New Phytologist

Research

Cytokinin concentrations and CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2)- and NaCHK3-mediated perception modulate herbivory-induced defense signaling and defenses in *Nicotiana attenuata*

Martin Schäfer[1], Ivan D. Meza-Canales[1], Christoph Brütting[1], Ian T. Baldwin[1] and Stefan Meldau[1,2,3]

[1]Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, Hans Knöll Str. 8, Jena 07745, Germany; [2]German Centre for integrative Biodiversity Research (iDiv), Deutscher Platz 5, Leipzig 04107, Germany; [3]KWS SAAT AG, Grimsehlstraße 31, Einbeck 37574, Germany

Author for correspondence:
Stefan Meldau
Tel: +49 0 5561 311 1391
Email: stefan.meldau@kws.com

Received: *19 August 2014*
Accepted: *11 March 2015*

New Phytologist (2015)
doi: 10.1111/nph.13404

Key words: cytokinin, herbivory, histidine kinase, isopentenyltransferase, jasmonic acid, *Manduca sexta*, *Nicotiana attenuata*, phenolamide.

Summary

• Herbivore attack elicits changes in cytokinins (CKs), but how these changes influence defense signaling remains poorly described. We investigated the influence of the CK pathway on the well-described inducible defense pathways of *Nicotiana attenuata* in response to wounding with and without elicitors from the specialist herbivore *Manduca sexta*.

• CK pathway manipulation often suffers from substantial side effects on plant growth and development. We therefore used multiple manipulation tools including spray application of CKs, chemically-inducible expression of the CK biosynthesis enzyme isopentenyltransferase, and transient and constitutive RNAi-mediated gene silencing of CK receptors to resolve the function of CKs in plant defense.

• The results demonstrated that CK concentrations in leaves and perception through CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2) and NaCHK3 were important for the accumulation of jasmonic acid (JA) and phenolamides and proteinase inhibitor activity. By contrast, the CK pathway did not promote the accumulation of the active JA-isoleucine conjugate and negatively regulated the release of specific green leaf volatile esters. Interestingly, CK signaling also promotes the systemic phenolamide accumulation.

• We conclude that the CK pathway is an important regulator of herbivory-inducible defense signaling and chemistry, which expands its reported participation in adjusting a plant's physiology to abiotic and biotic stress responses.

Introduction

In nature, plants must cope with a plethora of environmental factors, including abiotic conditions, but also biotic attackers, such as phytophagous insects. Research in the last few decades has steadily increased the number of small molecules, including various classes of phytohormones, that have been revealed to be involved in plant–herbivore interactions (Erb *et al.*, 2012). Classical plant growth regulators such as cytokinins (CKs) were reported to participate in plant–insect interactions. The most prominent examples are leaf mining insects and sawflies. The former have been shown to use CKs to modify the tissue surrounding their mines, resulting in the well-described phenomena of 'green islands' (Engelbrecht *et al.*, 1969), and the latter have been shown to induce abnormal growth leading to the formation of so-called 'leaf galls' (Elzen, 1983). However, in-depth investigations of the roles played by CKs in plant–herbivore signaling and the defenses they mediate are rare. This data deficit is probably related to the strong influence that CKs have on growth processes. Plant development itself is known to affect many processes involved in plant–herbivore interactions, including the induction of defense signaling (Diezel *et al.*, 2011), the distribution of defense chemicals (McKey, 1974) and nutritional value (Karley *et al.*, 2002). This makes it difficult to interpret the results from studies of the influence of CKs on plant defenses that are based on plants with altered patterns of development. Here, we used different CK pathway manipulation techniques to circumvent these problems and rigorously address the question of how CKs interact with the herbivory-induced defense signaling and the subsequently induced metabolic changes (Fig. 1).

It was proposed that insects and their associated microorganisms can hijack the CK pathway to gain control of CK-regulated processes, such as senescence inhibition, nutrient regulation and plant growth, to optimize the plant tissue according to the needs of the insects (reviewed in Giron *et al.*, 2013). It was also shown that elevated CK concentrations in *Nicotiana attenuata* leaves increase the damage inflicted by the mirid bug *Tupiocoris notatus* (Schäfer *et al.*, 2013). In contrast to the studies that suggest that CKs are manipulated presumably for the herbivore's fitness benefit, an increasing number of studies also propose an active,

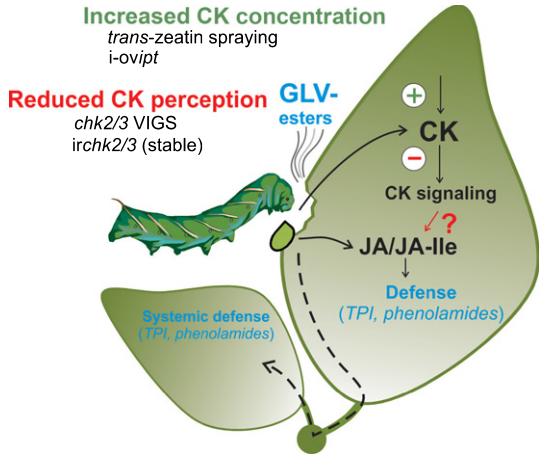

Fig. 1 Experimental logic used to analyze cytokinin (CK)-mediated effects on the herbivory-inducible defense response. Herbivore-derived elicitors in the oral secretions of *Manduca sexta* are well known to induce rapid increases in the concentrations of jasmonic acid (JA) and the active JA–isoleucine conjugate (JA-Ile) in *Nicotiana attenuata*, which elicit increases in direct defenses, for example the activities of phenolamides and trypsin proteinase inhibitors (TPIs). Indirect defenses, such as green leaf volatile (GLV) esters, are also triggered. Recently, herbivore attack has also been found to elevate CK concentrations. Here, we investigated the influence of the CK pathway on herbivory-induced defense responses by manipulating CK concentrations and signaling. To manipulate CK concentrations, we separately used external *trans*-zeatin application and transgenic plants with chemical-inducible expression of the CK biosynthesis gene isopentenyltransferase (i-ov*ipt*). To manipulate CK perception, we transiently and constitutively silenced the CK receptors CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2) and NaCHK3 by virus-induced gene silencing (*chk2/3* VIGS) and stable transformation (ir*chk2/3*), respectively.

plant-controlled role for CKs in their defense responses against herbivores (Giron *et al.*, 2013). Transcriptional studies in *N. attenuata* identified changes in the CK-related genes *CYTOKININ-INDUCED GENE 2* (*CIG2*) and *CYTOKININ-REGULATED KINASE 1* (*CRK1*) in response to *Manduca sexta* feeding and perception of specific herbivore-associated molecular patterns (HAMPs) from *M. sexta* oral secretions (OS), respectively (Hui *et al.*, 2003; Gilardoni *et al.*, 2010), indicating that the CK pathway might actively respond to herbivory. Recently, these inferences were verified with the demonstration of HAMP-specific transcript level changes of multiple genes involved in CK biosynthesis, degradation and signaling, as well as in the concentrations of CK metabolites themselves (Schäfer *et al.*, 2014a).

CKs were shown to be positive regulators of plant defense against pathogens (Choi *et al.*, 2010; Großkinsky *et al.*, 2011; Argueso *et al.*, 2012). Similarly, increased CK concentrations in a plant can also negatively affect herbivore performance (Smigocki *et al.*, 1993, 2000; Dervinis *et al.*, 2010). As CKs were shown to amplify the accumulation of secondary metabolites in tobacco (*Nicotiana tabacum*) leaves (Hino *et al.*, 1982; Großkinsky *et al.*, 2011) and carrot (*Daucus carota*) suspension cultures (Ozeki & Komamine, 1986), an effect of CKs in also mediating changes in the antiherbivore chemistry of a plant seems likely. Indeed, Smigocki *et al.* (2000) discovered CK-dependent insecticidal activity in surface extracts of *N. tabacum* and *Nicotiana plumbaginifolia* leaves. The activity was proposed to be associated with oxygen-containing aliphatic compounds, presumably diterpenes. Dervinis *et al.* (2010) showed, in accordance with Sano *et al.* (1996), that CKs were positive regulators of wound-induced increases in jasmonic acid (JA) concentrations and additionally that CKs increased the abundance of trypsin proteinase inhibitor (TPI) transcripts after induction. These studies provide a compelling case for CKs playing an active role in the elicitation of plant defense responses, but there are several limitations of the previous work that should be discussed.

One limitation of the previous work is that many results were derived only from experiments using external supplementation of high amounts of CKs for the manipulation (e.g. Dervinis *et al.*, 2010). Other studies increased endogenous CK concentrations by the heterologous expression of the CK biosynthesis enzyme isopentenyltransferase (IPT) driven by a wound-inducible promoter from a potato (*Solanum tuberosum*) TPI gene (Smigocki *et al.*, 1993, 2000; Mujer & Smigocki, 2001), but these suffered from unspecific promoter activity in the absence of herbivore attack, resulting in visually apparent changes in growth and development (Smigocki, 1995).

An additional limitation to the existing evidence is that no investigations have verified the results through down-regulation of the CK pathway and, as a consequence, the involvement of the classical CK signaling pathway remains an open question. Potential targets for CK signaling manipulations are the cyclases/histidine kinases associated sensing extracellular (CHASE)-domain-containing His kinases (CHKs) that are responsible for CK perception and that represent the primary elements of the pathway (Stolz *et al.*, 2011; Gruhn & Heyl, 2013). Many higher plants possess only a small set of CK receptors (*N. attenuata*, three; *Arabidopsis thaliana*, three; *Oryza sativa*, four; Pils & Heyl, 2009; Schäfer *et al.*, 2014a), but the signal specificity is retained, probably through variations in ligand affinities and different expression profiles (Stolz *et al.*, 2011). ARABIDOPSIS HISTIDINE KINASE 2 (AHK2) and AHK3, for example, were reported as predominant CK receptors in the leaf lamina of *A. thaliana* (Stolz *et al.*, 2011), which is concordant with their functions in chlorophyll retention (AHK3) and leaf cell formation (AHK2 and AHK3) (Riefler *et al.*, 2006). The CK-mediated regulation of plant defenses might therefore be mediated by a single CHK or specific CHK combinations.

Another limitation is the lack of mechanistic details about the interaction between CKs and the plant defense responses against herbivores. Smigocki *et al.* (2000), for example, could not identify the specific compound(s) responsible for CK-mediated herbivore resistance, and did not report any signaling events involved. Similarly, Dervinis *et al.* (2010) reported neither CK-mediated effects on active signaling compounds of plant defense, such as the JA-isoleucine conjugate (JA-Ile), nor activity measurements for the defense compound TPI. Additionally, the interaction between CKs and HAMP-induced responses was not further characterized.

In *N. attenuata*, wounding and perception of fatty acid–amino acid conjugates, which are specific HAMPs derived from *M. sexta* OS, rapidly induce the accumulation of JA and JA-Ile

(Kallenbach *et al.*, 2010). Subsequently, the binding of JA-Ile to the ubiquitin–E3 ligase complex protein CORONATINE INSENSITIVE1 (COI1) leads to the degradation of the negative regulator JASMONATE ZIM-DOMAIN (JAZ) (Chini *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*, 2007; Oh *et al.*, 2012), therefore allowing the induction of specific defense responses, such as TPIs (Van Dam *et al.*, 2001) and phenolamides, including caffeoylputrescine (CP; Kaur *et al.*, 2010; Onkokesung *et al.*, 2012), which have been demonstrated to function as defenses against natural herbivores such as *M. sexta* (Zavala *et al.*, 2004; Kaur *et al.*, 2010). In addition to their induction in tissues that are directly attacked by herbivores, these defenses can also be induced in undamaged systemic tissues (Green & Ryan, 1972; Kaur *et al.*, 2010) and thereby reduce the herbivore performance (Orozco-Cardenas *et al.*, 1993). In addition to these direct defenses, herbivore attack also induces indirect defenses such as the release of herbivory-induced plant volatiles (HIPVs), which increases a plant's fitness by attracting herbivore predators and deterring herbivore oviposition (De Moraes *et al.*, 1998; Kessler & Baldwin, 2001; Allison & Daniel Hare, 2009; Allmann & Baldwin, 2010; Schuman *et al.*, 2012). Among the best-studied HIPVs are the green leaf volatiles (GLVs), which are comprised of fatty acid-derived C_6 aldehydes, alcohols and esters, which are released by wounding in most, if not all plant species (Hatanaka *et al.*, 1978; Matsui, 2006).

Here, we combined the analysis of phytohormones and defense compounds, including JA, JA-Ile, CP and TPI, with different tools for CK concentration and signaling manipulation (Fig. 1) to thoroughly test the hypothesis that CK concentrations and signaling are positive regulators of wound- and HAMP-inducible defense signaling and defenses in both attacked and unattacked systemic tissues.

Materials and Methods

Plant material

For transient silencing of the CK receptor genes *NaCHK2*, *NaCHK3* and *NaCHK4*, we used the virus-induced gene silencing (VIGS) system optimized for *Nicotiana attenuata* (Torr. ex S. Wats.) described by Ratcliff *et al.* (2001) and by Saedler & Baldwin (2004). The sequences used for silencing are provided in Supporting Information Table S1.

Constitutive transformation with *Agrobacterium tumefaciens* (strain LBA 4404) was carried out as described by Krügel *et al.* (2002). The *NaCHK2/NaCHK3* silenced plants ir*chk2/3* (line number A-12-313) and ir*chk2/3*-2 (line number A-12-356) were generated by transformation with the pRESC8HK2HK3 vector shown in Fig. S1 and subsequent screening of two independently transformed lines as described by Gase *et al.* (2011). The silencing efficiencies for *NaCHK2* and *NaCHK3* are shown in Fig. S2. The i-ov*ipt* line (line number A-11-92 × A-11-61) contains the pOp6/LhGR expression system (Craft *et al.*, 2005; Samalova *et al.*, 2005), which is comprised of the steroid-controlled transcription activator LhGR and the target construct under the control of the LhGR-dependent pOp6 promoter. As a target construct we used the *A. tumefaciens* IPT coding gene *Tumor morphology root* (*Tmr*; Heidekamp *et al.*, 1983) to allow dexamethasone (DEX)-inducible manipulation of the endogenous CK concentration. The line was generated by crossing pSOL9LHGRC (GenBank JX185747) and pPOP6IPT (GenBank JX185749) containing plants as described by Schäfer *et al.* (2013). Plant germination and growth were carried out as described by Krügel *et al.* (2002).

Leaf treatments

For the standardized wound treatment, a fabric pattern wheel was rolled three times on each side of a leaf. Subsequently, 20 µl of water was applied to the puncture holes (W + W). To simulate herbivore feeding, instead of water 1 : 5 diluted OS was applied (W + OS). OS was obtained from *Manduca sexta* larvae of an inhouse colony. After 0.5, 1, 1.5, 2 or 48 h, as indicated in the figures and figure legends, the leaves were harvested, immediately frozen in liquid nitrogen and stored at −80°C. For analysis of systemic induced defenses, young untreated leaves adjacent to the treated leaves were collected. The treatments were performed in the morning between 09:00 and 10:00 h, if not stated otherwise.

CK spray application

For spray application, *trans*-zeatin (*tZ*) was dissolved in 80% EtOH (1 mg ml^{-1}) and diluted to 1 µM in an aqueous solution of 0.02% Tween-20. Spray application of *tZ* and the corresponding buffer control started 2 d before treatment and continued until samples were harvested. The treated leaves were sprayed three times per day until runoff. Between 0.4 and 0.6 ml of the solution was applied each time per leaf, depending on the leaf size.

DEX application

DEX was dissolved in dimethylsulfoxide (DMSO) and stored at −20°C until use. DEX-containing lanolin paste was prepared as described by Schäfer *et al.* (2013). Briefly, DEX stock solution was mixed with lanolin, partitioned in syringes (Omnifix-F 1 ml; B. Braun Melsungen AG, Melsungen, Germany; http://www.bbraun.de/) and then applied to the lower part of the respective leaf petiole. For the DEX treatments, only fully developed leaves were used. The final concentration of the lanolin paste was 5 µM DEX with a DMSO content of 1%. As a control, a 1% DMSO-containing lanolin paste without DEX was used (designated as 0 µM DEX). DEX applications were performed 1 d before the start of the experiments.

Quantitative (q)PCR analysis

RNA was extracted with TRIzol (Invitrogen, Darmstadt, Germany) according to the manufacturer's instructions. After cDNA synthesis by reverse transcription using oligo(dT) primer and RevertAid reverse transcriptase (Invitrogen), the qPCR was performed on a Stratagene Mx3005P qPCR machine using a SYBR Green-containing reaction mix (Eurogentec, Cologne, Germany;

qPCR Core kit for SYBR Green I No ROX). The primer sequences are provided in Table S2. As a standard we used actin, except for the analysis of *NaCHK2*/*NaCHK3* silencing in ir*chk2*/*3* and ir*chk2*/*3*-2 plants (Fig. S1), where glyceraldehyde-3-phosphate dehydrogenase (NaGAPDH) was used.

CK analysis

The CKs were extracted from the plant material with acidified aqueous methanol and purified in two steps of a solid-phase extraction as described by Dobrev & Kamínek (2002) with the modifications by Kojima *et al.* (2009) and Schäfer *et al.* (2013). For a step-by-step protocol, see Schäfer *et al.* (2014b). Measurements were performed using UHPLC coupled MS-MS operated in multi-reaction-monitoring mode. The instrument specifications and settings are described by Schäfer *et al.* (2014a).

JA and JA-Ile measurements

JA and JA-Ile were extracted and analyzed as described by Kallenbach *et al.* (2010). To quantify JA and JA-Ile, (9,10-^{2}H)dihydro-JA and (^{13}C6)JA-Ile were added as internal standards to each sample.

Secondary metabolite measurement and analysis

Secondary metabolite extraction was performed as described by Gaquerel *et al.* (2010), with the modifications that 80% MeOH in water was used as the extraction buffer (500 µl for 100 mg ground plant tissue) and that the extraction was carried out in 96-well BioTubes (1.1-ml individual tubes; Arctic White LLC, Bethlehem, PA, USA). For separation, an UltiMate 3000 system (Dionex, Sunnyvale, CA, USA), combined with a Dionex Acclaim 2.2-µm 120A 2.1 × 150 mm column was used. The mobile phase comprised solvent A (water, 0.1% acetonitrile and 0.05% formic acid) and solvent B (acetonitrile and 0.05% formic acid) with the elution profile: 0–0.5 min, 10% B in A; 0.5–6.5 min, 10–80% B in A; 6.5–8 min, 80% B in A. The flow rate was 0.4 ml min^{-1}. Measurements were performed on a Micro-ToF mass spectrometer (Bruker Daltonics, Bremen, Germany; http://www.bruker.com) equipped with an electrospray ionization source in positive mode as described by Gaquerel *et al.* (2010). The analysis was performed with QuantAnalysis 2.0 (Bruker Daltonics) according to Onkokesung *et al.* (2012), with the settings shown in Table S3.

TPI activity assay

TPI activity was determined using the radial diffusion assay described by Jongsma *et al.* (1994).

Protein measurement

Soluble proteins were extracted with 0.1 M Tris HCl, pH 7.6, and the protein content was determined by the method of Bradford (1976).

Ethylene measurements

To analyze the herbivory-induced ethylene release, correspondingly treated leaf discs were incubated in 4-ml glass vials for 5 h. Measurements were performed with a photo-acoustic ETD-300 ethylene detector (Sensor Sense, Nijmegen, the Netherlands; http://www.sensor-sense.nl/) similarly to the method described by von Dahl *et al.* (2007). The instrument was operated in sample mode, with a flow of 2 l h^{-1} and 7.5 min measurement time per sample.

Volatile measurements

Volatile measurements were performed as described by Kallenbach *et al.* (2014). In brief, after two applications of the W + OS treatment in the morning (between 09:00 and 10:00 h) and the evening (between 18:00 and 19:00 h) of the same day, volatiles were collected overnight and in the first 12 h of the subsequent photoperiod in a plastic cup (*c.* 400 ml) using 5-mm pieces of polydimethysiloxane (PDMS) tubing (Carl Roth: art. no. 9557.1, 25 m, 1.5 mm inner diameter × 3.5 mm outer diameter; Rotilabo-silicone tube; Carl Roth GmbH, Karlsruhe, Germany; http://www.carlroth.com/. Measurements were performed on a TD-20 thermal desorption unit (Shimadzu, Duisburg, Germany) connected to a quadrupole GC-MS-QP2010Ultra (Shimadzu). For separation, a Rtx-5MS column (30 m; 0.25 mm inner diameter; 0.25 µm film thickness; Restek, Bad Homburg, Germany) was used. As the carrier gas, helium (He) was used with a constant velocity of 40 cm s^{-1}. After 5 min at 40°C, the oven temperature was raised to 180°C (5°C min^{-1}) and subsequent to 280°C (30°C min^{-1}) where it was held for 0.83 min. Electron impact (EI) spectra were recorded at 70 eV in scan mode. Data were analyzed with the Shimadzu GCMS solutions software (v2.72). Settings for peak identification and integration are shown in Table S4. The identities of 3(*Z*)-hexenyl isobutyrate and 3(*Z*)-hexenyl butyrate were confirmed by comparison to an identical standard and that of 3(*Z*)-hexenyl isovalerate by a literature search and using the Kovats index (calculated: 1236; Ruther (2000): 1237).

Mitogen-activated protein kinase assay

Protein extraction was performed according to Wu *et al.* (2007) and kinase activity was measured as described by Zhang & Klessig (1997) using myelin basic protein as a substrate. After the reaction and washing steps, the gels were dried on a gel dryer (Bio-Rad, Munich, Germany; http://www.bio-rad.com). For image generation, a FLA-3000 phosphor imager system (FujiFilm Europe GmbH, Düsseldorf, Germany; http://www.fujifilm.eu/) was used. For each sample, at least three biological replicates were pooled.

Statistical analysis

The influence of CK pathway manipulations and treatment (C, W + W and W + OS) on defense signaling and chemistry was

analyzed using two-way ANOVAs. In the case of a significant interaction, a *post hoc* analysis using Tukey's honestly significant difference (HSD) test was performed. Because of heterogeneity in some data sets, these effects were analyzed using a generalized least squares model (GLS with nlme package; Pinheiro *et al.*, 2014) with the varIdent variance structure, which allows correction for different variances in each group. Statistical values for the main explanatory variables and their interactions were obtained by backward selection and comparison of the simpler with the more complex model using a likelihood ratio test (Zuur *et al.*, 2009). Factor-level reductions were used to reveal differences between factor levels. Data analyses using an independent (unpaired) samples *t*-test and two-way ANOVA were performed with SPSS STATISTICS 17.0 (http://www.01.ibm.com/software/de/analytics/spss/), whereas R version 3.1.1 (R Core Team, 2014) was used for GLS. If homoscedasticity could be achieved by transformation, two-way ANOVA was preferred over the GLS model. The statistical methods used and the number of biological replicates (n = number of independent plants per treatment, line and time-point) are indicated in the figure legends. The presented data are supported by at least two independently conducted experiments with similar results.

Results

Increased CK concentrations elevate herbivory-inducible defense responses

Dervinis *et al.* (2010) reported an amplification of induced defense responses after external CK application. To investigate if CK concentrations also regulate herbivory-inducible defense responses in *N. attenuata*, we sprayed leaves with 1 μM of the bioactive CK *tZ*, which occurs naturally in *N. attenuata* (Schäfer *et al.*, 2013), and analyzed the response to simulated herbivory. JA concentrations, as well as the well-established herbivore resistance traits CP and TPI activity, were elevated after *tZ* spraying, while JA-Ile concentrations were not affected (Fig. 2).

As spraying does not allow precise control of endogenous CK concentrations and spraying itself might induce unintended responses, including changes in leaf transpiration, experiments with transgenic plants with increased CK biosynthesis were conducted. We used plants transformed with the DEX-inducible pOp6/LhGR expression system (Craft *et al.*, 2005; Samalova *et al.*, 2005) to heterologously express the CK biosynthesis enzyme IPT. DEX treatments of these plants (i-ov*ipt*) allowed us to fine-tune concentrations of endogenous CKs in the treated leaves (Fig. 3; Schäfer *et al.*, 2013) and to study their effects on herbivore resistance responses upon OS elicitation (Figs 3, S3, S4). To date, no evidence for DEX-mediated effects on the development and physiology of *N. attenuata* has been found. Even after the application of 20× higher DEX concentrations than used in this investigation, no direct effects on the performance of *M. sexta* caterpillars feeding on these plants were found, indicating that herbivore resistance traits of the plant are not affected by DEX (Schäfer *et al.*, 2013). However, to avoid a potential influence of DEX, short-term treatments were used, DEX was applied

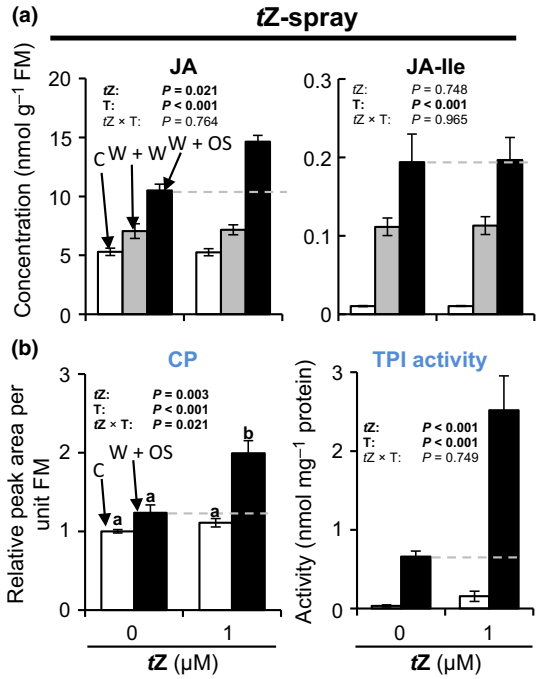

Fig. 2 Cytokinin application increases the herbivory-inducible defense response. (a) Jasmonic acid (JA) and JA–isoleucine conjugate (JA-Ile) accumulation in leaves of *Nicotiana attenuata* 1 h after wounding and application of water (W + W; gray bars) or *Manduca sexta* oral secretions (W + OS; black bars) to the puncture wounds and in untreated control leaves (C; white bars). (b) Caffeoylputrescine (CP) accumulation and trypsin proteinase inhibitor (TPI) activity were measured in leaves 2 d after W + OS treatment (black bars) and in untreated control leaves (C; white bars). Measurements were performed in leaves after spraying with 1 μM *trans*-zeatin (*tZ*) or mock solution until runoff. *tZ* and treatment (C, W + W and W + OS; T) effects and their interactions (*tZ* × T) were analyzed using two-way ANOVA. Different letters indicate significant differences (CP; Tukey's honestly significant difference (HSD) test: $P \leq 0.05$). Error bars are ± SE ($n \geq 3$). CP is shown relative to the 0 μM *tZ* control. FM, fresh mass.

in low concentrations and only fully developed leaves were used for experiments. No visible differences between DEX-treated and untreated leaves were observed under the experimental conditions. Fig. 3 shows that DEX treatment of rosette stage i-ov*ipt* plants led to 3.8-fold higher concentrations of CK free bases and ribosides, mainly through increases in the abundance of *tZ* and its riboside, which resulted in an increase in JA, but not JA-Ile concentrations. CP is strongly induced by herbivory in *N. attenuata* and has known defense functions against herbivores such as *M. sexta* (Kaur *et al.*, 2010; Onkokesung *et al.*, 2012). The W + OS-induced transcript levels of the CP biosynthesis gene *ACETYL TRANSFERASE 1* (*NaAT1*), as well as CP itself, were more than doubled in leaves with elevated CK concentrations. In *N. attenuata*, phenolamide biosynthesis is known to be regulated by the transcription factor NaMYB8, which regulates the transcription of the enzymes NaAT1, involved in the biosynthesis of acylated putrescines, as well as the acyltransferases NaCV86 and NaDH29, which are responsible for the biosynthesis of mono- and diacylated spermidines (Onkokesung *et al.*, 2012). Interestingly, higher CK concentrations did not increase the abundance of transcripts of *NaMYB8*, *NaDH29*, and *NaCV86* after 2 d (Fig. S3).

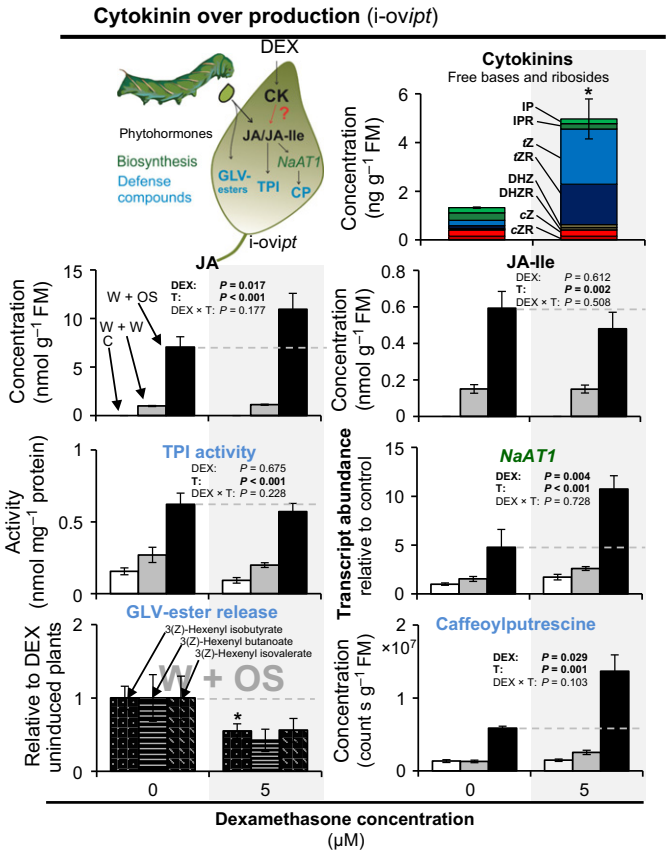

Fig. 3 Cytokinin concentrations regulate herbivory-inducible defense responses. Isopentenyladenine (IP), isopentenyladenosine (IPR), *trans*-zeatin (*t*Z), *trans*-zeatin riboside (*t*ZR), dihydrozeatin (DHZ), dihydrozeatin riboside (DHZR), *cis*-zeatin (*c*Z) and *cis*-zeatin riboside (*c*ZR) concentrations in leaves of *Nicotiana attenuata* were measured. Jasmonic acid (JA) and JA–isoleucine conjugate (JA-Ile) accumulation in leaves 60 min after wounding and treatment with water (W + W; gray bars) or *Manduca sexta* oral secretions (W + OS; black bars) and in untreated control leaves (C; white bars) were measured. Trypsin proteinase inhibitor (TPI) activity, the transcript level of *ACETYL TRANSFERASE 1* (*NaAT1*) and accumulation of the defense metabolite caffeoylputrescine (CP) were measured in leaves 2 d after W + W (gray bars) or W + OS (black bars) treatment and in untreated control leaves (white bars). Green leaf volatile (GLV)-ester release from leaves was measured during the night and for the next 12 h of the following photoperiod after two consecutive W + OS treatments. Measurements were performed with leaves of dexamethasone (DEX)-inducible isopentenyltransferase-overexpressing plants (i-ov*ipt*) that had been treated with 0 or 5 μM DEX-containing lanolin paste 1 d before the experiment. DEX and treatment (C, W + W and W + OS; T) effects and their interactions (DEX × T) were analyzed using two-way ANOVA. Cytokinins and GLV esters were analyzed using a *t*-test. Asterisks indicate significant differences between DEX-induced and uninduced i-ov*ipt* plants (independent samples *t*-test: *, $P \leq 0.05$). Error bars are ± SE ($n \geq 3$). For additional phenolamide-related transcripts and phenolamides, see Supporting Information Figs S3 and S4. FM, fresh mass.

To better understand the CK-specific regulation of the phenolamide pathway, we used a targeted metabolomics approach (Fig. S4). Intriguingly, most compounds that are specifically induced by simulated herbivory showed increased concentrations after an increase in CK concentrations. Higher CK concentrations particularly increased the accumulation of monoacylated putrescines and spermidines, approximately doubling their W + OS-induced concentrations. The diacylated spermidines, by contrast, were not affected or, in the case of dicaffeoyl spermidine, even tended to have slightly reduced concentrations ($P = 0.057$).

Herbivore-induced volatile compounds are known to function as very effective indirect defenses in *N. attenuata* (Kessler & Baldwin, 2001; Allmann & Baldwin, 2010; Schuman *et al.*, 2012). When we analyzed the HIPV bouquet of W + OS-induced DEX- and non-DEX-treated i-ov*ipt* plants, we found that the release of the GLV ester 3(*Z*)-hexenyl isobutyrate was reduced to *c.* 50% by elevated CK concentrations and also 3(*Z*)-hexenyl butanoate and 3(*Z*)-hexenyl isovalerate emissions tended to be decreased (Fig. 3).

The TPI activity per unit protein content was not affected in our short-term DEX-treated plants (Fig. 3). Also, W + OS-induced TPI transcript levels were not significantly elevated after DEX treatment (Fig. S5). However, compared with the respective control levels, TPI transcript levels increased after W + OS treatment significantly more in DEX-treated plants than in the non-DEX-treated plants (23.5× vs 3.1×; independent samples *t*-test; $P = 0.008$).

CK signaling is required for the full induction of herbivory-induced defense responses

Several studies used plants with impaired CK perception to investigate their role in specific physiological processes (Gonzalez-Rizzo *et al.*, 2006; Riefler *et al.*, 2006; Choi *et al.*, 2010), but such data are still elusive regarding the molecular responses after herbivore attack. To further investigate the role of CKs in the inducible herbivore resistance responses of *N. attenuata*, we transformed *N. attenuata* plants to silence CK perception. Three CK receptor homologs were cloned and used with transient VIGS technology to silence single receptors or combinations of two receptors (Fig. S6). CP was used as a marker to assess the plant's ability to mount a full defense response. The strongest reduction in W + OS-induced CP accumulation was observed in the case of the VIGS-mediated co-silencing of *NaCHK2* and *NaCHK3*. Silencing of *NaCHK2* and *NaCHK3* also reduced JA accumulation, as well as TPI activity, whereas JA-Ile was not affected (Fig. 4). To evaluate if the observed changes in JA were mediated by altered activity of mitogen-activated protein kinases, particularly those known to influence JA biosynthesis in *N. attenuata*, for example WOUND-INDUCED PROTEIN KINASE (WIPK) and SALICYLIC ACID-INDUCED PROTEIN KINASE (SIPK) (Wu *et al.*, 2007; Kallenbach *et al.*, 2010), an in-gel kinase activity assay was performed, but no obvious changes were observed (Fig. S7).

Despite its versatility and the advantage that it allows genetic manipulation after plants have been allowed to develop unhampered to the small rosette stage, the VIGS technology has the drawback that the virus may alter a plant's physiology, which could affect experimental outcomes. Therefore, stably transformed plants are necessary for the evaluation of the robustness of VIGS results. We used the same sequence fragments in an inverted repeat orientation as were used in the VIGS constructs to generate plants with stable, constitutive,

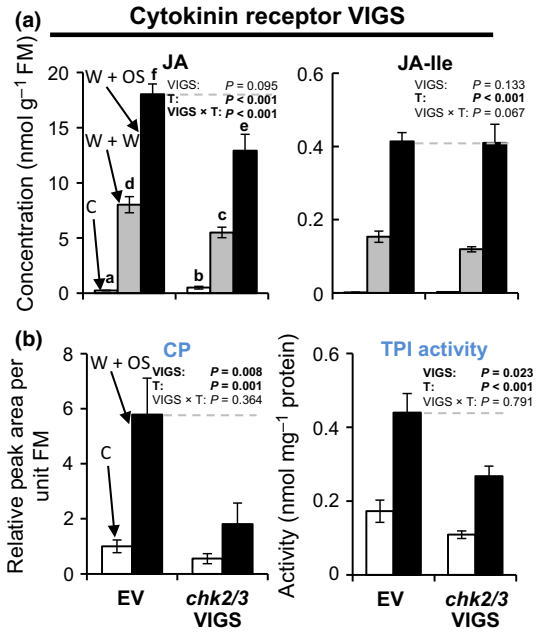

Fig. 4 Impaired cytokinin perception decreases herbivory-inducible defense responses. (a) Jasmonic acid (JA) and JA–isoleucine conjugate (JA-Ile) concentrations in leaves of *Nicotiana attenuata* 90 min after wounding and application of water (W + W; gray bars) or *Manduca sexta* oral secretions (W + OS; black bars) to the puncture wounds and in untreated control leaves (C; white bars) were measured. (b) Caffeoylputrescine (CP) accumulation and trypsin proteinase inhibitor (TPI) activity were measured in leaves 2 d after W + OS treatment (black bars) and in untreated control leaves (C; white bars). Measurements were performed with leaves of plants silenced in *CHASE-DOMAIN CONTAINING HIS KINASE 2* (*NaCHK2*) and *NaCHK3* expression by virus-induced gene silencing (VIGS) and empty vector control (EV) plants. VIGS and treatment (C, W + W and W + OS; T) effects and their interactions (VIGS × T) were analyzed using two-way ANOVA, except for JA data which were analyzed using a generalized least squares model. Different letters indicate significant differences (JA, factor-level reduction: $P \leq 0.05$). Error bars are ± SE ($n \geq 3$). FM, fresh mass.

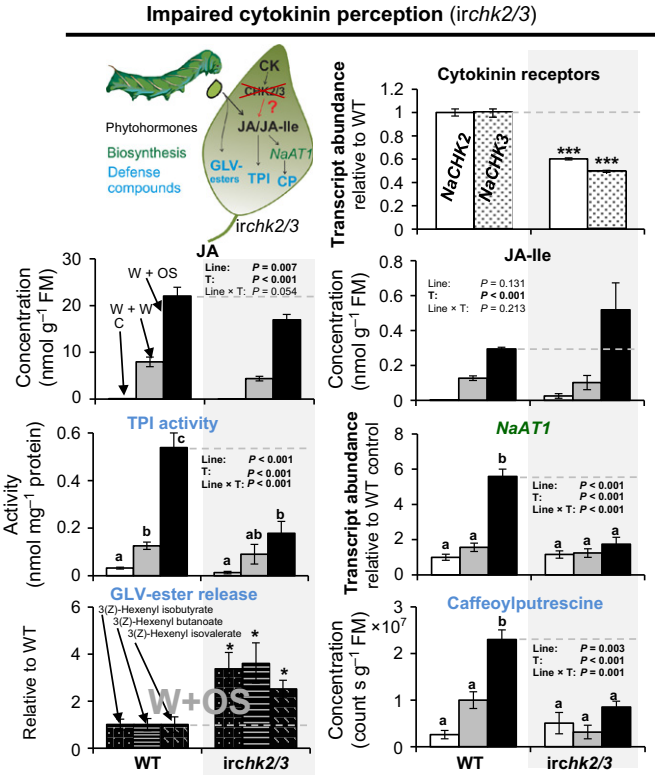

Fig. 5 Cytokinin perception regulates herbivory-induced defense responses. Transcript abundance of *CHASE-DOMAIN CONTAINING HIS KINASE 2* (*NaCHK2*) and *NaCHK3* in the shoots of 6-d-old *Nicotiana attenuata* seedlings was measured. Jasmonic acid (JA) and JA–isoleucine conjugate (JA-Ile) accumulation in leaves 60 min after wounding and treatment with water (W + W; gray bars) or *Manduca sexta* oral secretions (W + OS; black bars) and in untreated control leaves (C; white bars) was measured. Trypsin proteinase inhibitor (TPI) activity, the transcript level of *ACETYL TRANSFERASE 1* (*NaAT1*) and accumulation of the defense metabolite caffeoylputrescine (CP) were measured in leaves 2 d after W + W (gray bars) or W + OS treatment (black bars) and in untreated control leaves (white bars). Green leaf volatile (GLV)-ester release of leaves was measured during the night and in the next 12 h of the following photoperiod after a twice repeated W + OS treatment. Measurements were performed in leaves of wild-type (WT) plants and *NaCHK2*/*NaCHK3*-silenced plants (ir*chk2/3*). Line and treatment (C, W + W and W + OS; T) effects and their interactions (line × T) were analyzed using two-way ANOVA, except for JA-Ile data which were analyzed using a generalized least squares model instead. Different letters indicate significant differences (TPI activity, *NaAT1* and CP; Tukey's honestly significant difference (HSD) test: $P \leq 0.05$). Cytokinin receptor transcripts and GLV-esters were analyzed using a *t*-test. Asterisks indicate significant differences between WT and ir*chk2/3* plants (independent samples *t*-test: *, $P \leq 0.05$; ***, $P \leq 0.001$). Error bars are ± SE ($n \geq 4$). For the complete JA and JA-Ile kinetics, see Fig. S10 and for additional phenolamide-related transcripts and phenolamides, see Figs S11 and S12. Data for an independently transformed *NaCHK2*/*NaCHK3*-silenced line are shown in Figs S13 and S14. FM, fresh mass.

RNAi-mediated silencing of *NaCHK2* and *NaCHK3* (ir*chk2/3* and ir*chk2/3*-2). We chose lines with moderate silencing efficiency, in which plant development (Fig. S8) and CK concentrations (Fig. S9) were only mildly affected. Plants with more pronounced developmental differences were excluded from the experiments to ensure comparability with the WT. The experiments were performed with two independently transformed lines (ir*chk2/3* and ir*chk2/3*-2), which showed similar changes in herbivory-inducible responses (ir*chk2/3:* Figs 5, S10–S12, S15; ir*chk2/3*-2: Figs S13–S15).

Rosette-stage plants of the ir*chk2/3* line showed a reduced accumulation of JA (Figs 5, S10). By contrast, the JA-Ile concentrations were not decreased (Figs 5, S10). The W + OS-induced transcript abundance of the phenolamide biosynthesis gene *NaAT1* was reduced by nearly 70% when compared with WT plants (Fig. 5). Also, CP accumulation and TPI activity after W + OS treatments were reduced to *c.* 30% of WT levels (Fig. 5). W + OS-induced transcript levels of other phenolamide-related genes, including the transcription factor *NaMYB8* and the biosynthesis genes *NaDH29* and *NaCV86*, were reduced by 30–70% (Fig. S11). With the exception of diferuloyl spermidine, the concentrations of all analyzed phenolamides were reduced in the ir*chk2/3* plants (Fig. S12). By contrast, 2–4 times more 3(*Z*)-hexenyl isobutyrate, 3(*Z*)-hexenyl butanoate and 3(*Z*)-hexenyl isovalerate were emitted from W + OS-treated leaves of ir*chk2/3* plants, when compared with WT leaves treated in the same way

(Fig. 5). *NaCHK2*/*NaCHK3* silencing had only minor effects on plant defense in noninduced control leaves (Fig. 5).

CKs are known as positive regulators of ethylene production (Bertell & Eliasson, 1992; Cary *et al.*, 1995; Hansen *et al.*, 2009). Therefore, we analyzed ethylene release in leaves of plants with increased CK concentration (i-ov*ipt*), as well as in plants impaired in CK perception (ir*hk2/3* and ir*hk2/3*-2) after simulated herbivory. For all those CK pathway manipulations, we observed no differences in comparison with the respective control (Fig. S15).

CK signaling promotes herbivory-inducible systemic defense responses

Phenolamides and TPI activity are well-known herbivory-induced systemic defense responses (Green & Ryan, 1972; Kaur *et al.*, 2010). We analyzed the influence of CK signaling on these systemic responses. CP concentrations were significantly reduced in the ir*chk2/3* plants compared with the WT, resulting in *c.* 50% lower W + OS-induced CP accumulation (Fig. 6). Similar to the treated leaves, the systemic concentrations of most other tested monoacyl-putrescines and -spermidines were also reduced in the CK receptor silenced plants (Fig. S16). The diacyl-spermidines were less strongly affected. The systemic TPI activity was not affected (Fig. S17).

Discussion

CKs have been reported to amplify the antiherbivore defense of plants (Smigocki *et al.*, 1993, 2000; Dervinis *et al.*, 2010), but information on the plant defenses involved and the underlying regulation is limited. Here, we show that a functional CK pathway is a substantial component of the herbivory-induced defense signaling machinery and provide new information about the role of CK perception in local and systemic defense responses. Additionally, we show that specific indirect defense responses are suppressed by CKs.

The CK pathway regulates the phenolamide and TPI response

We used topical application of CKs (Fig. 2), chemically induced *IPT* expression (Figs 3, S3–S5, S15), VIGS-mediated CK receptor silencing (Figs 4, S6, S7) and stable CK receptor silencing (Figs 5, 6, S10–S17) to manipulate CK concentrations and perception in order to rigorously evaluate their effects on the defense responses of *N. attenuata* (for an overview, see Fig. 1). Consistently, we found CKs to be positive regulators of the accumulation of monoacyl-putrescines and -spermidines, including CP (Figs 2–5, S4, S6, S12, S14). It has been reported that after herbivore attack the JA pathway induces the expression of the transcription factor NaMYB8, which is responsible for the herbivory-induced accumulation of phenolamides, by regulating the expression of the acyltransferases NaAT1, NaDH29 and NaCV86 (Kaur *et al.*, 2010; Onkokesung *et al.*, 2012). CKs increased JA concentrations, but interestingly not the concentrations of its active conjugate JA-Ile (Figs 2–5, S10, S13). As JA-Ile concentrations were not reported in the previous investigations of CK-mediated effects on plant defense signaling (e.g. Sano *et al.*, 1996; Dervinis *et al.*, 2010), it is unclear if the lack of a CK-induced increase in JA-Ile concentrations also applies for other plant species. CKs are known to stimulate the development of chloroplasts (Volfová *et al.*, 1978), which are also the site of the first steps in JA biosynthesis (Wasternack, 2007), thereby potentially promoting JA formation. The lack of an increase in JA-Ile concentrations might be related to their cytoplasmic biosynthesis (Hsieh *et al.*, 2000; Staswick *et al.*, 2002). Additionally, the changes in JA accumulation might not influence JA-Ile formation, because the concentration of JA-Ile is *c.* 50-fold lower than that of JA (Figs 2–5, S10, S13), making it unlikely that JA is a limiting factor under our conditions. Still, the herbivory-induced accumulation of *NaMYB8* transcripts was strongly reduced in plants with impaired CK signaling (Figs S11, S13). *NaMYB8* transcript accumulation is accepted to be JA-Ile-dependent (Kaur

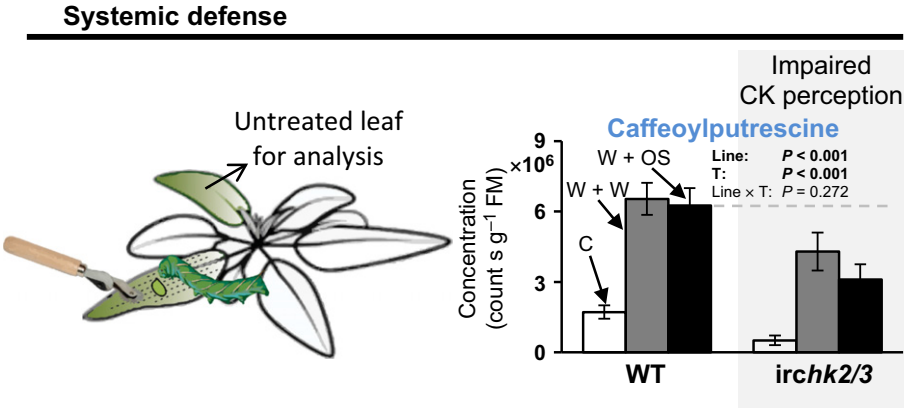

Fig. 6 Cytokinin (CK) perception regulates systemic caffeoylputrescine concentrations. Caffeoylputrescine accumulation was measured in untreated systemic leaves of *Nicotiana attenuata* 2 d after wounding and treatment with water (W + W; gray bars) or *Manduca sexta* oral secretions (W + OS; black bars) and in untreated control plants (C; white bars). Measurements were performed in leaves of wild-type (WT) plants and *CHASE-DOMAIN CONTAINING HIS KINASE 2* (*NaCHK2*) and *NaCHK3*-silenced plants (ir*chk2/3*), which were impaired in CK perception. Line and treatment (C, W + W and W + OS; T) effects and their interactions (line × T) were analyzed using two-way ANOVA. Error bars are ± SE ($n = 5$). For additional phenolamides, see Fig. S16. FM, fresh mass.

et al., 2010; Onkokesung *et al.*, 2012; Gaquerel *et al.*, 2014), and therefore the CK pathway might promote JA-Ile signaling.

Phenolamide biosynthesis is mediated by *NaAT1*, *NaDH29* and *NaCV86* in *N. attenuata*, which are all regulated by *NaMYB8*. The reduced *NaMYB8* transcript abundance in ir*chk2/3* plants could therefore sufficiently explain the reduced levels of *NaAT1*, *NaDH29* and *NaCV86* transcripts and thereby possibly the decreased phenolamide concentrations (Figs 5, S11–S14). Increasing CK concentrations in the i-ov*ipt* plants increased the accumulation of *NaAT1* transcripts, monoacyl-putrescines and monoacyl-spermidines (Figs 3, S4), confirming their regulation by the CK pathway. The transcripts of *NaMYB8* and *NaDH29*, by contrast, were not elevated (Fig. S3), although, according to previous reports (Onkokesung *et al.*, 2012), they were expected to up-regulate *NaAT1* expression and increase monoacyl-spermidine concentrations, respectively. Based on these observations, we propose that CKs regulate phenolamides by at least two independent mechanisms; by regulating *NaMYB8*-transmitted phenolamide induction, but also in another way. Most parts of the phenolamide pathway were almost unaffected in noninduced ir*chk2/3* and ir*chk2/3*-2 plants compared with the WT (Figs 5, S11–S14), but required simultaneous induction by herbivory, indicating CK signaling to be required for the full induction of increases in herbivory-induced phenolamide concentrations.

TPI activity, another JA-dependent anti-herbivore defense (Van Dam *et al.*, 2001; Paschold *et al.*, 2007), was also clearly dependent on CK perception by NaCHK2 and NaCHK3 (Figs 4, 5, S13), supporting the idea of CK-mediated amplification of JA signaling. However, the mild, short-term elevations of endogenous CKs in the DEX-treated i-ov*ipt* plants failed to further increase herbivory-induced TPI activity (Fig. 3). The missing increase might be related to the normalization of the TPI activity to the protein content, which itself is affected by CKs (Fig. S5a; Richmond & Lang, 1957) and could have masked potential differences in TPI activity. However, W + OS-induced TPI transcripts were also not significantly elevated in plants with increased CK concentrations (Fig. S5b). The lack of sensitivity of TPI activity to small CK concentration changes, such as in i-ov*ipt* plants, might also indicate that basal CK concentrations in the rosette-stage plants used were already sufficient to saturate these effects.

The concentrations of CKs, such as isopentenyladenosine and *cis*-zeatin riboside, were recently shown to increase in response to wounding and HAMP treatment in young rosette-stage plants (Schäfer *et al.*, 2014a). It is tempting to speculate that CKs therefore could be involved in the priming of defense responses observed after repeated elicitations (Stork *et al.*, 2009). Additionally, it would be interesting to investigate how far differing CK concentrations throughout the day (Novakova *et al.*, 2005) or between the various tissues within a plant are correlated with defense metabolite distributions (Meldau *et al.*, 2012).

From our observations and current knowledge about JAs and CKs, we propose three mechanisms to explain how CKs might amplify JA signaling and one mechanism that might allow CK-dependent phenolamide concentration elevation without concomitant amplification of JA/NaMYB8 signaling. (1) Signaling interactions: CK signaling may promote JA signaling downstream of JA-Ile; for example, CK signaling elements may interact at the level of JAZ repressors, as has been demonstrated for other hormonal pathways, including auxin and gibberellins (Grunewald *et al.*, 2009; Hou *et al.*, 2010). Similar interactions between the CK pathway and salicylic acid signaling are required for pathogen resistance in *A. thaliana*. In this plant, the CK response regulator ARABIDOPSIS RESPONSE REGULATOR 2 (ARR2) directly interacts with TGA3, which, in the presence of salicylic acid and NON-EXPRESSOR OF PR1 (NPR1), leads to increased PATHOGENESIS-RELATED GENE 1 (PR1) expression and higher pathogen resistance (Choi *et al.*, 2010). (2) Hormone concentration changes: CKs may increase the concentrations of active oxylipins other than JA-Ile. Some investigations reported JA-Ile-independent effects of JA and its related metabolites on plant defense responses. Wang *et al.* (2008), for example, showed in *N. attenuata* that silencing JA biosynthesis has a stronger effect on herbivore defense than only preventing JA-Ile formation. Similarly, van Doorn *et al.* (2011) showed that JA-Ile signaling is not required for defense responses in *Solanum nigrum* under natural conditions. (3) Indirect interactions: CKs might influence herbivory-inducible defense responses indirectly, by up- or down-regulating other hormonal pathways, such as auxins and gibberellins, which subsequently leads to changes in JA sensitivity (Meldau *et al.*, 2012). (4) Resource improvement: CKs are known to control the source–sink status of tissues (Kuiper, 1993; Balibrea Lara *et al.*, 2004) and the resources associated with strong sink strength can increase secondary metabolite production (Arnold *et al.*, 2004). CKs were shown to elevate the activity of the PHENYLALANINE AMMONIA-LYASE, an enzyme that is responsible for the first step in the phenylpropanoid pathway (Jones, 1984), thereby potentially providing substrates for phenolamide formation. Testing this hypothesis will require a detailed analysis of metabolites that act as precursors or supply energy for the synthesis of defense compounds. Future investigations should take into account the possibility that more than one of these mechanisms might be functioning.

The CK pathway suppresses GLV-ester emissions of herbivory-induced leaves

In *N. attenuata*, Halitschke *et al.* (2004) reported a competitive, probably substrate-mediated relationship between the JA and GLV pathways arising from the utilization of the same precursors. While JA concentrations are positively correlated with the activity of the CK pathway, the release of GLV esters was negatively correlated, suggesting that CKs control the balance between these two oxylipin classes. As GLVs are known to function as feeding stimulants for some herbivores (Halitschke *et al.*, 2004; Meldau *et al.*, 2009), reduced GLV-ester concentrations might lower tissue consumption. GLVs are also potent attractants for predators and ovipositing moths (Halitschke *et al.*, 2008; Allmann & Baldwin, 2010; Schuman *et al.*, 2012), which have opposite fitness effects on plants. By contrast, we observed no consistent CK effects on the emissions of the sesquiterpene *trans*-α-bergamotene, which is another herbivory-associated indirect

defense compound of *N. attenuata* (Kessler & Baldwin, 2001; Schuman *et al.*, 2009). Analysis of the interactions of plants that have altered CK concentrations or signaling with herbivores and predators in the plant's natural habitat will be required to reveal the ecological significance of altered volatile emission by these plants.

CK perception regulates specific systemic defenses

Within-plant movement is an important strategy for herbivores to avoid inducible plant defenses (Paschold *et al.*, 2007). Probably as a counterstrategy, plants evolved defenses that are induced not only in the attacked leaf but also in adjacent leaves, thereby reducing herbivore performance (Green & Ryan, 1972; Orozco-Cardenas *et al.*, 1993). Similar to results reported by Dervinis *et al.* (2010) for poplar, manipulation of the CK pathway did not affect induced systemic TPI concentrations in *N. attenuata* (Fig. S17). Interestingly, CP, another well-known systemic herbivore defense of wild tobacco (Kaur *et al.*, 2010), was attenuated in CK receptor-silenced plants (Fig. 6). It is still not clear from which part of the plant the CK pathway contributes to the systemic CP induction. The induced increases in CP concentrations in systemic leaves reported by Kaur *et al.* (2010) decreased with the age of the plants. As CKs are growth regulators and strong suppressors of senescence processes (Richmond & Lang, 1957), the CK receptor-silenced plants (ir*chk2/3*) might mimic the CK pathway of a plant in a later developmental stage. Therefore, the CK pathway might be a missing link in the developmental regulation of herbivore defense responses. Future experiments on defense-related functions of CKs should be designed to test mechanisms of well-established ecological theories about tissue-specific and ontogenic regulation of defense distributions, such as the optimal defense theory or the growth-differentiation balance hypothesis (McKey, 1974; Herms & Mattson, 1992; Ohnmeiss *et al.*, 1997).

CKs do not promote ethylene release after herbivory

Another defense signal that was shown to be regulated by CKs is ethylene. Ethylene interacts with the JA pathway and regulates herbivory-induced responses (Rojo *et al.*, 1999; Kahl *et al.*, 2000; Adie *et al.*, 2007) and its production can be regulated by CKs (Bertell & Eliasson, 1992; Cary *et al.*, 1995; Hansen *et al.*, 2009). We found that HAMP-induced ethylene emissions in leaves were affected neither by increased CK concentrations nor by the inhibition of CK perception (Fig. S15). This might be explained by the report of Zd'arska *et al.* (2013), which showed that CKs mediate ethylene production in the root, but not in the shoot, indicating that CK–ethylene cross-talk might be tissue- and stress-specific.

Ecological implications of CKs in plant defense

CKs have been shown to be positive regulators of plant defense against herbivores, resulting in reduced herbivore survival and growth (Smigocki *et al.*, 1993, 2000; Dervinis *et al.*, 2010), but also to be advantageous for the performance of certain highly specialist insect attackers, such as leaf miners (Engelbrecht *et al.*, 1969), sawflies (Elzen, 1983) and a specialist mirid bug (Schäfer *et al.*, 2013). Similar reports also exist for the interaction between plants and pathogens. CKs are important factors for a successful *A. tumefaciens* infection but they can also strengthen plant defense against other pathogens, including *Pseudomonas syringae* (Jameson, 2000; Choi *et al.*, 2010; Großkinsky *et al.*, 2011; Argueso *et al.*, 2012; Giron *et al.*, 2013). The contradiction between positive and negative effects of CKs on plant defense might be explained by a tradeoff between CK-associated factors beneficial for insects and pathogens, such as increased nutritional value, and negative factors, including elevated defense metabolites. Various organisms interacting with a plant may differ in their nutritional needs and their sensitivities to certain defense compounds. Because CK concentration changes are regulated by herbivore and pathogen attack (López-Carbonell *et al.*, 1998; Giron *et al.*, 2013; Schäfer *et al.*, 2014a), a change in CKs might be an important environmental response variable that shapes the interaction of various phytophagous or pathogenic organisms that may co-colonize a plant at a given time. As CKs are well known to play an important role in adapting plants to abiotic

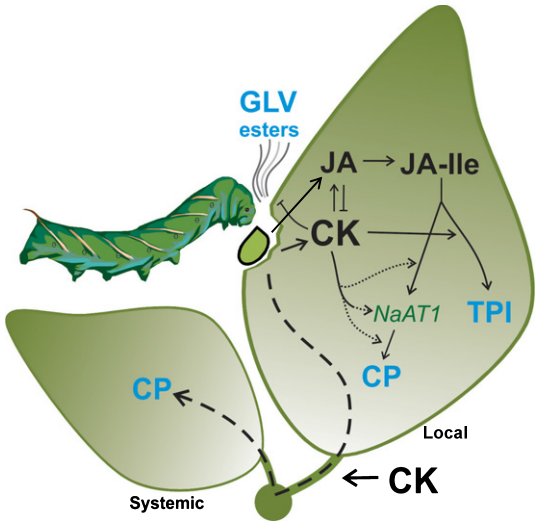

Fig. 7 The roles of cytokinins (CKs) in the herbivore defense responses of *Nicotiana attenuata*. CK metabolism and signaling are regulated by wounding and perception of herbivore-derived elicitors from the oral secretions of *Manduca sexta*. Jasmonic acid (JA) suppresses the herbivory-induced CK signaling changes. By contrast, CK concentrations and perception slightly elevate the accumulation of JA, but not that of the JA–isoleucine conjugate (JA-Ile), which is thought to mediate most JA-related responses according to the canonical model of JA signaling. The events downstream of the JA pathway, leading to the production of trypsin proteinase inhibitors (TPIs) and the accumulation of phenolamides, such as caffeoylputrescine (CP), were positively regulated by CKs. The regulation of CP might be mediated by the up-regulation of its biosynthetic enzyme ACETYL TRANSFERASE 1 (NaAT1) and/or the CK-dependent accumulation of its phenylpropanoide precursors, which is strongly suggested by the literature. In addition to the local responses elicited in attacked tissues, functional CK perception was also important for a substantial systemic accumulation of CP. By contrast, the production of green leaf volatile (GLV) esters, which function as indirect defenses, was suppressed by the CK pathway.

factors such as drought stress and nutrient availability (reviewed in Werner & Schmülling, 2009), CKs could also function as integrators of different biotic and abiotic stress responses. Analyzing the performance of plants that are altered in their CK concentrations or signaling in their natural environment, in which the plant faces a variety of natural enemies and abiotic conditions, will help to elucidate the role of CKs in regulating diverse ecological interactions.

Conclusions

Here, we provide new data on the role of CKs in plant–herbivore interactions (for an overview, see Fig. 7). Previous investigations demonstrated herbivory-induced CK pathway changes, which were partially dependent on the interaction with the JA pathway (Schäfer *et al.*, 2014a). Here we show that elevated CK concentrations amplify JA-mediated defense signaling against herbivores and identify two CK receptors that are important for local and systemic defense responses. Future investigations should focus on the ecological consequences of CK pathway changes and the identification of downstream signaling elements.

Acknowledgements

We thank Radomira Vanková and Mario Kallenbach for helpful scientific comments; Michael Reichelt, Mario Kallenbach, Klaus Gase, Matthias Schöttner, Thomas Hahn, Susanne Kutschbach and Wibke Kröber for technical assistance; Tamara Krügel, Andreas Weber and Andreas Schünzel from the glasshouse team for plant cultivation; Grit Kunert for help with the statistical analysis, and Karl Pioch for suggestions on the manuscript. M.S. and I.T.B receive funding from the Max Planck Society, and I.D.M-C. receives funding from the German Academic Exchange Service (DAAD). S.M and C.B. receive funding through Advanced Grant No. 293926 of the European Research Council to I.T.B.

References

Adie B, Chico JM, Rubio-Somoza I, Solano R. 2007. Modulation of plant defenses by ethylene. *Journal of Plant Growth Regulation* **26**: 160–177.

Allison JD, Daniel Hare J. 2009. Learned and naive natural enemy responses and the interpretation of volatile organic compounds as cues or signals. *New Phytologist* **184**: 768–782.

Allmann S, Baldwin IT. 2010. Insects betray themselves in nature to predators by rapid isomerization of green leaf volatiles. *Science* **329**: 1075–1078.

Argueso CT, Ferreira FJ, Epple P, To JPC, Hutchison CE, Schaller GE, Dangl JL, Kieber JJ. 2012. Two-component elements mediate interactions between cytokinin and salicylic acid in plant immunity. *PLoS Genetics* **8**: e1002448.

Arnold T, Appel H, Patel V, Stocum E, Kavalier A, Schultz J. 2004. Carbohydrate translocation determines the phenolic content of *Populus* foliage: a test of the sink–source model of plant defense. *New Phytologist* **164**: 157–164.

Balibrea Lara ME, Garcia MCG, Fatima T, Ehness R, Lee TK, Proels R, Tanner W, Roitsch T. 2004. Extracellular invertase is an essential component of cytokinin-mediated delay of senescence. *Plant Cell* **16**: 1276–1287.

Bertell G, Eliasson L. 1992. Cytokinin effects on root growth and possible interactions with ethylene and indole-3-acetic acid. *Physiologia Plantarum* **84**: 255–261.

Bradford MM. 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248–254.

Cary AJ, Liu W, Howell SH. 1995. Cytokinin action is coupled to ethylene in its effects on the inhibition of root and hypocotyl elongation in *Arabidopsis thaliana* seedlings. *Plant Physiology* **107**: 1075–1082.

Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, Garcia-Casado G, Lopez-Vidriero I, Lozano FM, Ponce MR *et al.* 2007. The JAZ family of repressors is the missing link in jasmonate signalling. *Nature* **448**: 666–671.

Choi J, Huh SU, Kojima M, Sakakibara H, Paek KH, Hwang I. 2010. The cytokinin-activated transcription factor ARR2 promotes plant immunity via TGA3/NPR1-dependent salicylic acid signaling in *Arabidopsis*. *Developmental Cell* **19**: 284–295.

Craft J, Samalova M, Baroux C, Townley H, Martinez A, Jepson I, Tsiantis M, Moore I. 2005. New pOp/LhG4 vectors for stringent glucocorticoid-dependent transgene expression in Arabidopsis. *Plant Journal* **41**: 899–918.

von Dahl CC, Winz RA, Halitschke R, Kuhnemann F, Gase K, Baldwin IT. 2007. Tuning the herbivore-induced ethylene burst: the role of transcript accumulation and ethylene perception in *Nicotiana attenuata*. *Plant Journal* **51**: 293–307.

De Moraes CM, Lewis WJ, Pare PW, Alborn HT, Tumlinson JH. 1998. Herbivore-infested plants selectively attract parasitoids. *Nature* **393**: 570–573.

Dervinis C, Frost CJ, Lawrence SD, Novak NG, Davis JM. 2010. Cytokinin primes plant responses to wounding and reduces insect performance. *Journal of Plant Growth Regulation* **29**: 289–296.

Diezel C, Allmann S, Baldwin IT. 2011. Mechanisms of optimal defense patterns in *Nicotiana attenuata*: flowering attenuates herbivory-elicited ethylene and jasmonate signaling. *Journal of Integrative Plant Biology* **53**: 971–983.

Dobrev PI, Kamínek M. 2002. Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. *Journal of Chromatography A* **950**: 21–29.

van Doorn A, Bonaventure G, Rogachev I, Aharoni A, Baldwin IT. 2011. JA-Ile signalling in *Solanum nigrum* is not required for defence responses in nature. *Plant, Cell & Environment* **34**: 2159–2171.

Elzen GW. 1983. Cytokinins and insect galls. *Comparative Biochemistry and Physiology Part A: Physiology* **76**: 17–19.

Engelbrecht L, Orban U, Heese W. 1969. Leaf-miner caterpillars and cytokinins in the "green islands" of autumn leaves. *Nature* **223**: 319–321.

Erb M, Meldau S, Howe GA. 2012. Role of phytohormones in insect-specific plant reactions. *Trends in Plant Science* **17**: 250–259.

Gaquerel E, Gulati J, Baldwin IT. 2014. Revealing insect herbivory-induced phenolamide metabolism: from single genes to metabolic network plasticity analysis. *Plant Journal* **79**: 679–692.

Gaquerel E, Heiling S, Schoettner M, Zurek G, Baldwin IT. 2010. Development and validation of a liquid chromatography-electrospray ionization-time-of-flight mass spectrometry method for induced changes in *Nicotiana attenuata* leaves during simulated herbivory. *Journal of Agricultural and Food Chemistry* **58**: 9418–9427.

Gase K, Weinhold A, Bozorov T, Schuck S, Baldwin IT. 2011. Efficient screening of transgenic plant lines for ecological research. *Molecular Ecology Resources* **11**: 890–902.

Gilardoni P, Schuck S, Jungling R, Rotter B, Baldwin I, Bonaventure G. 2010. SuperSAGE analysis of the *Nicotiana attenuata* transcriptome after fatty acid-amino acid elicitation (FAC): identification of early mediators of insect responses. *BMC Plant Biology* **10**: 66.

Giron D, Frago E, Glevarec G, Pieterse CMJ, Dicke M. 2013. Cytokinins as key regulators in plant–microbe–insect interactions: connecting plant growth and defence. *Functional Ecology* **27**: 599–609.

Gonzalez-Rizzo S, Crespi M, Frugier F. 2006. The *Medicago truncatula* CRE1 cytokinin receptor regulates lateral root development and early symbiotic interaction with *Sinorhizobium meliloti*. *Plant Cell* **18**: 2680–2693.

Green TR, Ryan CA. 1972. Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. *Science* **175**: 776–777.

Großkinsky DK, Naseem M, Abdelmohsen UR, Plickert N, Engelke T, Griebel T, Zeier J, Novák O, Strnad M, Pfeifhofer H *et al.* 2011. Cytokinins mediate

resistance against *Pseudomonas syringae* in tobacco through increased antimicrobial phytoalexin synthesis independent of salicylic acid signaling. *Plant Physiology* **157**: 815–830.

Gruhn N, Heyl A. 2013. Updates on the model and the evolution of cytokinin signaling. *Current Opinion in Plant Biology* **16**: 569–574.

Grunewald W, Vanholme B, Pauwels L, Plovie E, Inze D, Gheysen G, Goossens A. 2009. Expression of the *Arabidopsis* jasmonate signalling repressor *JAZ1/TIFY10A* is stimulated by auxin. *EMBO Reports* **10**: 923–928.

Halitschke R, Stenberg JA, Kessler D, Kessler A, Baldwin IT. 2008. Shared signals – "alarm calls" from plants increase apparency to herbivores and their enemies in nature. *Ecology Letters* **11**: 24–34.

Halitschke R, Ziegler J, Keinanen M, Baldwin IT. 2004. Silencing of hydroperoxide lyase and allene oxide synthase reveals substrate and defense signaling crosstalk in *Nicotiana attenuata*. *Plant Journal* **40**: 35–46.

Hansen M, Chae HS, Kieber JJ. 2009. Regulation of ACS protein stability by cytokinin and brassinosteroid. *Plant Journal* **57**: 606–614.

Hatanaka A, Sekiya J, Kajiwara T. 1978. Distribution of an enzyme system producing *cis*-3-hexenal and *n*-hexanal from linolenic and linoleic acids in some plants. *Phytochemistry* **17**: 869–872.

Heidekamp F, Dirkse WG, Hille J, van Ormondt H. 1983. Nucleotide sequence of the *Agrobacterium tumefaciens* octopine Ti plasmid-encoded *tmr* gene. *Nucleic Acids Research* **11**: 6211–6223.

Herms DA, Mattson WJ. 1992. The dilemma of plants: to grow or defend. *The Quarterly Review of Biology* **67**: 283–335.

Hino F, Okazaki M, Miura Y. 1982. Effects of kinetin on formation of scopoletin and scopolin in tobacco tissue cultures. *Agricultural and Biological Chemistry* **46**: 2195–2202.

Hou X, Lee LY, Xia K, Yan Y, Yu H. 2010. DELLAs modulate jasmonate signaling via competitive binding to JAZs. *Developmental Cell* **19**: 884–894.

Hsieh HL, Okamoto H, Wang M, Ang LH, Matsui M, Goodman H, Deng XW. 2000. *FIN219*, an auxin-regulated gene, defines a link between phytochrome A and the downstream regulator COP1 in light control of *Arabidopsis* development. *Genes and Development* **14**: 1958–1970.

Hui D, Iqbal J, Lehmann K, Gase K, Saluz HP, Baldwin IT. 2003. Molecular interactions between the specialist herbivore *Manduca sexta* (lepidoptera, sphingidae) and its natural host *Nicotiana attenuata*: V. Microarray analysis and further characterization of large-scale changes in herbivore-induced mRNAs. *Plant Physiology* **131**: 1877–1893.

Jameson PE. 2000. Cytokinins and auxins in plant–pathogen interactions – an overview. *Plant Growth Regulation* **32**: 369–380.

Jones DH. 1984. Phenylalanine ammonia-lyase: regulation of its induction, and its role in plant development. *Phytochemistry* **23**: 1349–1359.

Jongsma M, Bakker P, Visser B, Stiekema W. 1994. Trypsin inhibitor activity in mature tobacco and tomato plants is mainly induced locally in response to insect attack, wounding and virus infection. *Planta* **195**: 29–35.

Kahl J, Siemens DH, Aerts RJ, Gabler R, Kuhnemann F, Preston CA, Baldwin IT. 2000. Herbivore-induced ethylene suppresses a direct defense but not a putative indirect defense against an adapted herbivore. *Planta* **210**: 336–342.

Kallenbach M, Alagna F, Baldwin IT, Bonaventure G. 2010. *Nicotiana attenuata* SIPK, WIPK, NPR1, and fatty acid-amino acid conjugates participate in the induction of jasmonic acid biosynthesis by affecting early enzymatic steps in the pathway. *Plant Physiology* **152**: 96–106.

Kallenbach M, Oh Y, Eilers EJ, Veit D, Baldwin IT, Schuman MC. 2014. A robust, simple, high-throughput technique for time-resolved plant volatile analysis in field experiments. *Plant Journal* **78**: 1060–1072.

Karley AJ, Douglas AE, Parker WE. 2002. Amino acid composition and nutritional quality of potato leaf phloem sap for aphids. *Journal of Experimental Biology* **205**: 3009–3018.

Kaur H, Heinzel N, Schöttner M, Baldwin IT, Gális I. 2010. R2R3-NaMYB8 regulates the accumulation of phenylpropanoid-polyamine conjugates, which are essential for local and systemic defense against insect herbivores in *Nicotiana attenuata*. *Plant Physiology* **152**: 1731–1747.

Kessler A, Baldwin IT. 2001. Defensive function of herbivore-induced plant volatile emissions in nature. *Science* **291**: 2141–2144.

Kojima M, Kamada-Nobusada T, Komatsu H, Takei K, Kuroha T, Mizutani M, Ashikari M, Ueguchi-Tanaka M, Matsuoka M, Suzuki K *et al.* 2009. Highly sensitive and high-throughput analysis of plant hormones using MS-probe modification and liquid chromatography-tandem mass spectrometry: an application for hormone profiling in *Oryza sativa*. *Plant and Cell Physiology* **50**: 1201–1214.

Krügel T, Lim M, Gase K, Halitschke R, Baldwin IT. 2002. *Agrobacterium*-mediated transformation of *Nicotiana attenuata*, a model ecological expression system. *Chemoecology* **12**: 177–183.

Kuiper D. 1993. Sink strength: established and regulated by plant growth regulators. *Plant, Cell & Environment* **16**: 1025–1026.

Balibrea Lara ME, Garcia MCG, Fatima T, Ehness R, Lee TK, Proels R, Tanner W, Roitsch T. 2004. Extracellular invertase is an essential component of cytokinin-mediated delay of senescence. *Plant Cell* **16**: 1276–1287.

López-Carbonell M, Moret A, Nadal M. 1998. Changes in cell ultrastructure and zeatin riboside concentrations in *Hedera helix*, *Pelargonium zonale*, *Prunus avium*, and *Rubus ulmifolius* leaves infected by fungi. *Plant Disease* **82**: 914–918.

Matsui K. 2006. Green leaf volatiles: hydroperoxide lyase pathway of oxylipin metabolism. *Current Opinion in Plant Biology* **9**: 274–280.

McKey D. 1974. Adaptive patterns in alkaloid physiology. *The American Naturalist* **108**: 305–320.

Meldau S, Erb M, Baldwin IT. 2012. Defence on demand: mechanisms behind optimal defence patterns. *Annals of Botany* **110**: 1503–1514.

Meldau S, Wu J, Baldwin IT. 2009. Silencing two herbivory-activated MAP kinases, SIPK and WIPK, does not increase *Nicotiana attenuata*'s susceptibility to herbivores in the glasshouse and in nature. *New Phytologist* **181**: 161–173.

Mujer CV, Smigocki AC. 2001. Cytokinin- and wound-inducible cytochrome P450 from *Nicotiana plumbaginifolia*. *Physiologia Plantarum* **111**: 172–181.

Novakova M, Motyka V, Dobrev PI, Malbeck J, Gaudinova A, Vankova R. 2005. Diurnal variation of cytokinin, auxin and abscisic acid levels in tobacco leaves. *Journal of Experimental Botany* **56**: 2877–2883.

Oh Y, Baldwin IT, Galis I. 2012. NaJAZh regulates a subset of defense responses against herbivores and spontaneous leaf necrosis in *Nicotiana attenuata* plants. *Plant Physiology* **159**: 769–788.

Ohnmeiss TE, McCloud ES, Lynds GY, Baldwin IT. 1997. Within-plant relationships among wounding, jasmonic acid, and nicotine implications for defence in *Nicotiana sylvestris*. *New Phytologist* **137**: 441–452.

Onkokesung N, Gaquerel E, Kotkar H, Kaur H, Baldwin IT, Galis I. 2012. MYB8 controls inducible phenolamide levels by activating three novel hydroxycinnamoyl-coenzyme A: polyamine transferases in *Nicotiana attenuata*. *Plant Physiology* **158**: 389–407.

Orozco-Cardenas M, McGurl B, Ryan CA. 1993. Expression of an antisense prosystemin gene in tomato plants reduces resistance toward *Manduca sexta* larvae. *Proceedings of the National Academy of Sciences, USA* **90**: 8273–8276.

Ozeki Y, Komamine A. 1986. Effects of growth regulators on the induction of anthocyanin synthesis in carrot suspension cultures. *Plant and Cell Physiology* **27**: 1361–1368.

Paschold A, Halitschke R, Baldwin IT. 2007. Co(i)-ordinating defenses: NaCOI1 mediates herbivore-induced resistance in *Nicotiana attenuata* and reveals the role of herbivore movement in avoiding defenses. *Plant Journal* **51**: 79–91.

Pils B, Heyl A. 2009. Unraveling the evolution of cytokinin signaling. *Plant Physiology* **151**: 782–791.

Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team. 2014. *nlme: linear and nonlinear mixed effects models*. R package, version 3.1-117. [WWW document] URL http://CRAN.R-project.org/package=nlme [accessed 26 March 2013].

R Core Team. 2014. *R: a language and environment for statistical computing*. Vienna, Austria: Foundation for Statistical Computing. [WWW document] URL http://www.R-project.org/ [accessed 9 June 2014].

Ratcliff F, Martin-Hernandez AM, Baulcombe DC. 2001. Technical advance. Tobacco rattle virus as a vector for analysis of gene function by silencing. *Plant Journal* **25**: 237–245.

Richmond AE, Lang A. 1957. Effect of kinetin on protein content and survival of detached *Xanthium* leaves. *Science* **125**: 650–651.

Riefler M, Novak O, Strnad M, Schmülling T. 2006. *Arabidopsis* cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. *Plant Cell* **18**: 40–54.

Rojo E, Leon J, Sanchez-Serrano JJ. 1999. Cross-talk between wound signalling pathways determines local versus systemic gene expression in *Arabidopsis thaliana*. *Plant Journal* **20**: 135–142.

Ruther J. 2000. Retention index database for identification of general green leaf volatiles in plants by coupled capillary gas chromatography–mass spectrometry. *Journal of Chromatography A* **890**: 313–319.

Saedler R, Baldwin IT. 2004. Virus-induced gene silencing of jasmonate-induced direct defences, nicotine and trypsin proteinase-inhibitors in *Nicotiana attenuata*. *Journal of Experimental Botany* **55**: 151–157.

Samalova M, Brzobohaty B, Moore I. 2005. pOp6/LhGR: a stringently regulated and highly responsive dexamethasone-inducible gene expression system for tobacco. *Plant Journal* **41**: 919–935.

Sano H, Seo S, Koizumi N, Niki T, Iwamura H, Ohashi Y. 1996. Regulation by cytokinins of endogenous levels of jasmonic and salicylic acids in mechanically wounded tobacco plants. *Plant and Cell Physiology* **37**: 762–769.

Schäfer M, Brütting C, Gase K, Reichelt M, Baldwin I, Meldau S. 2013. "Real time" genetic manipulation: a new tool for ecological field studies. *Plant Journal* **76**: 506–518.

Schäfer M, Meza-Canales ID, Navarro-Quezada A, Brütting C, Vanková R, Baldwin IT, Meldau S. 2014a. Cytokinin levels and signaling respond to wounding and the perception of herbivore elicitors in *Nicotiana attenuata*. *Journal of Integrative Plant Biology* **57**: 198–212.

Schäfer M, Reichelt M, Baldwin IT, Meldau S. 2014b. Cytokinin analysis: sample preparation and quantification. *Bio-Protocol* **4**: e1167.

Schuman MC, Barthel K, Baldwin IT. 2012. Herbivory-induced volatiles function as defenses increasing fitness of the native plant *Nicotiana attenuata* in nature. *Elife* **1**: e00007.

Schuman MC, Heinzel N, Gaquerel E, Svatos A, Baldwin IT. 2009. Polymorphism in jasmonate signaling partially accounts for the variety of volatiles produced by *Nicotiana attenuata* plants in a native population. *New Phytologist* **183**: 1134–1148.

Smigocki A, Heu S, Buta G. 2000. Analysis of insecticidal activity in transgenic plants carrying the *ipt* plant growth hormone gene. *Acta Physiologiae Plantarum* **22**: 295–299.

Smigocki A, Neal JW Jr, McCanna I, Douglass L. 1993. Cytokinin-mediated insect resistance in *Nicotiana* plants transformed with the *ipt* gene. *Plant Molecular Biology* **23**: 325–335.

Smigocki AC. 1995. Expression of a wound-inducible cytokinin biosynthesis gene in transgenic tobacco: correlation of root expression with induction of cytokinin effects. *Plant Science* **109**: 153–163.

Staswick PE, Tiryaki I, Rowe ML. 2002. Jasmonate response locus *JAR1* and several related Arabidopsis genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. *Plant Cell* **14**: 1405–1415.

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 Journal* **67**: 157–168.

Stork W, Diezel C, Halitschke R, Gális I, Baldwin IT. 2009. An ecological analysis of the herbivory-elicited JA burst and its metabolism: plant memory processes and predictions of the moving target model. *PLoS ONE* **4**: e4697.

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu GH, Nomura K, He SY, Howe GA, Browse J. 2007. JAZ repressor proteins are targets of the SCF^{COI1} complex during jasmonate signalling. *Nature* **448**: 661–662.

Van Dam N, Horn M, Mareš M, Baldwin I. 2001. Ontogeny constrains systemic protease inhibitor response in *Nicotiana attenuata*. *Journal of Chemical Ecology* **27**: 547–568.

Volfová A, Chvojka L, Friedrich A. 1978. The effect of kinetin and auxin on the chloroplast structure and chlorophyll content in wheat coleoptiles. *Biologia Plantarum* **20**: 440–445.

Wang L, Allmann S, Wu J, Baldwin IT. 2008. Comparisons of LIPOXYGENASE3- and JASMONATE-RESISTANT4/6-silenced plants reveal that jasmonic acid and jasmonic acid-amino acid conjugates play different roles in herbivore resistance of *Nicotiana attenuata*. *Plant Physiology* **146**: 904–915.

Wasternack C. 2007. Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Annals of Botany* **100**: 681–697.

Werner T, Schmülling T. 2009. Cytokinin action in plant development. *Current Opinion in Plant Biology* **12**: 527–538.

Wu JQ, Hettenhausen C, Meldau S, Baldwin IT. 2007. Herbivory rapidly activates MAPK signaling in attacked and unattacked leaf regions but not between leaves of *Nicotiana attenuata*. *Plant Cell* **19**: 1096–1122.

Yan Y, Stolz S, Chételat A, Reymond P, Pagni M, Dubugnon L, Farmer EE. 2007. A downstream mediator in the growth repression limb of the jasmonate pathway. *Plant Cell* **19**: 2470–2483.

Zavala JA, Patankar AG, Gase K, Hui DQ, Baldwin IT. 2004. Manipulation of endogenous trypsin proteinase inhibitor production in *Nicotiana attenuata* demonstrates their function as antiherbivore defenses. *Plant Physiology* **134**: 1181–1190.

Zd'arska M, Zatloukalova P, Benitez M, Sedo O, Potesil D, Novak O, Svacinova J, Pesek B, Malbeck J, Vasickova J *et al.* 2013. Proteome analysis in Arabidopsis reveals shoot- and root-specific targets of cytokinin action and differential regulation of hormonal homeostasis. *Plant Physiology* **161**: 918–930.

Zhang SQ, Klessig DF. 1997. Salicylic acid activates a 48-kD MAP kinase in tobacco. *Plant Cell* **9**: 809–824.

Zuur AF, Ieno EN, Walker NJ, Saveliev AA, Smith GM. 2009. *Mixed effects models and extensions in ecology with R.* New York, NY, USA: Springer.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Plasmid vector for cytokinin receptor silencing.

Fig. S2 ir*chk2/3* and ir*chk2/3*-2 plants are silenced in *CHASE-DOMAIN CONTAINING HIS KINASE 2* (*NaCHK2*) and *NaCHK3* expression.

Fig. S3 Increased cytokinin concentrations are not sufficient to up-regulate the herbivory-induced transcript accumulation of *NaMYB8*, *NaDH29* and *NaCV86*.

Fig. S4 Increased cytokinin concentrations increase the accumulation of monoacyl-putrescines and -spermidines.

Fig. S5 Increased cytokinin concentrations affect the content of soluble proteins and the transcript accumulation of *TRYPSIN PROTEASE INHIBITOR* (*NaTPI*).

Fig. S6 Impaired cytokinin perception by CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2) and NaCHK3 attenuates caffeoylputrescine accumulation.

Fig. S7 CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2) and NaCHK3 mediated cytokinin perception is not necessary for SIPK and WIPK activation after wounding and herbivory.

Fig. S8 *CHASE-DOMAIN CONTAINING HIS KINASE 2* (*NaCHK2*) and *NaCHK3*-silenced plants show only minor phenotypic differences compared with wild-type plants.

Fig. S9 *CHASE-DOMAIN CONTAINING HIS KINASE 2* (*NaCHK2*) and *NaCHK3*-silenced plants show only minor changes in the concentration of active cytokinins compared with wild-type plants.

Fig. S10 Impaired cytokinin perception reduces jasmonic acid accumulation, but not jasmonic acid–isoleucine conjugate accumulation.

Fig. S11 Impaired cytokinin perception reduces the herbivory-induced accumulation of *NaMYB8*, *NaDH29* and *NaCV86* transcripts.

Fig. S12 Impaired cytokinin perception reduces the herbivory-induced accumulation of phenolamides.

Fig. S13 The independently transformed *CHASE-DOMAIN CONTAINING HIS KINASE 2* (*NaCHK2*) and *NaCHK3*-silenced plants (ir*chk2/3*-2) confirm cytokinin signaling-mediated effects on herbivory-induced defense responses.

Fig. S14 The independently transformed *CHASE-DOMAIN CONTAINING HIS KINASE 2* (*NaCHK2*) and *NaCHK3*-silenced plants (ir*chk2/3*-2) confirm cytokinin signaling-mediated effects on the herbivory-induced accumulation of phenolamides.

Fig. S15 Cytokinin concentrations and their perception by CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2) and NaCHK3 do not regulate herbivory-induced ethylene release.

Fig. S16 Impaired cytokinin perception reduces the systemic accumulation of monoacyl-putrescines and -spermidines.

Fig. S17 Cytokinin perception by CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2) and NaCHK3 does not regulate the systemic increase of trypsin proteinase inhibitor (TPI) activity.

Table S1 Sequences used for gene silencing of *Nicotiana attenuata* cytokinin receptors

Table S2 Sequences of primers used for quantitative (q)PCR

Table S3 Settings for MicroToF *post* run analysis for phenolamide quantification in positive ionization mode

Table S4 Settings for gas chromatography–thermal desorption–mass spectrometry (GC-TD-MS) analysis

Please note: Wiley Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.