marker gene in this investigation.

JA affects CK levels and signaling

Many herbivory-induced responses in N. attenuata (and most other plant species) are JA dependent (Halitschke and Baldwin 2003; Paschold et al. 2007; Stitz et al. 2011). We tested if JA also regulates the CK pathway. We analyzed different transgenic lines impaired in JA and JA-Ile biosynthesis and perception and performed JA supplementation experiments (Figure 5). Our results revealed that JAs partially regulate CK levels and signaling in W+OS-treated leaves. Transgenic lines with an impaired JA pathway showed more pronounced NaRRA5 transcript accumulations, while treatments with methyl-JA (MeJA) attenuated NaRRA5 transcript levels (Figure 5C, D). The iraoc line, which is silenced in the allene oxide cyclase (AOC) an early step of the JA biosynthesis and the ircoi1 line, which is deficient in JA signaling, showed mild reductions in IPR and stronger reductions in czR and tzROG levels (Figure 5A). MeJA treatment strongly reduced the herbivory-elicited accumulation of IPR, but significantly increased czR and tended to increase tzROG levels (Figure 5B). The control and wound-induced CK levels and NaRRA5 transcripts were regulated in similar ways by the treatments (Figure 5).

Figure 2. Wounding and herbivory regulate transcript accumulations of cytokinin-related genes

Transcript accumulations were measured in leaves of Nicotiana attenuata at different time points after wounding and application of water (W+W; dashed line; 1, 5, and 17 h) or Manduca sexta oral secretions (W+OS; solid line; 0.5, 1, 5, 9, 13, 17, and 21 h) to the puncture wounds, as well as in untreated control leaves (C; dotted line; 0.5, 1, 5, 9, 13, 17, and 21 h). Data are obtained from kinetic analysis conducted with microarrays. Time and treatment (C and W+OS; T) effects and their interaction (Time*T) were analyzed by univariate ANOVA, except for NaLOG4, NaHPT2, NaRRA5, NaCKX5, and NaZOG2 data which were analyzed by generalized least squares model instead. Asterisks indicate significant differences between W+W and W+OS-treated samples at the same time point (independent samples t-test: *P ≤ 0.05, ***P ≤ 0.001). Error bars are standard errors (n = 3). For overview of transcript accumulation of additional cytokinin-related N. attenuata genes and their phylogeny see Figures S1–S8.
Wounding and simulated herbivory induce systemic CK pathway changes

In addition to local CK pathway changes, we also found wound and herbivory-induced alterations in the abundance of CK-related transcript (Figures 6, S13, S14) and CK levels (Figure 7) in systemic leaves and root tissues. Figure 6 shows some representative changes and additional information can be found in Figures S13 and S14. In addition to the changes in the transcripts of the CK biosynthesis genes like *NaLOG4*, we also observed changes in the transcripts of the CK signaling elements and CK-inactivation/degradation enzymes, including *NaRRA5* and *NaCKX5*, respectively (Figures 6, S13, S14). The levels of cZR slightly increased systemically after wounding, whereas tZR levels decreased (Figure 7).

Wounding and HAMP-mediated CK level changes in *Arabidopsis*

To investigate if HAMP-induced CK levels are also induced in other plants, we wounded leaves of *A. thaliana* and treated them with water or grasshopper OS (OSGH). While *A. thaliana* leaves do not respond to FACs, they do perceive OSGH, which results in an amplification of wound-induced defense responses (Schäfer et al. 2011). Wounding alone increased the IPR levels in leaves by 35%, cZ level by 115%, cZR level by 86%, and cZROG level by 73%, when compared to untreated leaves. OSGH application to puncture wounds significantly elevated the W+OSG treated samples at the same time point (independent samples t-test: *P* ≤ 0.05). Error bars are standard errors (n = 5). For additional cytokinins see Figure S12. FM, fresh mass.
pathway. These results establish CKs as new components of the herbivore response in plants.

The CK pathway responds to wounding and simulated herbivory

More than 90 years ago, Haberlandt (1921) reported that wound-induced hormones trigger cell division. Cell division is well known to be regulated by CKs (Miller et al. 1955) and wound-induced increases in CK activities, which likely promote tissue healing, were reported, for example, for potato and cucumber (Conrad and Kohn 1975; Mitchell and van Staden 1983; Crane and Ross 1986); however, due to limitations in analytical chemistry at the time of these studies, a comprehensive analysis of changes in CK metabolites was not possible. Here, we confirm that wounding rapidly changes the levels of CKs, including active CKs (IP, IPR, and cZR) and inactivation products (tZROG, cZROG, and tZ7G) (Figure 3).

Interestingly, OS elicitation specifically amplified many of the wound-induced increases. Changes in CK levels were accompanied by a dramatic regulation of many genes involved in CK metabolism and signaling. We found that wounding and FAC application was sufficient to mimic W + OS-induced changes in CK levels and expression of the CK marker gene NaRRA5 (Figure 4), demonstrating that regulation of the CK pathway is one of the earliest HAMP-induced hormone responses in plants.

Connecting specific changes in transcript levels of genes involved in CK metabolism with concomitant changes in CK levels is intricate, since the transcript levels are expected to be influenced by multiple factors, including tissue disruption and FAC perception, CK-mediated feedback regulation (Brenner et al. 2012; Bhargava et al. 2013) and by the interaction with other herbivory induced phytohormones (Erb et al. 2012; El-Showk et al. 2013). In addition, changes in CK levels could also be a result of changes in their transport rates away from or to the specific tissue. Posttranslational regulation might also play an important role, as reported for the CK-dependent increase in CKX activity (Motyka et al. 1996). Increased levels of NaRRA5 (Figure 2) are likely related with the rapid changes in the active CKs IP, IPR, and cZR (Figures 3, S1). Additionally, we assume that the rapid increases in some LOG transcripts (Figures 2, S1) might play a role in elevating CK levels and sustaining them by counteracting CK inactivation processes, which are indicated by the elevation in CK-glucoside levels (Figure 3). Based on the literature (Brenner et al. 2012; Bhargava et al. 2013) and the timing of increases in ZOG and CKX transcript accumulation (Figures 2, S7, S8), these genes could be also induced by the observed increase in IP, IPR, and cZR (Figure 3) levels. That CKs themselves are mediating some of the observed transcript changes is supported by Figure S11, showing similar changes in NaIPT5, NaRRA5, and NaCKX9 transcript accumulation after external CK application. Changes in ZOG expression might account for the increase in CK inactivation products (Figure 3). Additionally changes in cytochrome P450 monooxygenases involved in the conversion of IP-type to CZ-type CKs (Takei et al. 2004), the postulated zeatin cis-trans isomerase (Bassil et al. 1993) or β-glucosidases responsible for the release of CKs from CK O-glucosides (Brzobohaty et al. 1993) could play a role, but were not further addressed in this study.

Since CKs have been shown to increase a plant’s resistance to pathogens (Choi et al. 2010; Großkinsky et al. 2011; Argueso...
Figure 5. Continued.
Figure 6. Wounding and herbivory regulate systemic transcript accumulation of cytokinin-related genes
Transcript accumulation was measured in roots and systemic leaves of *N. attenuata* at different time points after wounding and application of water (W+W; dashed line; 1, 5 and 17 h) or *M. sexta* oral secretions (W+OS; solid line; 1, 5, 9, 13, 17 and 21 h) to the puncture wounds, as well as in untreated control leaves (C; dotted line; 1, 5, 9, 13, 17 and 21 h). Data are obtained from kinetic analysis conducted with microarrays. Time and treatment (C and W+OS; T) effects and their interaction (Time*T) were analyzed by a generalized least squares model. Asterisks indicate significant differences between W+W and W+OS-treated samples at the same time point (independent samples t-test: *P* ≤ 0.05, **P** ≤ 0.01, ***P** ≤ 0.001). Error bars are standard errors (N=3). For additional transcript information see Figure S13 and S14.

Cytokinin responses after herbivory
Regulation of the CK pathway after herbivory

CKs cross-talk with other hormones, including auxins, gibberellins, abscisic acid, and ethylene (*Naseem et al. 2012; El-Showk et al. 2013*), but aside from a few reports about CK-mediated effects on JAs (*Sano et al. 1996; Dervinis et al. 2010*), very little is known about the JA-CK interaction. JAs are widely accepted as one of the main regulators of wound- and HAMP-induced responses (*Erb et al. 2012*). We found that JA pathway manipulations had an influence on CK levels and signaling; however, a functional JA pathway was not required for the induction of W+W and W+OS-induced CK responses. Instead of increasing CK responses JA had a suppressive effect on CK signaling after simulated herbivory (*NaRRA5*; Figure S5C, D). The elevated CK signaling response (*NaRRA5*; Figures 5C) in JA pathway-impaired plants is not explained by the levels of the measured active CKs (Figures S5A, S15); therefore in addition to changes in CK metabolism, CK signaling might also be affected, as reported for the JA-interaction with other phytohormones.

Figure 5. Herbivory-induced cytokinin levels and signaling are regulated by jasmonates

(A) Isopentenyladenosine (IPR), cis-zeanin riboside (cZR) and trans-zeatin riboside O-glucoside (tZROG) levels in leaves of *Nicotiana attenuata* 30 min after wounding and application of water (W+W; white bars with blue lines) or *Manduca sexta* oral secretions (W+OS; lime-green bars) to the puncture wounds and in untreated control leaves (C; purple bars). Measurements were performed in leaves of wild-type (WT) plants and RNAi lines silenced in AOC or COI1 expression. (B) IPR, cZR, and tZROG levels in leaves of *N. attenuata* 30 min after W+W (white bars with blue lines) or W+OS treatment (lime-green bars) and in untreated control leaves (C; purple bars). Measurements were performed in leaves of WT plants with and without 24 h methyl-jasmonate (MeJA; 150 µg per leaf) pretreatment. (C) Response regulator 5 (*NaRRA5*) transcript abundance under the conditions mentioned in (A). (D) *NaRRA5* transcript abundance under the conditions mentioned in (B). Line/MeJA and treatment (C, W+W and W+OS; T) effects and their interaction (Line*T and MeJA*T, respectively) were analyzed by univariate ANOVA, except for *NaRRA5* (C) data which were analyzed by a generalized least squares model. Asterisks indicate significant differences between same treatments from RNAi lines and WT plants, and plants with and without a MeJA pretreatment (independent samples t-test: *P* ≤ 0.05, **P** ≤ 0.01, ***P** ≤ 0.001). Error bars are standard errors (A, C n ≥ 4; B, D n ≥ 5). FM, fresh mass.
such as auxin or abscisic acid (Pauwels et al. 2010). JA supplementation resulted in downregulation of the shoot derived active CK IPR, while it promoted the accumulation of cZR (Figure 5A, B), a CK associated with stress responses (Gajdošová et al. 2011). JAs also trigger CK inactivation processes, as indicated by O-glucosylation of cZ. While the JA supplementation experiments (Figure 5B) indicate a negative effect of JA on the IPR accumulation, IPR levels were not increased, but slightly reduced in iraoc and ici1 plants, which are impaired in the JA pathway (Figure 5A). This might be explained by the very low levels of active JA-Ile in the absence of wound or HAMP induction and the chronological order of events after W + OS treatment: that IPR accumulates before JA-Ile in N. attenuata (maximum accumulation: IPR, 30 min after wounding and application of water (W) or grasshopper oral secretions (OSGH) to the puncture wounds, as well as in untreated control leaves (C). Asterisks indicate significant differences between W and OSGH application to wounding sites or between wounding and W application compared to C, as indicated (independent samples t-test: \*P ≤ 0.05, \**P ≤ 0.01, \***P ≤ 0.001). Error bars are standard errors (n ≥ 4). FM, fresh mass. [Correction made on 23 January 2015, after first online publication: An asterisk, which has been erroneously added to Figure 8, has been removed from the figure.]

Figure 7. Wounding and herbivory regulate systemic changes in cytokinin levels
Cis-zeatin riboside (cZR) and trans-zeatin riboside (tZR) levels were measured in roots and systemic leaves of Nicotiana attenuata at different time points after wounding and application of water (W + W; dashed line) or Manduca sexta oral secretions (W + OS; solid line) to the puncture wounds, as well as in untreated control leaves (C; dotted line). Time and treatment (C and W + OS; T) effects and there interaction (Time*T) were analyzed by a generalized least squares model. Asterisks indicate significant differences between W + W and W + OS-treated samples at the same time point (independent samples t-test: \*P ≤ 0.05). Error bars are standard errors (n ≥ 4).

Figure 8. Wounding and herbivory induce changes in cytokinin levels in leaves of Arabidopsis thaliana
Isopentenyladenosine (IPR), cis-zeatin (cZ), cis-zeatin riboside (cZR), and cis-zeatin riboside O-glucoside (cZROG) levels in leaves 30 min after wounding and application of water (W) or grasshopper oral secretions (OSGH) to the puncture wounds, as well as in untreated control leaves (C). Asterisks indicate significant differences between W and OSGH application to wounding sites or between wounding and W application compared to C, as indicated (independent samples t-test: \*P ≤ 0.05, \**P ≤ 0.01, \***P ≤ 0.001). Error bars are standard errors (n ≥ 4). FM, fresh mass.
Cytokinin responses after herbivory

lines and after MeJA treatments. Kinetic measurements are required to identify the critical steps in CK metabolism that are regulated by JAs. Additionally, the effect of multiple elicitations should be investigated since JA accumulation from previous elicitations might influence CK response, thereby representing a potential mechanism to distinguish between early and late herbivory-induced responses or attack frequency-dependent responses. Interestingly JA-mediated effects on the CK pathway were also observed in untreated control leaves (Figure 5B, D) indicating that herbivory is not required for the observed JA-CK interaction.

Cytokinins are long known for their role in inhibiting leaf senescence (Richmond and Lang 1957), for growth promotion of leaf tissue (Miller 1961) and to induce cell division (Miller et al. 1955). JAs were shown to counteract the senescence-inhibiting activities of the CK kinetin, to induce premature senescence (Ueda and Kato 1980; He et al. 2002), suppress leaf growth (Attaran et al. 2014), as well as to repress cell cycle propagation (Noir et al. 2013), which indicates a more general negative effect of JA on the CK pathway activity than our data on herbivory-induced signaling suggests.

The CK pathway in long distance systemic responses

Cytokinins not only play a role in regulating local processes, but were also found as important systemic regulators, for example, for the adjustment of the nitrogen-foraging strategy (Ruffel et al. 2011) and nitrogen-dependent root-shoot signaling (Takei et al. 2001). We observed that W+W and W+OS treatment of leaves resulted in CK pathway changes in other untreated leaves and the roots of the same plants (Figures 6, 7). Therefore, our data indicate Cks as an integral component of the systemic response after herbivore attack. The wound and herbivory-induced transcript changes shown in Figures 6, S13, and S14 indicate that the CK-biosynthesis and the CK-inactivation/degradation could be affected systemically after W+W and W+OS treatment, as well as the CK signaling output (NaRRAs). Effects on CK sensitivity or signal transduction might explain why the OS-mediated effects differ between CK-related transcripts and CK metabolites, but it is also possible that the systemic CK response is mainly wound-dependent. Since Cks are known to be specifically transported either by the phloem (IP-type Cks) or the xylem (IZ-type Cks; Kudo et al. 2010) between different plant parts, changed CK transport might also be involved in shaping systemic CK patterns.

With regard to the work of Dervinis et al. (2010), systemic CK level changes might play a role in systemic priming of plant-defense responses after herbivore attack. Additionally, Cks are known to be involved in many abiotic stress responses (Jeon et al. 2010; Nishiyama et al. 2011) and therefore could play a role in integrating these different signals on a whole plant scale.

The CK pathway proteins show diverse evolutionary patterns

The response of the CK pathway to HAMPs, which are only perceived by particular plant species (Erb et al. 2012) raised the question if the genes in the CK pathway of N. attenuata went through a similar evolutionary history as observed in other plant species. To answer this we constructed a phylogeny of the CK pathway and analyzed it in respect to established concepts of the CK pathway evolution.

Evolutionary analyses have previously been used to support CK signaling models and gene function analysis (Gruhn and Heyl 2013). We used our available 454 sequences for data mining and confirmed most of the evolutionary patterns found in previous studies (Pils and Heyl 2009; Tsai et al. 2012). We found that HPTs have notably expanded in Poplar (10 copies) in comparison to the remaining dicot sequences available, where a stable basal number of five copies is found, as described by Pils and Heyl (2009). We corroborated that HPTs, together with CKXs and CHKs have stable numbers across all green plant species analyzed and further observed that each gene has a homolog in monocots and dicots, indicating conserved evolution after duplication of these genes in the flowering plants. Our findings for HPTs contrast with the results from Tsai et al. (2012), which described gene expansion for this gene family. The discrepancy may result from the fact that we analyzed more species than they did. A higher similarity of CK receptors to the homologs in monocot/dicot species had also been shown by Tsai et al. (2012) and Pils and Heyl (2009). Both studies also showed that RRs had expanded after the separation of lower land plants and flowering plants. In addition to the confirmation of results from previous publications, we observed a similar gene expansion pattern in the CK biosynthesis genes (IPTs and LOGs) and in the first step of CK inactivation (ZOGs).

Rapid evolution due to duplication can result in functional diversification. We expect this to occur in RRs, which are reported to play roles as both negative and positive stress regulators (Wohlbach et al. 2008). Interestingly, gene number expansion also appears to play a role in CK deactivation enzymes, such as the glucosyltransferases that do not have homologs in green algae and lower land plants, which indicates that these proteins duplicated after their emergence in flowering plants, allowing them to develop specialized functions. A higher specificity in N-glucosylation enzymes had already been described by Sakakibara (2006). In the case of IPTs, evidence for functional diversification also exists (Miyawaki et al. 2006), which might be partly explained by maintaining different gene copies of these genes in the genome. Relaxed selection might also result in amplification of gene numbers of one family due to a process called genomic drift (Nei 2007), which occurs if gene dosage effects can be neglected. We cannot rule out relaxed selection in case of LOGs, although this family most probably is undergoing changes in gene birth-and-death dynamics, as has been shown for similar enzymes in A. thaliana (Kuroha et al. 2009). From this analysis, we confirm that N. attenuata’s CK pathway genes have evolved in similar ways to homologous genes in other species.

CKs are also regulated by herbivore perception in Arabidopsis

From the gene phylogenetic analysis we observe that among other important model plants for the analysis of plant defense against herbivores, A. thaliana, has similar evolutionary patterns for CK biosynthesis and regulation genes. Therefore, an analysis of the CK response in this plant is interesting, especially as it was previously shown to be unresponsive to FACs, but to respond instead to lipases and other unknown elicitors present in OSGH (Schmelz et al. 2009; Schäfer et al. 2011). We found that A. thaliana showed similar qualitative
changes in CK levels when treated with OS$_{GH}$ as observed for N. attenuata treated with OS from M. sexta (Figure 8). The elevation of IPR and c2R in W + OS$_{GH}$-treated leaves, as compared to those elicited by wounding alone, was particularly noteworthy. This indicates that the patterns of HAMP-induced CK changes are likely conserved among different plant families. A broader analysis of herbivore-induced CK responses in plants from additional plant families and ecological backgrounds could be useful to understand the conservation patterns of the CK-pathway genes and their potential functional implication.

**MATERIALS AND METHODS**

**Plant material and growth**

The establishment of the stable transformed irao (line number A-07-457) and ircoir (line number A-04-249) plants was described by Kallenbach et al. (2012) and Paschold et al. (2007), respectively. As WT we used plants from an inbred “Utah” line of Nicotiana attenuata (Torr. ex S. Wats.). N. attenuata seed germination and growth under greenhouse conditions was performed as described elsewhere (Krügel et al. 2002). In brief, after 10 d on Gamborg's B5 medium (Sigma-Aldrich, Taufkirchen, Germany, [http://www.sigmaaldrich.com]) with phytagel (Sigma) and 12 d in TEKU JP 3050 104 pots plants were transferred to 1 L pots filled with sand. Plants were kept under greenhouse conditions with 16–28 °C and 80% humidity with 10 h light/day with an intensity of 190 W/m$^2$ per s.

**Leaf treatments**

Standardized wound treatments in N. attenuata were performed by rolling a fabric pattern-wheel three times on each side of the leaf and subsequently applying 20 μL water to the punctured holes ($W + W$). Herbivory was simulated by applying herbivore OS instead of water ($W + OS$) and for FAC treatments, N-linolenoyl-l-glutamate at a concentration similar to 1:5 diluted M. sexta OS was used (27.6 ng/μL; Hettenhausen et al. 2013). These treatments were performed in the morning (09.00–10.00 hours). In contrast to N. attenuata, A. thaliana leaves were treated with only 5 μL water or OS$_{GH}$. After incubation for the indicated time, the leaf tissue was harvested and immediately frozen in liquid nitrogen and stored at −80 °C.

**Manduca sexta and Schistocerca gregaria**

Manduca sexta larvae were obtained from in-house colonies and S. gregaria from Bugs International, Irsingen/Unterfeld, Germany ([http://www.bugs-international.com]). M. sexta larvae were fed on N. attenuata and S. gregaria on A. thaliana before OS/OS$_{GH}$ collection, which was performed according to Turlings et al. (1993) with the modifications of Alborn et al. (2003). OS and OS$_{GH}$ were diluted 1:5 in water before application.

**CK spray application**

For spray application, tZ and c2R were dissolved in 80% (v/v) EtOH (tZ, 1 mg/mL; c2R, 5 mg/mL) and diluted in an aqueous solution of 0.02% (v/v) Tween-20 to 1 μmol/L and 5 μmol/L, respectively. Spray application of tZ, c2R and the corresponding buffer control was done three times per day over 3 d.

**MeJA pretreatment**

MeJA treatment was done according to Baldwin (1996), by applying 20 μL of a 7.5 μg/μL MeJA containing lanolin paste to the adaxial side of the base of a leaf. Lanolin without MeJA was applied as control. MeJA applications were performed 1 d before the start of the experiments.

**Phylogenetic analysis**

Cloned CK biosynthesis and receptor gene sequences from cDNA were confirmed using sequences of N. attenuata 454 sequenced transcripts obtained as described in Case and Baldwin (2013). Confirmed sequences were then used to perform a blastx search to the proteomes of all green plants (Viridiplantae) at NCBI. For illustration purposes, we show proteins present in at least one (ideally all) of the representative species for each phyletic group: green algae (Volvox carteri, Micromonas pusilla, Ostreococcus lucimarinus, Ostreococcus tauri, and Chlamydomonas reinhardtii), lower-lower plants (P. patens, Selaginella mollendorfii), monocots (O. sativa cv. Japonica, Sorghum bicolor, and Z. mays), and dicots (Vitis vinifera, Solanum lycopersicum, A. thaliana, G. max, Ricinus communis, and Populus trichocarpa).

For each protein family, multiple sequence alignments were generated using MUSCLE (Edgar 2004). The phylogenetic relationships were analyzed using the Neighbor-Joining method (Saitou and Nei 1987) as implemented in MEGA5 (Tamura et al. 2011). We used 1000 bootstrap replicates for tree support. Evolutionary distances were computed using the JTT matrix-based method (Jones et al. 1992) with a gamma distribution (shape parameter = 1) for rate site variation. For easier understanding, we display only the topology of the trees. Genes were named according to Heyl et al. (2013).

**Microarray analysis**

Microarray analysis was done as described by Onkokesung et al. (2012). Analysis was done with leaves treated for 1, 5, and 17 h with W + W or 0.5 (only local leaves), 1, 5, 9, 13, 17, and 21 h with W + OS, as well as in untreated control leaves. For hybridization we used a N. attenuata-specific Agilent microarray platform (GEO microarray repository, GPL13527). Microarray data (Figures 2, 51–58) were confirmed by qPCR analysis of representative genes in samples from an independent experiment (Figures 59, 610).

**qPCR analysis**

RNA extraction was performed with TRIzol (Invitrogen, Darmstadt, Germany), according to the manufacturer’s instructions. cDNA was synthesized by reverse transcription...
Cytokinin responses after herbivory

using oligo(dT) primer and RevertAid reverse transcriptase (Invitrogen). qPCR was performed using actin as standard on a Stratagene MX3000P qPCR machine using a SYBR Green containing reaction mix (Eurogentec, Cologne, D, http://www.eurogentec.com); qPCR Core kit for SYBR Green I No ROX). The primer sequences are provided in Table S2.

CK analysis
Cytokinins were extracted from plant tissue with acidified aqueous methanol followed by two solid-phase extraction (SPE) steps. Measurement was done by LC-MS/MS. The method was adapted according to Dobrev and Kami’nek (2002) with the modifications by Kojima et al. (2009) and Schäfer et al. (2013).

In brief, 100 mg frozen plant material was extracted twice with 800 μL MeOH: H2O: HCOOH (15:4:1) at −20 °C. Labeled internal standards were supplemented in the first extraction step. The first SPE step was performed on a Multi 96 HR-X column (96 × 25 mg; Macherey-Nagel, Düren, Germany, http://www.mn-net.com) conditioned with extraction buffer. The methanol in the column eluent was evaporated and after replenishment with 850 μL 1 N HCOOH a second SPE step was performed using a Multi 96 HR-XC column (96 × 25 mg; Macherey-Nagel) conditioned with 1 N HCOOH. After several washing steps (consecutively 1 mL 1 N HCOOH, 1 mL MeOH, and 1 mL 0.35 M NH4OH) the CK-ribosides, free bases, and glucosides were eluted with 1 mL 0.35 M NH4OH in 60% (v/v) MeOH. After evaporation, samples were reconstituted in 50 μL 0.1% (v/v) acetic acid.

Extraction was performed in 96-well BioTubes (1.1 mL individual tubes, Arctic White LLC, Bethlehem, PA, USA, http://www.arcticwhitesusa.com/) and Nunc 96-Well Deep Well Plates (Thermo Scientific, Waltham, MA USA, https://www.thermoscience.com), evaporation under constant nitrogen flow in an Evaporator system (Glas-Col, Terre Haute, IN, USA, http://www.glascol.com) and SPE using a Chromabond Multi 96 vacuum chamber (Macherey).

Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA, http://www.home.agilent.com/). For separation a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 μm, Agilent Technologies) was used. The mobile phase comprised solvent A (water, 0.05% (v/v) HCOOH) and solvent B (acetonitrile) with the elution profile: 0–0.5 min, 95% A; 0.5–5 min, 5%–31.5% B in A; 5.01–6.5 min 100% B and 6.51–9.9 min 95% A, with a flow rate of 1.1 mL/min. The column temperature was maintained at 25 °C. The liquid chromatography was coupled to an API 5000 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany, http://www.invitrogen.com/site/us/en/home/brands/Applied-Biosystems.html?CID=fl-AppliedBiosystems) equipped with a Turbospray ion source. For detection, the mass spectrometer was operated in positive ionization mode multi-reaction-monitoring modus to monitor analyte parent ion → product ion (Table S3). Settings were as follows: ion spray voltage, 5,500 eV; turbo gas temperature, 700 °C; nebulizing gas, 70 psi; curtain gas, 25 psi; heating gas, 60 psi; and collision gas, 6 psi. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. tZ, tZR, tZROG, tZG, cZ, cZR, cZROG, cZGc, DHZ, DHZR, IP, and IPR were quantified by using deuterated internal standards (Table S3; Olchemim, Olomouc, CZ, http://www.olchemim.cz/).

Chemicals
ETOH and MeOH were purchased by Merck, acetonitrile by VWR (Darmstadt, D, http://www.vwr.com), lanolin, Tween-20, as well as MeJA by Sigma–Aldrich and HCOOH for chromatography by Fisher Scientific (Schwerte, D, http://www.de.fishersci.com/de/), otherwise by Riedel-de Haen (Seelze, D, http://www.reideldehaen.de/), acetic acid by Carl Roth (Karlsruhe, D, http://www.carlo Roth.com) and CK standards by Olchemim.

Statistical analysis
Data were analyzed by SPSS Statistics 17.0 (IBM, Ehningen, D, http://www.spss.de) with independent (unpaired) samples t-test and univariate ANOVA. If homoscedasticity could not be achieved by transformation, datasets were analyzed by a generalized least squares (GLS) model using R 3.0.1 (http://www.r-project.org). The used statistical analysis methods are indicated in the figure legends. In Figures 3 and S12, the 0 h control was also used for the 15 and 30 min time point comparisons. In Figures 2, S1–S8, S10, S13, and S14, the W + W treatment was excluded from the analysis by univariate ANOVA and GLS model. The number of biological replicates per experiment is indicated in the figure legends. The presented data are supported by at least two independent experiments with similar results.

Accession numbers
The data from the Transcriptome Shotgun Assembly project have been deposited at DDBJ/EMBL/GenBank under the accession GBGF00000000. The version described in this paper is the first version, GBGF01000000.

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**SUPPORTING INFORMATION**

Additional supporting information can be found in the online version of this article:

**Figure S1.** Wounding and herbivory regulate the expression of LOG-family genes

**Figure S2.** Wounding and herbivory regulate the expression of IPT-family genes

**Figure S3.** Wounding and herbivory regulate the expression of cytokinin receptor genes

**Figure S4.** Wounding and herbivory regulate the expression of cytokinin metabolism in systemic leaves

**Figure S5.** Wounding and herbivory regulate the expression of type-A RR-family genes

**Figure S6.** Wounding and herbivory regulate the expression of type-B RR-family genes

**Figure S7.** Wounding and herbivory regulate the expression of CKX-family genes

**Figure S8.** Wounding and herbivory regulate the expression of ZOG-family genes

**Figure S9.** Confirmation of microarray expression data

**Figure S10.** Confirmation of NaPT5 microarray data

**Figure S11.** Cytokinin-spraying changes NaIPT5, NaRRA5, and NaCKX5 transcript accumulation

**Figure S12.** Wounding and herbivory-induced changes in cytokinin levels

**Figure S13.** Wounding and herbivory regulate transcript accumulation of cytokinin-related genes in the root

**Figure S14.** Wounding and herbivory regulate transcript accumulation of cytokinin-related genes in systemic leaves

**Figure S15.** Trans-zeatin riboside levels are reduced in jasmonic acid pathway impaired transgenic plants

**Table S1.** Abbreviations

**Table S2.** Sequences of primers used for qPCR

**Table S3.** Multi-reaction-monitoring settings for cytokinin quantification in positive ionization mode