Cytokinins shape plant-herbivore interactions in *Nicotiana attenuata*

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1 GENERAL INTRODUCTION

Plants make up the basis of most terrestrial food webs due to their function as primary producers. Plants are capable of using light energy and inorganic matter to produce biomass which is then consumed by herbivores. Vegetation is defining and framing ecosystems and drastically shapes the appearance of our planet. Regarding the multitude of herbivores, it is not self-evident that our planet is as green as it is. The total depletion of plants by herbivores is usually an exception. Although cases where plant populations are erased completely are possible, most plants remain more or less intact during their life. In 1960, a theory about community structure, population control and competition became famous as the "Green-World-Hypothesis"(Hairston *et al.* 1960). Hairston claimed that our world would not be as green as it is, if there would be no top-down limitation of herbivores by predators. Although predation may be the major limiting factor for most herbivores, plants themselves are not as defenseless as they seem at first sight. They can promote the attraction of predators by production of herbivore induced plant volatiles (HIPVs; Kessler and Baldwin 2001, Schuman *et al.* 2012) and have developed a variety of defense and tolerance mechanisms against herbivores (Schuman and Baldwin 2016).

Phytophagous insects make up the biggest and probably most heterogeneous group amongst herbivores. Often their relationship with the host plant is highly interwoven and characterized by a high specialization of the insect. The relationship of plants with herbivores is not only a hostile relation. Many plants are indeed dependent on some of their herbivores to produce fertile offspring. Herbivores are often not only devastating consumers or transmitters of diseases but at the same time also function as pollinators or disperse seeds of the plants.

The multiple interactions with insects require a tight control of attractants for pollinators, defense metabolites against herbivores and investment in growth and seed-production. In that

regard, phytohormones play a key role in the regulation of a plant's metabolism and its responses to herbivorous insects (Erb *et al.* 2012).

In my thesis I will use the model plant *Nicotiana attenuata* and two of its most abundant herbivores *Manduca sexta* and *Tupiocoris notatus* to demonstrate how plant-insect interactions and in particular plant-herbivore interactions are shaped by cytokinins (CKs), a group of plant hormones. I will show, how CKs are involved in the modulation of anti-herbivore defenses and that they can shape the optimal distribution of defense metabolites. I will show that CKs are able to even influence feeding preferences of insects and that a free living, sap feeding insect is capable of manipulating the host plant's metabolism by injecting CKs.

1.1 Plant-herbivore interactions

Plants are constantly attacked by phytophagous insects. We can assume that the history of insects consuming plants is almost as old as the history of plants and insects itself. The first evidence of terrestrial arthropod herbivores dates back to around 400 million years ago (Labandeira 2007). Since then, herbivorous insects and plants have been in a constant evolutionary arms race leading to specializations and adaptations on both sides.

Plants developed defense mechanisms to defend against herbivores, which range from physical barriers, like thick cuticles and thorns, over the production of toxic or anti-digestive plant metabolites to the indirect defense by attraction of predators through the emission of HIPVs (Schuman and Baldwin 2016).

The production of defenses is energy consuming and resource demanding, and can impair a plant's growth and seed production in the case that the plants do not face an attack by herbivores; however it may provide an advantage in the presence of herbivores (Baldwin 1998). This drawback of defense production is partially circumvented, as some defense metabolites are not constitutively expressed, but only produced on demand after herbivore perception.

The distinction of herbivory from mechanical wounding, which would not require defense activation, occurs through herbivory associated elicitors (HAEs) which are present in oral secretions, oviposition secretions or are degradation products of plant tissue (Mithofer and Boland 2008, Hilker and Meiners 2010, Bonaventure 2014). Several compounds have been shown to act as HAEs, such as fatty acid amino acid conjugates (FACs; Alborn *et al.* 1997, Bonaventure *et al.* 2011), disulfoxy fatty acids(caelifierins; Alborn *et al.* 2007), peptides released from digested plant proteins, like from the γ-subunit of the chloroplast ATPase (Schmelz *et al.* 2006), pectins and oligogalacturonides from cell wall degradation (Bishop *et al.* 1981), as well as enzymes like β-glucosidase (Hopke *et al.* 1994, Mattiacci *et al.* 1995) or lipases (Schäfer *et al.* 2011). The perception of HAEs leads to changes in several signaling pathways in the plant (Wu and Baldwin 2010, Bonaventure 2014), including changes in membrane potential and Ca^{2+} influx (Maffei *et al.* 2004), reactive oxygen species (Orozco-Cardenas and Ryan 1999), mitogen activated protein

kinases (MAPK; Wu *et al.* 2007) as well as in the levels of phytohormones like jasmonic acid (JA), salicylic acid and ethylene (Schmelz *et al.* 2009).

JA and JA-dependent signaling cascades have been identified as the key players in responses to herbivore attack (reviewed in Howe and Jander 2008, Wu and Baldwin 2010). Levels of JA are increased after herbivory and converted to a bioactive conjugate with isoleucine (JA-Ile) through JASMONATE RESISTANT (JAR) enzymes (Wang *et al.* 2007, Suza and Staswick 2008). JA-Ile binds to CORONATIN INSENSITIVE 1 (COI1) of the Skp/Cullin/F-box complex - SCF^{COI1} -leading to the ubiquitination of JASMONATE ZIM-DOMAIN (JAZ) proteins and their subsequent degradation by the 26S proteasome (Chini *et al.* 2007, Yan *et al.* 2007, Oh *et al.* 2012). The degradation of JAZ proteins leads then to an activation of JA responsive genes, as they are repressors of transcription activators like MYC2 (Kazan and Manners 2008).

Plants are capable of detecting different types of damage and different types of HAEs and respond differently to it (Voelckel and Baldwin 2004, Diezel *et al.* 2009, Erb, et al. 2012). The differentiated answer to different herbivores requires more than a simple unidirectional response cascade, but more likely a regulatory network connecting the JA signaling with other signaling cascades involving also other phytohormones (reviewed in Erb, et al. 2012). Considering this, we showed in **manuscript I,** how CK concentrations and CK related transcripts are influenced by herbivore feeding. In **manuscript II,** we show how CKs are modulating transcripts and levels of herbivore induced defenses and how they influence the JA signaling.

The variability of responses to herbivory may also be due to adaptations to the huge variability of feeding strategies and specializations of insects. The most obvious cases of herbivores are chewing herbivores which remove parts of the plant tissue like leaves, flowers, seeds, stem or roots. Piercing-sucking insects do not cause such an obvious damage, even though its effect can be as devastating as those of chewing herbivores. Some of them, like aphids are specialized phloem feeders that extract nutrient rich photosynthate, others are feeding on cell-content or the apoplast by regurgitating digestive enzymes and sucking on solved plant content.

The majority of the chewing, as well as the piercing sucking insects, are able to move more or less freely on the plant, between plants and sometimes even between species. However, some insects have evolved an endophytic lifestyle within the plant tissue itself. Those endophytic insects usually spend their larval stages, within the plant. This live style requires high specialization as the insect is highly dependent on the chosen host plant. There are insects living in the stem pith of the plant, like *Trichobaris mucorea* in tobacco stalks (Diezel *et al.* 2011b). Some are living in leaf tissue, like leaf-miners. And others are living in specialized organs, called galls, whose development was triggered by the endophytic insects itself. At least in the case of leaf-miners and gall forming insects, it has been demonstrated that the insects are able to increase the quality of their nearby plant habitat (reviewed in Giron *et al.* 2016): Leaf-miners are known to cause the phenomenon called green islands around their feeding sites, which are green areas of

increased photosynthetic activity and higher nutrient content in senescent leaves (Engelbrecht 1968, Behr *et al.* 2010, Kaiser *et al.* 2010, Body *et al.* 2013). Gall-forming insects are even capable of creating a new plant organ, which is metabolically active, rich in nutrients and usually features lower levels of defense compounds by reprogramming the expression of plant genes (e.g. Hartley 1998, Nabity *et al.* 2013). Both types of insects, gall formers and leaf-miners, have been shown to manipulate the plant metabolism to their own benefit via phytohormone-dependent processes (Engelbrecht 1968, Engelbrecht *et al.* 1969, Elzen 1983, Giron, et al. 2016).

In **manuscript VI,** I show that the strategy of manipulating the plant metabolism via phytohormones might not only be a strategy used by endophytic insects, but also of piercing-sucking herbivores. I show that the free living piercing-sucking herbivore *T. notatus* manipulates its host plant *N. attenuata* likely via direct injection of CKs.

1.2 Plant defense theories

To budget resources, plants have to regulate and minimize their investment in costly defense production. I just laid out in the last section of this introduction, how defense metabolites are only produced after induction by herbivore attack and adapted to the attacker. Besides their production on demand, plant defenses undergo further developmental regulation. To maximize its fitness in nature, a plant always has to balance its limited resources between growth, development and reproduction on one hand and plant defenses on the other hand. Several theories have been established in the past to explain this developmental regulation of defenses (reviewed in Stamp 2003, Meldau *et al.* 2012, Schuman and Baldwin 2016) These theories do not exclude each other but overlap and focus on different aspects.

Besides other important theories like the carbon:nutrient balance (CNB) hypothesis (Bryant *et al.* 1983, Tuomi *et al.* 1988) or the growth rate (GR) hypothesis (Coley *et al.* 1985), the most influential theories have been the growth-differentiation-balance (GDB) hypothesis (Herms and Mattson 1992) and the optimal defense (OD) theory (McKey 1974, Rhoades 1976). CNB, GR and GDB focus on the physiological parameters and resource availability, whereas the OD theory focuses on the functional aspect of defense distribution.

The CNB hypothesis states that nutrient availability from the environment determines the form and ratio of defense metabolites via the carbon:nitrogen ratio (Bryant, et al. 1983, Tuomi, et al. 1988).

The GR hypothesis (Coley, et al. 1985) states that the production of defense metabolites is dependent on the inherent growth rates of the plant, which is in turn dependent on the resource availability. If resources are limited, slow growth rates are favored over fast growth rates. Slow to intermediate growth rates in turn favor large investments in defenses, whereas fast growth rates are associated to low investment in defense.

The GDB hypothesis (Herms and Mattson 1992) divides plant activity on a cellular level in growth related processes and differentiation related processes, which include the production of defense metabolites. Growth and differentiation need to be balanced in a trade-off, as differentiation processes divert resources from the production of new leaf area and differentiation processes on the other side are constrained by cell-division and enlargement processes. As resource availability constrains both, growth and differentiation processes, the largest investment should occur at intermediate growth rates.

Other than CNB, GR and GDB theories, the OD theory includes a functional level and evolution theory to explain the deployment and distribution of plant defenses (Rhoades 1979). The OD theory claims that the kind of defenses and its distribution that evolved in a particular plant might reflect the threats a particular plant or plant part faces or has faced during evolution (McKey 1974, Rhoades 1976). It claims that 1) production of plant defenses is costly as it diverts resources from growth and reproduction and 2) that defenses and their allocation evolved in a way that maximizes a plant's fitness in a specific environment. Focusing on a single plant, the main observation is that plant defenses are unequally distributed in different plant parts. The OD theory predicts that the investment in defenses in a particular plant part is positively correlated with its risk of facing herbivore attack and its value for the plants fitness. Regarding leaves of an annual plant, this usually means that younger leaves should be better protected than older leaves. Younger leaves usually provide a higher value to the plant, as their remaining contribution to a plants carbon fixation at a given time is bigger than those of older leaves (Harper 1989, Barto and Cipollini 2005).

Distributions of defenses predicted by the OD theory have been reported for many plant species and different types of defense metabolites. In various plant species it has been shown that alkaloids like nicotine follow an OD distribution (James 1950, Mothes 1955, Ohnmeiss *et al.* 1997, Ohnmeiss and Baldwin 2000, Kariñho-Betancourt *et al.* 2015). Other direct defenses like iridoid glycosides in *Plantago lanceolata* (Bowers and Stamp 1992), cyanogenic glycosides in *Eucalyptus cladocalyx* (Gleadow and Woodrow 2000), xanthotoxin in *Pastinaca sativa* (Zangerl and Rutledge 1996), terpenoids in *Solidago altissima* (Heath *et al.* 2014), terpenoid aldehydes in cotton plants (Anderson and Agrell 2005), glucosinolates in *Arabidopsis thaliana* (Brown et al. 2003) as well as other phenolic compounds in the seagrass *Posidonia oceanica* (Agostini et al. 1998) and *N. attenuata* (Onkokesung et al. 2012) all show within plant or within developmental patterns supporting the OD predictions: more defenses in more important tissues, like reproductive tissue and young leaves. Also the distribution of indirect defenses, namely volatile organic compounds in *Phaseolus lunatus* and *Ricinus communis* have been shown to be higher in younger leaves (Radhika *et al.* 2008).

Although there have been many confirmations of the OD theory, the underlying mechanisms regulating the developmental distribution of defenses remained elusive due to the

lack of possible manipulations (Meldau, et al. 2012). In **manuscript III**, I show how inducible defenses in *N. attenuata* follow a distribution predicted by the OD theory and how these distributions can be changed by genetically changing natural distributions of CKs. This study may provide a possible tool to further explore the mechanisms behind OD. In **manuscript IV**, we show that Myb8 an R2/R3 MYB transcriptional activator that was known to regulate phenolamides (Kaur *et al.* 2010), which follow OD predictions, is also involved in the regulation of other defenses following OD predictions like trypsin proteinase inhibitors (TPI), as well as a threonine deaminase (TD). Myb8 might play an important role in the regulation of defenses following an optimal distribution.

1.3 Cytokinins

CKs are a group of plant hormones that are involved in the control of numerous fundamental biological processes in plants (Werner and Schmülling 2009, Kaminek 2015). Their discovery goes back to the observations that certain compounds in the phloem sap of several plant-species could induce the division – or cytokinesis - of potato parenchyma cells (Haberlandt 1913). It took more than 40 years to isolate and identify the first CK, kinetin from herring sperm (Miller *et al.* 1955a, Miller *et al.* 1955b, Miller *et al.* 1956) which is a secondary oxidation product from DNA (Hall and Deropp 1955, Barciszewski *et al.* 1997). The first natural plant CK discovered was *trans*-zeatin, named after the plant species *Zea mays* where it was isolated from immature maize kernels (Letham 1963, Miller 1965, Letham 1966). Since then several members of this group of hormones have been discovered (see for example Sakakibara 2006).

Natural plant CKs are adenine derivatives carrying a sidechain at the N6-position (see figure 1). The most commonly found plant CKs are carrying an isprenoid sidechain, although in some cases also CKs with an aromatic sidechain are reported (Horgan *et al.* 1973, Strnad 1997). The naturally occurring, isoprene containing CK bases are *trans*-zeatin (*t*Z), *cis*-zeatin (*c*Z), dihydrozeatin (DHZ) and N6-isopentenyladenine (IP). Plant tissue also contains the ribosylated forms of those CKs, *t*ZR, *c*ZR, DHZR and IPR, as well as various glycosylated forms which are CK inactivation and degradation products (Sakakibara 2006).

CKs and especially the free bases occur in tiny amounts, down to a femto-molar range, making it hard to quantify them. Effective and economic ways for quantification with mass spectrometry have developed in the last two decades. In my studies, I used a CK extraction and quantification method based on the method published by Dobrev and Kaminek (2002) and Kojima *et al.* (2009). This method is based on a solid-phase extraction procedure and detection using LC-MS/MS. We developed this extraction method further and combined it with the detection of more than 100 plant metabolites in a high-throughput extraction procedure on a 96 sample scale (Schäfer *et al.* 2016).

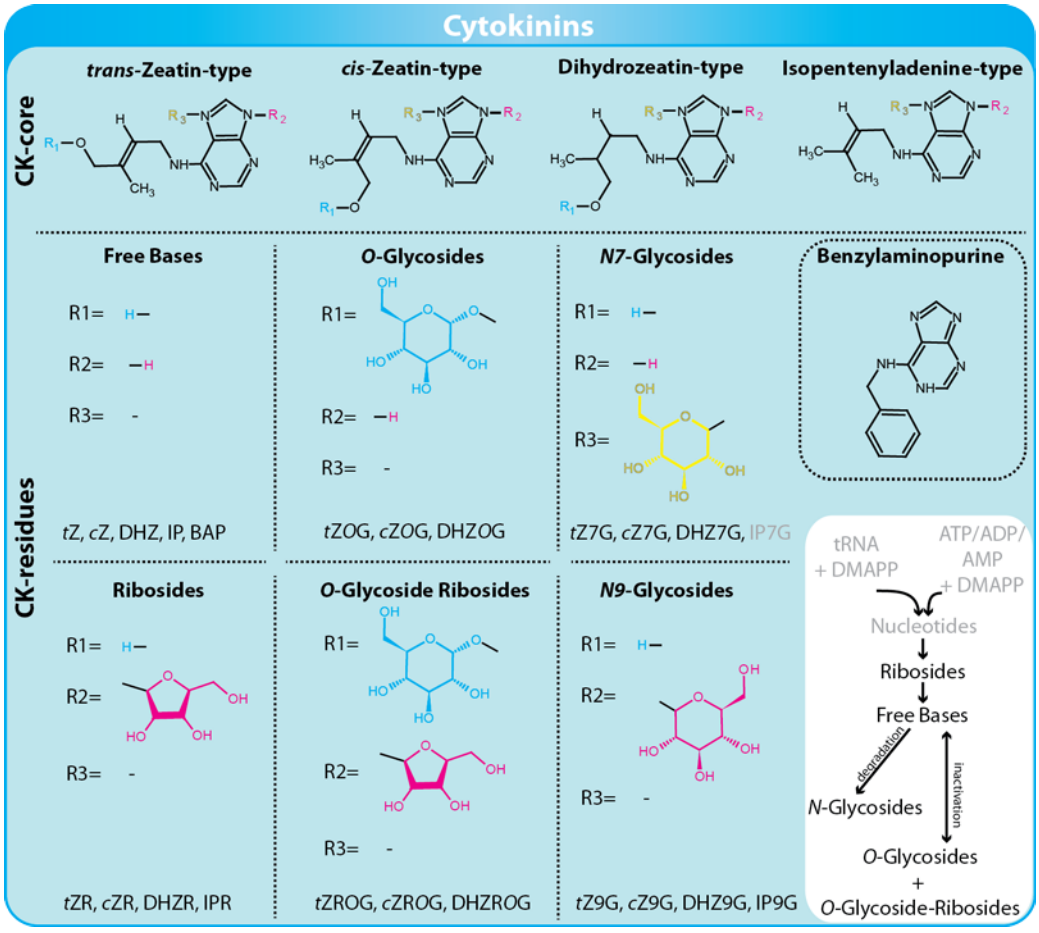

Figure 1: Overview over the cytokinins and the cytokinin-pathway

Known isoprenoid plant cytokinins (CKs) and the synthetic CK benzylaminopurine. The top line shows the CK core and the position of possible residues on the core. Below there is an overview on the different CKs that base on each of these four CK cores. The bottom right shows an overview of the CK-pathway. This figure is part of a figure that I designed for the manuscript of Schäfer, et al. (2016).

Isoprenoid CKs consist of an adenine and an isoprene moiety. The adenine-part can originate from AMP, ADP or ATP or from tRNAs (Sakakibara 2006). If AMP, ADP and ATP are used as isoprene acceptors, adenosine phosphate-isopentenyltransferases (IPTs) catalyze the addition of the isoprene moiety to adenosine and forms CK nucleotides (di-, tri-, and monophosphates; Kakimoto 2001, Takei *et al.* 2001, Miyawaki *et al.* 2006). If tRNA is the

isoprene acceptor, tRNA-isopentenyltransferases are catalyzing the addition of the isoprene (Miyawaki, et al. 2006). *c*ZR and its base *c*Z are thought to be synthesized from tRNA whereas *t*Z, DHZ and IP are synthesized from adenosine (Miyawaki, et al. 2006). Nevertheless, also IPR and *t*ZR have been found in tRNA hydrolysates (Taller 1994). The isoprene moiety can originate either from the methylerythriol phosphate (MEP) or the mevalonate pathway (MVA). Isoprenes used by tRNA-IPT usually originate from MVA pathway, whereas those used by adenosine-IPTs originate mostly from MEP pathway (Kasahara *et al.* 2004).

The di- and tri-phosphates originating from isoprenylation are dephosphorylated by phosphatases subsequently to monophosphates. The monophosphates are then converted by the cytokinin nucleoside 5'-monophosphate phosphoribohydrolases (LOGs) to the ribosides (Kurakawa *et al.* 2007), which can be converted to the free bases.

CKs can be degraded by CK-oxidases (CKX) to adenine or adenosine and the corresponding aldehyde of the side-chain (Whitty and Hall 1974, Brownlee *et al.* 1975, Mok and Mok 2001). Inactivation of CKs occurs reversibly to the *O*-glucoside by the zeatin-*O*-glucosyltransferase (ZOGT) and irreversibly to *N*-glucosides by the CK-*N*-glucosyltransferase (CK-N-GT; Brzobohaty *et al.* 1993). The occurring CK glucosides have so far not been proven to have a biological activity and are therefore generally considered to be inactive.

The free bases have been shown to have the highest binding affinity to the CK receptors, making it by definition the active forms of CKs (Lomin *et al.* 2015). In classical tests for CK activity also some of the ribosides have shown biological activity and there are also reports that ribosides can bind to the receptors as well (Spichal *et al.* 2004, Yonekura-Sakakibara *et al.* 2004, Stolz *et al.* 2011). In activity tests *t*Z and IP have been identified as the most active CKs, whilst DHZ and *c*Z show lower to almost no activity. The binding affinities of particular CKs to specific receptors vary amongst different plant species and receptors (Lomin *et al.* 2012). Nevertheless, it became clear that also *c*Z and *c*ZR, which are often much more abundant than other CKs, might play important roles in plants as well (Gajdosova *et al.* 2011a, Gajdosova *et al.* 2011b, Großkinsky *et al.* 2013). In a review article we summarized the current knowledge about the role of *cis*-type CKs in plant growth regulation and its role in responses to environmental factors (Schäfer *et al.* 2015). My contribution was to review the current knowledge about the role of *c*Z in plant growth and development.

Changes in CK levels are perceived through CHASE (cyclase/histidine kinase associated sensing extracellular) domain containing hisitidine kinases (CHKs; Yamada *et al.* 2001) as part of a His-to-Asp phosphorelay (Gruhn and Heyl 2013). Via hisitidine-containing phosphotransfer proteins (HPTs) type-B response regualtors (RRB) are activated and act as transcription factors (Hwang *et al.* 2012) to induce the expression of CK responsive genes, including type-A response regulators (RRAs).

CKs are named after the first discovered function, the cytokininesis or promotion of cell division. This is what has been defined as the "classical" function of CKs and where many tests of CK-function, for example with oat leaves or tobacco pith, are based on (reviewed in Gyulai and Heszky 1994). Due to their cell-division stimulating function, synthetic CKs like 6-benzylaminopurine are widely used in plant cell culture. Until now, CKs have been associated to many other biological processes in plants, especially in growth and development (Werner and Schmülling 2009). CKs have been shown to regulate cell-cycle control (Frank and Schmülling 1999), regulate apical dominance (Werner *et al.* 2003) and meristem function (Kurakawa, et al. 2007, Kyozuka 2007), they inhibit senescence (Richmond and Lang 1957, Gan and Amasino 1995, Ori *et al.* 1999), are involved in nutrient homeostasis and source-sink relationships (Roitsch and Ehness 2000, Mok and Mok 2001) and have been shown to play important roles in responses to abiotic and biotic stresses including responses to pathogens and herbivores (Smigocki *et al.* 2000, Argueso *et al.* 2009, Dervinis *et al.* 2010, Werner *et al.* 2010, Reguera *et al.* 2013). CKs often act in concert with and mainly as antagonists of auxins (Müller and Leyser 2011). Other studies have revealed crosstalk of CKs and CK-dependent signaling pathways with several other plant hormones (Robert-Seilaniantz *et al.* 2011, Durbak *et al.* 2012, Naseem *et al.* 2012, El-Showk *et al.* 2013, Meza-Canales *et al.* 2016).

The fact that CKs have such tremendous effects on a plants development and growth makes manipulations, especially transgenic manipulations very difficult. CK manipulations often cause massive developmental effects and often lead to lethal genotypes (e.g. Klee *et al.* 1987). Also the fact that CKs are used in cell-culture and callus-induction makes a manipulation of the CK perception critical, as this already interferes with established transformation techniques. In the past, some strategies have been developed to circumvent these obstacles. Besides external application of CKs, IPT genes have been expressed under the control of stress- or developmentally induced promoters (Smigocki *et al.* 1993, Gan and Amasino 1995, Jordi *et al.* 2000, Qin *et al.* 2011). In my studies, I used three different transgenic approaches to achieve CK manipulations with a minimal interference with the plants development: In **manuscript II and VI,** I used *N. attenuata* plants with two of the three known CK-receptors silenced (ir*chk2-3*) to explore the role of CK perception and signaling on plant herbivore interactions. In **manuscript III**, I was using transgenic *N. attenuata* plants with a senescence activated IPT from *Arabidopsis* (*SAG-IPT4*). This construct is inactive until the plants start their reproductive phase. This enables a normal development of the plant until flowering. With this construct we could examine the effects of increased CK production in old plant parts that usually feature low CK levels, on its interaction with herbivores. In **manuscript III and V,** I used transgenic plants with an IPT under the control of a chemically-inducible pOp6/LhGR expression system (i-ov*ipt*). This construct is inactive until induced by the elicitor dexamethasone (DEX). I used these plants to examine the

influence of locally and temporally restricted increase in CK production on the interaction with herbivores.

1.4 Cytokinins and insects - a special case for endophytes?

After the first natural CKs have been identified (Letham 1963), it only took a few years, until the first evidence for the involvement of CKs in plant-insect interactions were found in the late sixties. CK activity has been detected in leaf-gall tissue and later confirmed to be caused by high levels of CKs in this tissue (Matsubar.S and Nakahira 1967, Elzen 1983). Almost at the same time increased levels of CKs have been found in green islands around the feeding sites of leaf-miners (Engelbrecht 1968, Engelbrecht, et al. 1969). Green islands and leaf-galls share that they can be caused by endophytic insects. Endophytic insects are tightly bound to their hostplant and need to control the conditions in the hostplant, as they are unable to move to other plants and need to assure their nutrient supply. The assurance of their nutrition requires a metabolically active tissue locally around their feeding sites. It has been shown that leaf-miners and gall-forming insects are able to hijack the plants metabolism and locally increase the levels of photosynthesis and nutrients and – in case of gall-forming insects – even cause the formation of an entirely new plant organ (Stone and Schönrogge 2003, Shorthouse *et al.* 2005, Behr, et al. 2010, Body, et al. 2013, Giron, et al. 2016, Zhang *et al.* 2016). As processes like inhibition of senescence, increase of photosynthesis and generation of sinks have been accounted to CKs, it seems obvious that CKs are considered as a main factor in this manipulation.

Indeed higher levels of CKs have not only been found in gall-tissue and green-islands, but also have been found in high amounts in the body and salivary glands of gall-forming insects and leaf-mining insects (Engelbrecht, et al. 1969, Matsui *et al.* 1975, Mapes and Davies 2001, Dorchin *et al.* 2009, Straka *et al.* 2010, Yamaguchi *et al.* 2012, Body, et al. 2013, Tanaka *et al.* 2013). As some bacteria and microorganisms were also known to produce CKs, and gall- and green island formation can also be caused by microorganisms, it was hypothesized that CKs in insects might be produced by endosymbiotic bacteria (Kaiser, et al. 2010). Studies with *Phyllonorycter blancardella* demonstrated that antibiotic treatment of the insects eliminated the green islands effect caused by the insect (Body, et al. 2013). This provides strong evidence, that endosymbiotic bacteria in the insects provide high amounts of CKs that are transferred by the insect to the plant to manipulate the host-plants metabolism. The transfer of CKs to the plant has been generally assumed, but a rigorous test has been elusive so far.

Furthermore, studies on the CK-mediated manipulation of plant metabolism have so far only focused on endophytic herbivores, which represent some very specialized cases. Only little is known about the role of CKs in the interaction of plants with free living herbivorous insects. It has been shown that CKs can influence plant defenses against herbivores and pathogens (Smigocki, et al. 1993, Smigocki, et al. 2000, Choi *et al.* 2010, Grosskinsky *et al.* 2011, Argueso

et al. 2012, Grosskinsky *et al.* 2016) and also prime plant-defense responses against herbivores (Dervinis, et al. 2010). But nothing was known about the capability of free-living insects to manipulate the plants metabolism similarly to an endophytic insect. In my thesis, I analyzed the multiple roles of CKs in the interaction between plants and herbivores using the ecological model plant *N. attenuata* and two free living natural herbivores: *M. sexta* and *T. notatus*. In **manuscript I,** we show how that wounding and herbivore-associated elicitors are modulating the plants CK levels and CK signaling responses. In **manuscript II**, we show that changes in CK levels or CK signaling in the plant influences the plants anti-herbivore defense. In **manuscript III**, I show that CKs are sufficient to alter developmental gradients of defense metabolites in the plant and therefore play an important role in the establishment of optimal defense regulation. In **manuscript V,** I show that increased levels of CKs do not only increase levels of defense metabolites, but also increase the attractiveness to *T. notatus*. In **manuscript VI,** I demonstrate that the free living insect *T. notatus* contains high amounts of the CK IP similar to endophytic insects. With ^{15}N-labeling experiments I could show that these insects are able to transfer IP to the leaves through their oral secretions. I hypothesize that *T. notatus* is also able to manipulate its host plant and that CK mediated manipulation of the host plant is a far more widespread phenomenon that does not only occur in endophytes.

1.5 Nicotiana attenuata

In my studies, I used the model plant *N. attenuata,* a wild tobacco species native to the southwestern part of North-America (figure 2). This plant naturally grows in arid habitats and germinates in nitrogen rich soils in the first years after fires as its germination is triggered by smoke-derived germination cues (Baldwin *et al.* 1994). As a pioneering plant in post-fire habitats, it is exposed to a variety of different herbivores and has evolved a great arsenal of anti-herbivore defenses. Due to its exceptional ecological niche and its untouched genome that never underwent plant breeding by humans, this plant has evolved as a model plant for ecological studies and in particular for plant-herbivore interactions.

This annual plant has been well studied in its molecular and ecological interactions with herbivores, especially the specialist *M. sexta* (Baldwin 1998, Baldwin 1999, Ohnmeiss and Baldwin 2000, Halitschke *et al.* 2001, Kessler and Baldwin 2002, Paschold *et al.* 2007, Wu and Baldwin 2010). The plant became famous for the research done on HIPVs as indirect defenses (Kessler and Baldwin 2001, Schuman, et al. 2012), as well as the production of toxic and anti-digestive compounds upon induction by herbivore attack. *N. attenuata* produces a set of various defense metabolites that have been proven to feature a protective function against herbivores. The best studied defense metabolite is nicotine (e.g. Steppuhn *et al.* 2004). Furthermore anti-digestive petides, like trypsin proteinase inhibitors (TPI; Zavala *et al.* 2004) or *N*-acetylated polyamines

(phenolamides; PAs) like caffeoylputrescine (CP; Kaur, et al. 2010) have been characterized in *N. atteunata*.

In the past two decades, more than 200 transgenic plants have been created and a genome has been sequenced (Xu *et al.* 2017). Many genomic, metabolomics and transcriptomic tools are available (Brockmöller *et al.* 2017), including transcriptomes of the most abundant herbivores (Crava *et al.* 2016) and recombinant inbred lines from different ecotypes. This toolbox together with the possibility for experiments with transgenic plants in the natural habitat made it a useful tool in exploring a plant's ecological interactions with its abiotic and biotic environment.

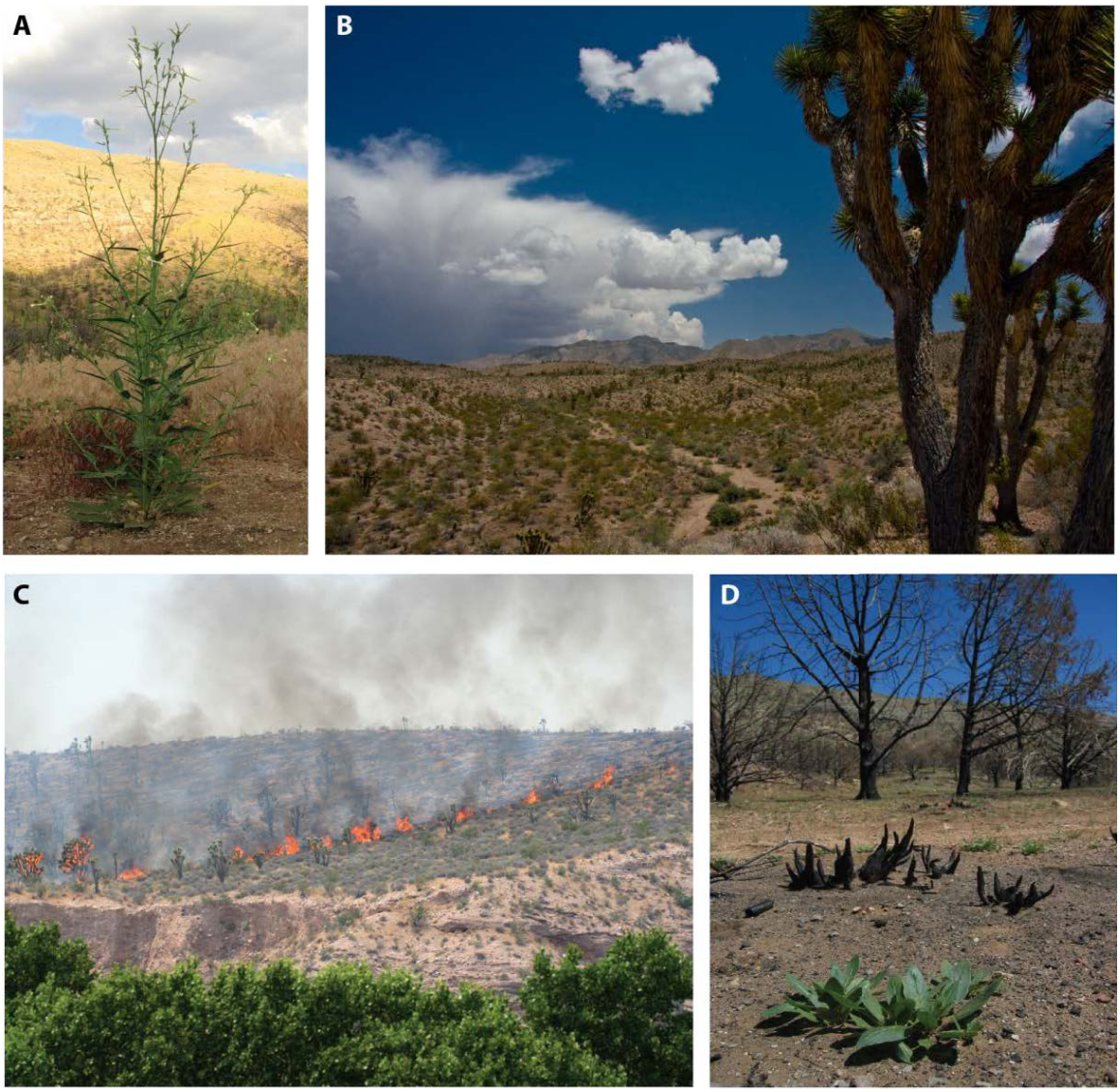

Figure 2: *Nicotiana attenuata* and its natural habitat

A shows a flowering *N. attenuata* plant in its natural environment. **B** shows a typical arid habitat in the Great Basin desert in south-western Utah, USA**. C** shows a bushfire that is necessary to promote the germination of *N. attenuata*. **D** shows a young *N. attenuata* plant in a post-fire environment. Pictures: A, C, D: Danny Kessler, B: Christoph Brütting

1.6 Manduca sexta *and* Tupiocoris notatus

N. attenuata faces the attack of a tremendous amount of different phytophagous insects in nature. Due to its character as a pioneering plant in post-fire environments it provides a potential food-source for many insects. In previous studies, many of those interactions with specialized insects like *M. sexta*, leafhoppers, mirids, stem borers, negro bugs and others have been described (Heidel and Baldwin 2004, Kessler and Baldwin 2004, Kallenbach *et al.* 2012, Lee *et al.* 2016, Stanton *et al.* 2016, Adam *et al.* 2017). Amongst those herbivores, *T. notatus* is probably the most abundant herbivore in the natural environment and *M. sexta* is, together with its close relative *M. quinquemaculata,* the insect species which can cause the most severe damage.

N. attenuata's interaction with *M. sexta,* the tobacco hawk moth or Carolina sphinx moth, is certainly the best explored interaction. *M. sexta* is a sphingid Lepidopteran moth (figure 3) and plays a two-sided role for *N. attenuata.* Whilst the adult moth functions as pollinator for the plant, the larvae, named tobacco hornworms, are feeding on the plants. The eggs get laid on the abaxial side of leaves of *N. attenuata,* as well as other tobacco and solanaceous plants like tomato or *Datura.* The larvae grow from about 1 mg up to around 5 g in about two weeks, passing 5 larval stages. In the early larval stages the caterpillars are especially prone to predation. Predators of the larvae are attracted by HIPVs that are emitted in response to herbivory, making HIPVs a form of indirect defense against *M. sexta* (Schuman, et al. 2012).

The larvae are inducing JA dependent defenses triggered by compounds present in their oral secretions, the FACs (Halitschke, et al. 2001, Halitschke *et al.* 2003, Kallenbach *et al.* 2010). *M. sexta* is resistant or tolerant to some toxic compounds produced by *N. attenuata,* including nicotine (Wink and Theile 2002). Nonetheless if defense compounds like nicotine (Voelckel *et al.* 2001, Steppuhn, et al. 2004), PAs (Kaur, et al. 2010) or TPIs (Zavala, et al. 2004) are not produced or JA is not perceived by the plant (Paschold, et al. 2007), the growth rates of the larvae are increased. Studies have demonstrated that *M. sexta* larvae can metabolize and excrete nicotine and can even use nicotine for their own protection against predators (Kumar *et al.* 2014).

Due to the size of the later larval instars and the adults, as well as the relative ease of rearing in laboratories, *M. sexta* has established as a model system not only in plant-herbivore interactions but also in neurobiology. I used *M. sexta* or *M. sexta* oral secretions to induce herbivore elicited defense mechanism in *N. attenuata* in **manuscripts I, II, III, IV and V**.

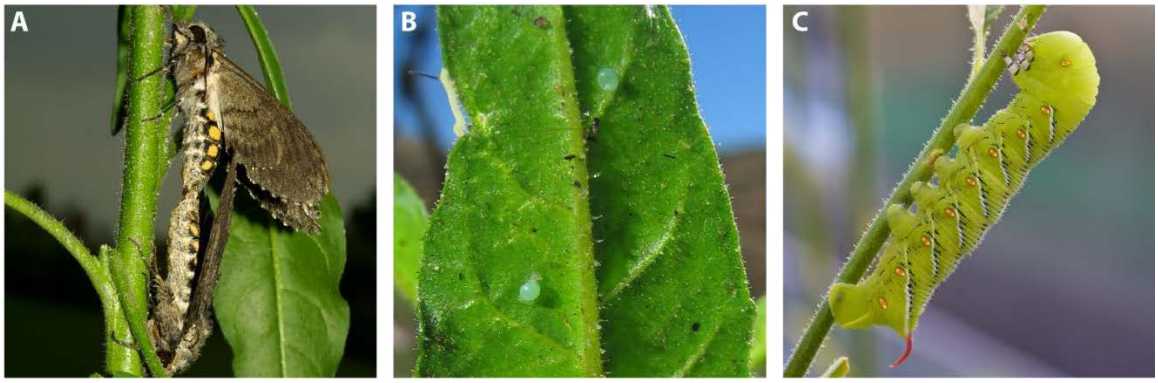

Figure 3: *Manduca sexta* in different developmental stages

A shows mating adult moths. **B** shows eggs and a neonate larva on the abaxial side of a leaf from *N. attenuata***. C** shows an almost fullygrown caterpillar. Pictures: A, B: Danny Kessler, C: Christoph Brütting

The other herbivore I worked with in **manuscripts V and VI**, *T. notatus* is a small mirid bug (figure 4), feeding exclusively on tobacco and sometimes closely related solanaceous plants like *Datura.* It is part of the family Miridae in the suborder Heteroptera. Adult insects reach a length of about 3 mm and a weight of about 0.5 mg. Typical for Heteroptera mirids have a hemimetabolous development with five nymphal stages. Eggs are laid under the epidermal layer of stem and midveins (figure 4). All nymph stages as well as adults are piercing sucking sap-feeders. The total life cycle of the insects is – depending on the conditions - around 30 days. Using field observations and camera monitoring, I could find out that *T. notatus* is mainly night active in nature (Joo *et al.* submitted). Mirids are feeding mainly on the abaxial side of the leaf and are very mobile on the plant, with adults are even able to fly. If disturbed they are hiding on the bottom of the stem below the rosette leaves and come back to their feeding sites later. They prefer to feed on young growing leaf tissue such as the young stem leaves, apical buds as well as axial buds as I will show in **manuscript VI**. Generally, their interaction with *N. attenuata* is less explored as the interaction of *M. sexta*. Nonetheless, it is known that they are inducing defense metabolites similar to *M. sexta* (Kessler and Baldwin 2004). So far only few traits that affect *T. notatus'* performance on *N. attenuata* have been discovered, like UV dependent 17-hydroxygeranyllinalool diterpene glycoside production (Dinh *et al.* 2013). Generally, mirids are colonizing plants independently from the plants JA production (Kessler and Baldwin 2001). In a recent study, we could show using an RNAseq approach, that *T. notatus* is expressing genes for detoxification (Crava, et al. 2016) which could provide an explanation for their resistance. In **manuscript V**, we found another trait that influences mirid feeding preferences. We show that increasing levels of CKs using a DEX-inducible IPT construct (i-ov*ipt*) is attractive to mirids and suffers from more damage in the field. In **manuscript VI,** we then also show that impairing CK perception using transgenic ir*chk2/3* plants is decreasing the attractiveness to mirids.

T. notatus damage is less obvious and less detrimental to the plant than damage by *M. sexta*. In fact, in studies in the natural environment *T. notatus* feeding did not decrease the plants seed production (Kessler and Baldwin 2004). The feeding leads to chlorotic spots on the leaves but the loss of photosynthetic tissue seems to be compensated by a higher photosynthetic rate in undamaged tissue which is induced by a compound in the oral secretions of the insect (Halitschke *et al.* 2011). In **manuscript VI,** I could show that mirids contain the CK IP in their body and oral secretions and are able to transfer it to the plant. I hypothesize that this might be the compound leading to the previously observed increase in photosynthesis and that this strategy to actively increase the food quality is a strategy previously only known from endophytes.

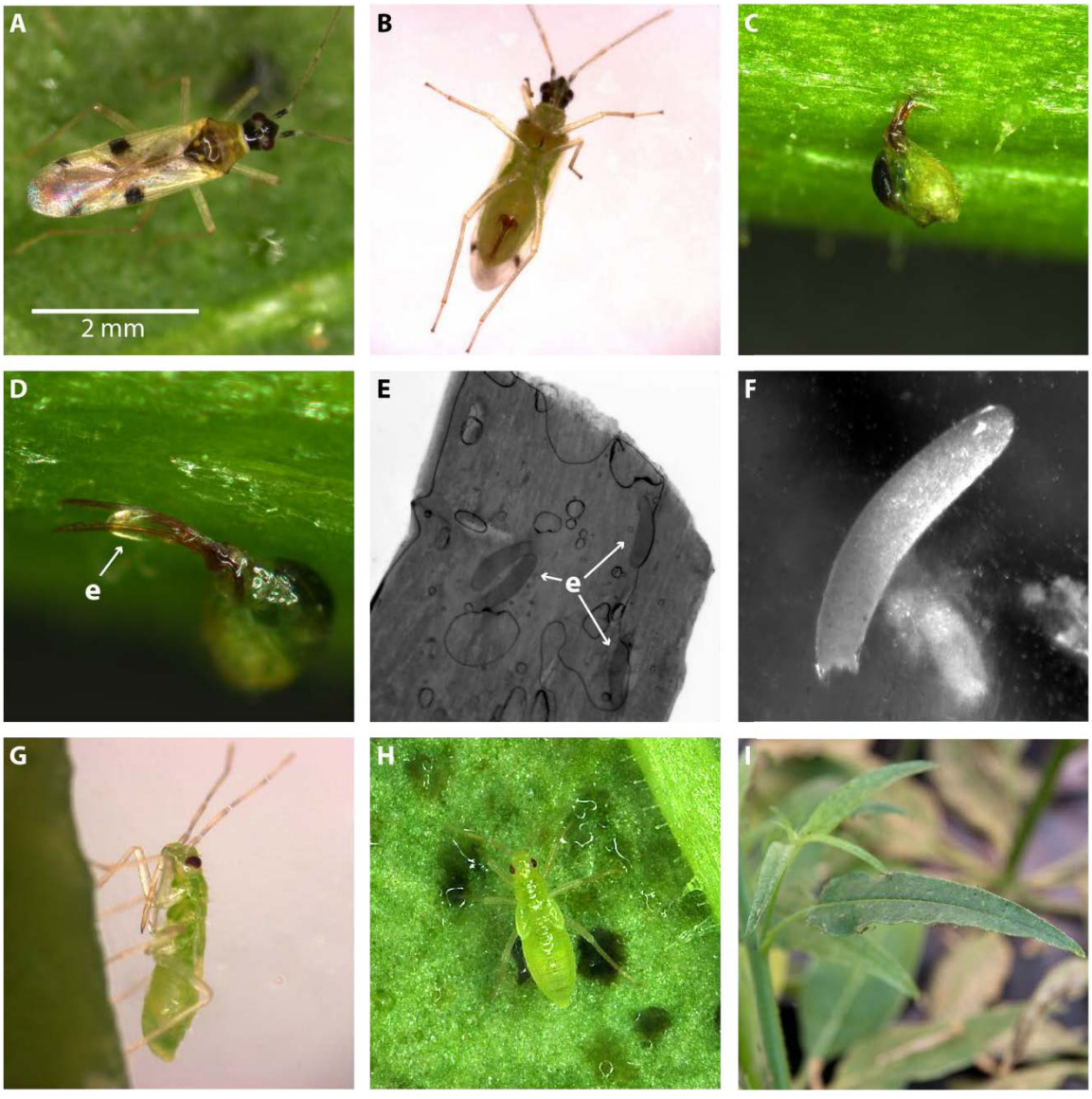

Figure 4: *Tupiocoris notatus*

A Adult from the top and **B** adult female from below**. C** shows the ovipositor sticking in the epidermal layer of the stem of a *N. attenuata* plant. **D** shows an egg (**e**) still sticking in the ovipositor. **E** shows eggs in a preparation of the epidermal layer of the stem of *N. attenuata*. **F** shows an egg dissected from an insect. **G** and **H** show nymphs of *T. notatus*. **I** shows typical damage caused by *T. notatus* feeding on leaves of *N. attenuata*. Pictures: Christoph Brütting

1.7 Objective of the thesis

This thesis will shed light on the variety of influences CKs and CK-dependent signaling has on the interaction of *N. attenuata* and two of its major herbivores, *M. sexta* and *T. notatus*. Specifically, I will show how CKs are modulating defense responses upon herbivore attack by *M. sexta* and how they can alter optimal developmentally regulated distributions of defense metabolites in the plant. I will show that high CK levels in leaves increase its attractiveness to *T. notatus* and that the insect itself is capable of injecting CKs in the plant. For this purpose, I used state of the art analytical and molecular biological tools to give answers to classical questions from plant-herbivore research like the regulation of optimal defense patterns in plants and the role of phytohormones in regulation of plant-insect interactions.

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2 MANUSCRIPT OVERVIEW AND AUTHOR'S CONTRIBUTIONS

Manuscript I:

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

Martin Schäfer, Ivan D. Meza-Canales, Aura Navarro-Quezada, Christoph Brütting, Radomira Vanková, Ian T. Baldwin and Stefan Meldau

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In manuscript I, we describe the cytokinin (CK) pathway of *N. attenuata* and its response to wounding and herbivore perception. We found complex changes throughout the CK pathway after wounding and herbivore perception in treated local leaves, as well as in the adjacent, untreated leaves and the roots. JA pathway manipulations revealed that this key regulator of herbivory-induced responses was not necessary for herbivory-induced CK pathway changes but even suppressed the CK-signaling response. Interestingly, CK pathway responses to herbivory were also found in *A. thaliana*.

MS designed and performed experiments, analyzed data and drafted the manuscript. IDMC designed and performed experiments for systemic CK pathway changes, analyzed data and corrected the manuscript. ANQ designed and performed the phylogenetic experiments and helped with drafting the manuscript. CB contributed to the experimental design, analyzed data and corrected the manuscript. RV contributed analytical tools for the CK measurement and corrected the manuscript. ITB coordinated the study and helped with drafting the manuscript. SM initiated and coordinated the study, performed experiments, analyzed data and helped with drafting the manuscript.

Manuscript II:

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, Ivan D. Meza-Canales, Christoph Brütting, Ian T. Baldwin and Stefan Meldau

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In manuscript II, we analyzed the influence of the CK pathway on the herbivory-induced defenses of *N. attenuata*. Increased CK levels enhanced the herbivory-induced accumulation of phenolamides (Pas), such as caffeoylputrescine (CP) and partially also the induced trypsin proteinase inhibitor (TPI) activity. The analysis of transgenic plants silenced in two of the three histidin kinases that are involved in CK perception (NaCHK2 and NaCHK3) revealed that a functional CK pathway is indispensable for the regular induction of these defense responses. Interestingly, impaired CK signaling also attenuates the systemic accumulation of CP in response to simulated herbivory.

MS designed and performed experiments, analyzed data and drafted the manuscript. IDMC designed and performed experiments with the stable CK receptor silenced plants, analyzed data and corrected the manuscript. CB contributed to the experimental design, performed external CK application experiments, analyzed data and corrected the manuscript. ITB coordinated the study, designed experiments and helped with drafting the manuscript. SM initiated and coordinated the study, performed experiments, analyzed data and helped to draft the manuscript.

Manuscript III:

Changes in cytokinins are sufficient to alter developmental patterns of defense metabolites in *Nicotiana attenuata*

Christoph Brütting, Martin Schäfer, Radomira Vanková, Klaus Gase, Ian T. Baldwin and Stefan Meldau

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In manuscript III, we demonstrate that CKs modulate ontogeny-dependent defenses in *N. attenuata*. We found that distribution of inducible defense metabolites like CP and TPI and associated transcripts following predictions made by the optimal defense theory (ODT) with higher levels in young leaves and low levels in old leaves. Interestingly, CK levels highly correlated with inducible defenses. We genetically manipulated the developmental patterns of two different cytokinin classes by using senescence- and chemically-inducible expression of cytokinin biosynthesis genes. Genetically modifying the levels of different cytokinins in leaves was sufficient to alter ontogenic patterns of defense metabolites: We could recover inducibility of defenses in old leaves.

CB designed and performed the experiments, analyzed the data and drafted the manuscript. MS contributed to the experimental design, helped with experiments with chemically-inducible plants and CK measurements and helped with drafting the manuscript. RV measured CKs of the senescence inducible plants and corrected the manuscript. KG generated the transgenic constructs and corrected the manuscript. ITB contributed to the design of the study, and helped with drafting the manuscript. SM initiated and coordinated the project, contributed to the experimental design and helped with drafting the manuscript.

Manuscript IV:

***NaMYB8* regulates distinct, optimally distributed herbivore defense traits**

Martin Schäfer, Christoph Brütting, Shuqing Xu, Zihao Ling, Anke Steppuhn, Ian T. Baldwin and Meredith C. Schuman

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In manuscript IV, we show that multiple defenses regulated by the R2/R3 MYB transcriptional activator NaMYB8 meet predictions by the ODT. NaMYB8 has been described before as a specific regulator of PA accumulation. Interestingly, we discovered that transcriptional regulation of biochemically very distinct TPIs and a threonine deaminase (TD) also depend on MYB8 expression. Induced distributions of PAs, TD and TPIs all meet predictions of optimal defense theory: their leaf concentrations are highest in young tissues, which have the highest fitness value and probability of attack. We suggest that these defensive compounds have evolved to be co-regulated by MYB8.

MS designed and performed experiments, analyzed data and drafted the manuscript. CB designed and performed experiments, analyzed data and helped to draft the manuscript. SX conducted promoter-motif and microarray analysis and helped to draft the manuscript. ZL conducted promoter-motif analysis and corrected the manuscript. AS provided first data on a regulation of TPI by MYB8 and helped to draft the manuscript. ITB initiated the project, contributed to the design of the study and helped to draft the manuscript. MCS coordinated the project, performed experiments and helped to draft the manuscript.

Manuscript V:

'Real time' genetic manipulation: a new tool for ecological field studies

Martin Schäfer, Christoph Brütting, Klaus Gase, Michael Reichelt, Ian T. Baldwin and Stefan Meldau

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In manuscript V, we established a method for chemically-inducible gene expression and gene silencing in *N. attenuata* that is also applicable under field conditions. The method was evaluated by spatial, temporal and quantitative controlled expression, among others of an isopentenyltransferase, thereby also providing the tool for fine-tuned manipulation of endogenous CK levels used in manuscript II, III and VI. The analysis of CK-mediated effects on the natural herbivore community revealed a positive correlation between the CK level and the damage inflicted by the specialist herbivore *T. notatus.*

MS designed and performed experiments, established the method for CK measurements, analyzed data and drafted the manuscript. CB designed and performed experiments with *T. notatus*, helped with CK measurement development, analyzed data and helped to draft the manuscript. KG coordinated the transformation and screening of the plants and corrected the manuscript. MR established the method for CK measurements (mass spectrometry) and corrected the manuscript. ITB initiated and coordinated the study and helped to draft the manuscript. SM initiated and coordinated the study, performed experiments, established the method for CK measurements and helped to draft the manuscript.

Manuscript VI:

Cytokinin transfer by the free living insect *Tupiocoris notatus* to its host-plant *Nicotiana attenuata* recapitulates a strategy of endophytic insects

Christoph Brütting, Cristina M. Crava, Martin Schäfer, Meredith C. Schuman, Stefan Meldau and Ian T. Baldwin

In preparation for eLIFE

In manuscript VI, we analyzed the CK-dependent interaction of the free-living cell-content feeding herbivore *Tupiocoris notatus* with its hostplant *N. attenuata*. *T. notatus* attack elicits increases in transcripts related to CK degradation and decreases in biosynthetic genes suggesting active CK manipulation. Surprisingly also high levels of the CK 6-isopentenyladenine (IP) were found in *T. notatus* bodies and saliva. Using ^{15}N-isotope labeling experiments we could prove that IP could be transferred from the insect to the plant in high amounts. Stable nutrient levels in attacked leaves, as well as a reduced attractiveness of plants with silenced CK receptors to mirids suggest an important role of CK injection to mirids; a strategy so far only known from endophytic insects.

CB initiated the project, designed and performed experiments, analyzed data and drafted the manuscript. CMC contributed to the experimental design and performed experiments, analyzed data and helped to draft the manuscript. MS contributed to the experimental design performed experiments and helped to draft the manuscript. MCS and SM contributed to the experimental design and corrected the manuscript. ITB coordinated the project, contributed to the experimental design and helped to draft the manuscript.

3 MANUSCRIPTS

3.1 *Manuscript I*

JIPB Journal of Integrative Plant Biology

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

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Research Article

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*-zeatin 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*; cytokinin; 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). Transcriptional studies in *N. attenuata* identified the transcripts of the CK-induced gene 2 (*CIG2*) and CK-regulated kinase 1 (*CRK1*) to be induced by *M. sexta* feeding and FACs, respectively, indicating that the CK pathway may play a role in plant responses to herbivores (Hui et al. 2003; Gilardoni et al. 2010). Although *CRK1* was previously shown to be negatively regulated by CKs, it was also reported to be responsive to auxin and abscisic acid and the function of this receptor kinase in hormone signaling was only hypothesized (Schäfer and Schmülling 2002). *CIG2*, in contrast, is not responsive to other tested phytohormones except CKs, which act as positive regulators (Kimura et al. 2001). It is questionable how far the information about *CIG2* and *CRK1*, which were derived from hormone application experiments with cell cultures reflect the *in vivo* responses to endogenous CK dynamics. Most claims in the literature on the response of the CK pathway to HAMP perception or defoliation by insect herbivores are based on the indirect evidence of Hui et al. (2003) and Gilardoni et al. (2010), whereas less is known about actual changes in CK biosynthesis, metabolites and signaling elements.

Cytokinin metabolism and signaling is complex and a simplified overview is provided in Figure 1 (abbreviations are summarized in Table S1). In brief, CKs are synthesized by the transfer of an isopentenyl moiety to an adenosine (di/tri) phosphate or tRNA. This rate-limiting step is catalyzed by isopentenyltransferases (IPTs), whereas the CK 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), dihydrozeatin (DHZ), and their ribosides (IPR, tZR, cZR, and DHZR, respectively) can bind to specific CK receptors (Stolz et al. 2011; Lomin et al. 2012; Shi and Rashotte 2012); a class of partially redundant CHASE (cyclase/histidine kinase associated sensing extracellular) domain-containing histidine kinases (CHKs). After CK binding and auto-phosphorylation of a conserved His, the phosphoryl group is transferred to another conserved Asp and transmitted to histidine-containing phosphotransfer proteins (HPTs), which finally phosphorylate-specific response regulators (RRs) responsible for the output of the CK-pathway (Hwang et al. 2012). While the type-B RRs (RRB) function as transcription factors, the type-A RRs (RRA) are known as negative feedback regulators of the CK pathway (Hwang et al. 2012). Various glucosyltransferases (e.g., ZOG and UGT) are responsible for the formation of reversible inactivation products like *O*-glucoside (~OG) and riboside *O*-glucoside (~ROG) or irreversible inactivation products such as N^7-glucoside (~7G) and N^9-glucoside (~9G), whereas CK degradation is carried out by CK oxidase/dehydrogenases (CKX; Mok and Mok 2001; Schmülling et al. 2003). Many of these genes involved in CK metabolism and signaling were found to be transcriptionally regulated in response to CKs,

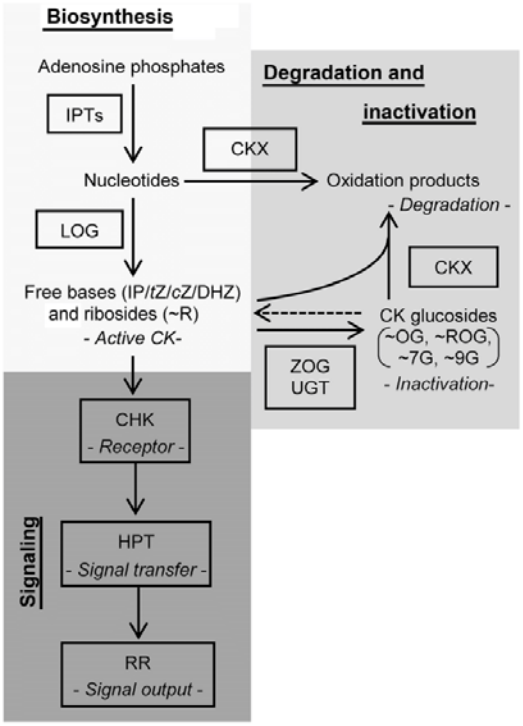

Figure 1. Cytokinin metabolism and signaling
Cytokinins (CK) biosynthesis is initiated by the activity of isopentenyltransferases (IPT). Monophosphorylated CK nucleotides can be directly converted into free bases by the activity of cytokinin nucleoside 5′-monophosphate phosphoribohydrolases (LOG). Active CKs such as isopentenyladenine (IP), *trans*-zeatin (tZ), *cis*-zeatin (cZ), and dihydrozeatin (DHZ) and their respective ribosides (~R) can be inactivated by CK oxidases/dehydrogenases (CKX) or by the formation of sugar conjugates like *O*-glucosides (~OG), riboside *O*-glucosides (~ROG), N^7-glucosides (~7G), and N^9-glucosides (~9G) through the activity of glycosyltransferases, 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.

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*, *LOG*), 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 phosphorelay. 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 (Halitschke 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 for 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 strong 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.

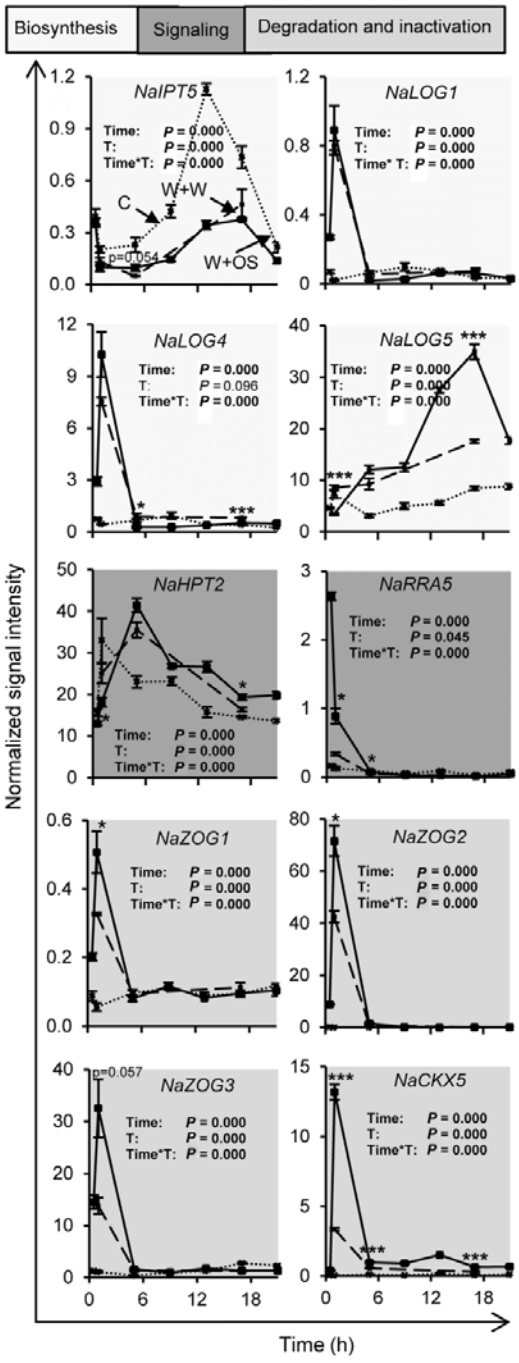

Figure 2. Continued.

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: $^{*}P \leq 0.05$, $^{***}P \leq 0.001$). 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.

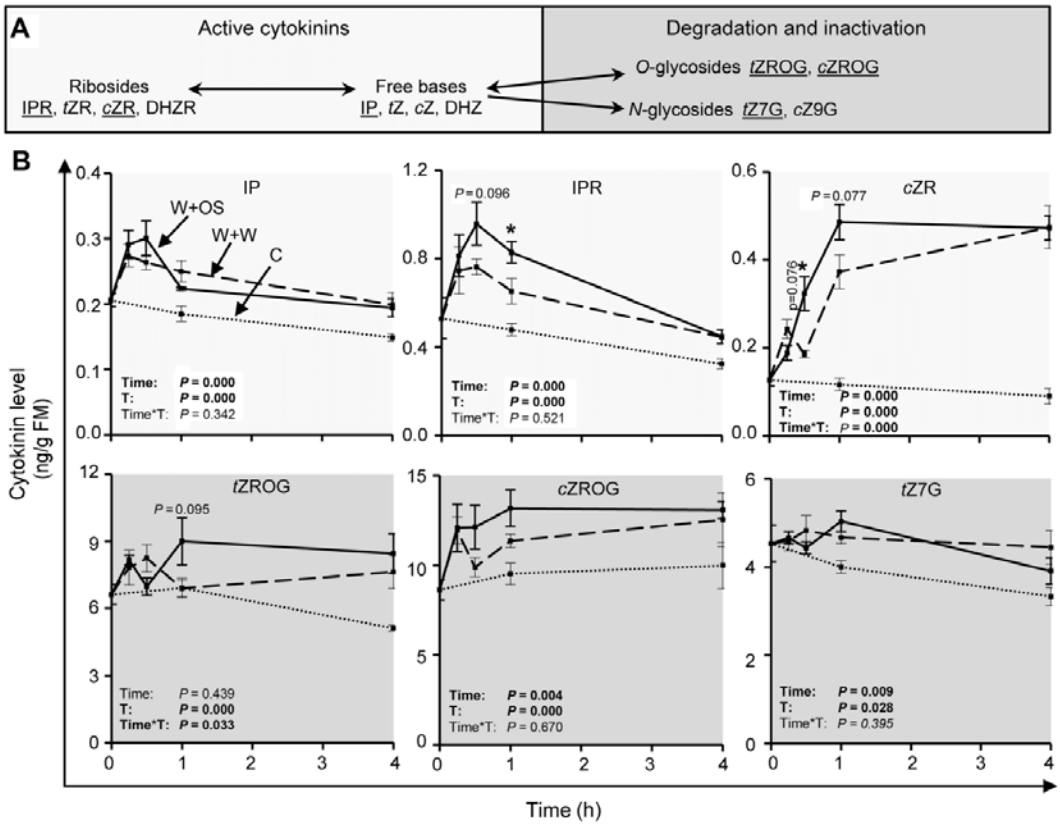

Figure 3. Wounding- and herbivory-induced changes in cytokinin levels
(**A**) Cytokinin metabolism overview. (**B**) Isopentenyladenine (IP), isopentenyladenosine (IPR), *cis*-zeatin riboside (cZR), *trans*-zeatin riboside *O*-glucoside (tZROG), *cis*-zeatin riboside *O*-glucoside (cZROG), and *trans*-zeatin *N*7-glucoside (tZ7G) levels in 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, W + W and W + OS; T) effects and their interaction (Time*T) were analyzed by univariate ANOVA, except for cZROG data which 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: $*P \leq 0.05$). Error bars are standard errors ($n = 5$). For additional cytokinins see Figure S12. FM, fresh mass.

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 (OS_{GH}). While *A. thaliana* leaves do not respond to FACs, they do perceive OS_{GH}, 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. OS_{GH} application to puncture wounds significantly elevated the W + W-induced changes in IPR, cZ, and cZR levels and marginally increased cZROG levels. W + OS_{GH} treatment resulted in 2.3, 18.1, 7.3, and 2.1 times as much IPR, cZ, cZR,

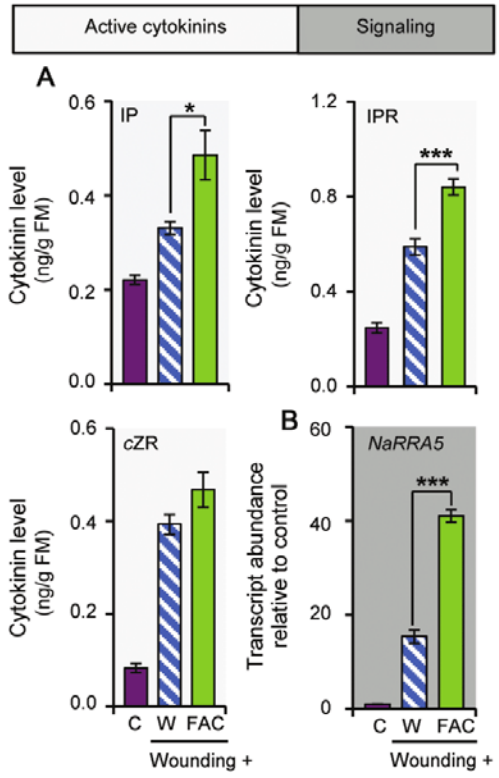

Figure 4. Fatty acid-amino acid conjugates increase active cytokinin levels and signaling
(**A**) Isopentenyladenine (IP), isopentenyladenosine (IPR), and *cis*-zeatin riboside (cZR) levels in *Nicotiana attenuata* 30 min after wounding and application of water (W) or the fatty acid-amino acid conjugate (FAC) *N*-linolenoyl-glutamate to the puncture wounds, as well as in untreated control leaves (C). (**B**) Response regulator 5 (*NaRRA5*) transcript accumulation under the conditions described in (**A**). Asterisks indicate significant differences between FAC application compared to water applications to wounded leaves (independent samples *t*-test: $*P \leq 0.05$, $***P \leq 0.001$). Error bars are standard errors ($n = 5$). FM, fresh mass.

and cZROG accumulation, respectively, when compared to control levels (Figure 8).

DISCUSSION

Here, we addressed previous speculations about the responsiveness of CKs to herbivore attack (Giron et al. 2013), by conducting an analysis of the CK pathway in the Solanaceous plant, *N. attenuata*. We show that the CK pathway responds to wounding and perception of HAMPs in local and systemic tissues and demonstrate its intimate interaction with the JA 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, S11). 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 *NaCKX5* 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 tZ-type CKs (Takei et al. 2004), the postulated zeatin *cis-trans* isomerase (Bassil et al. 1993) or ß-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

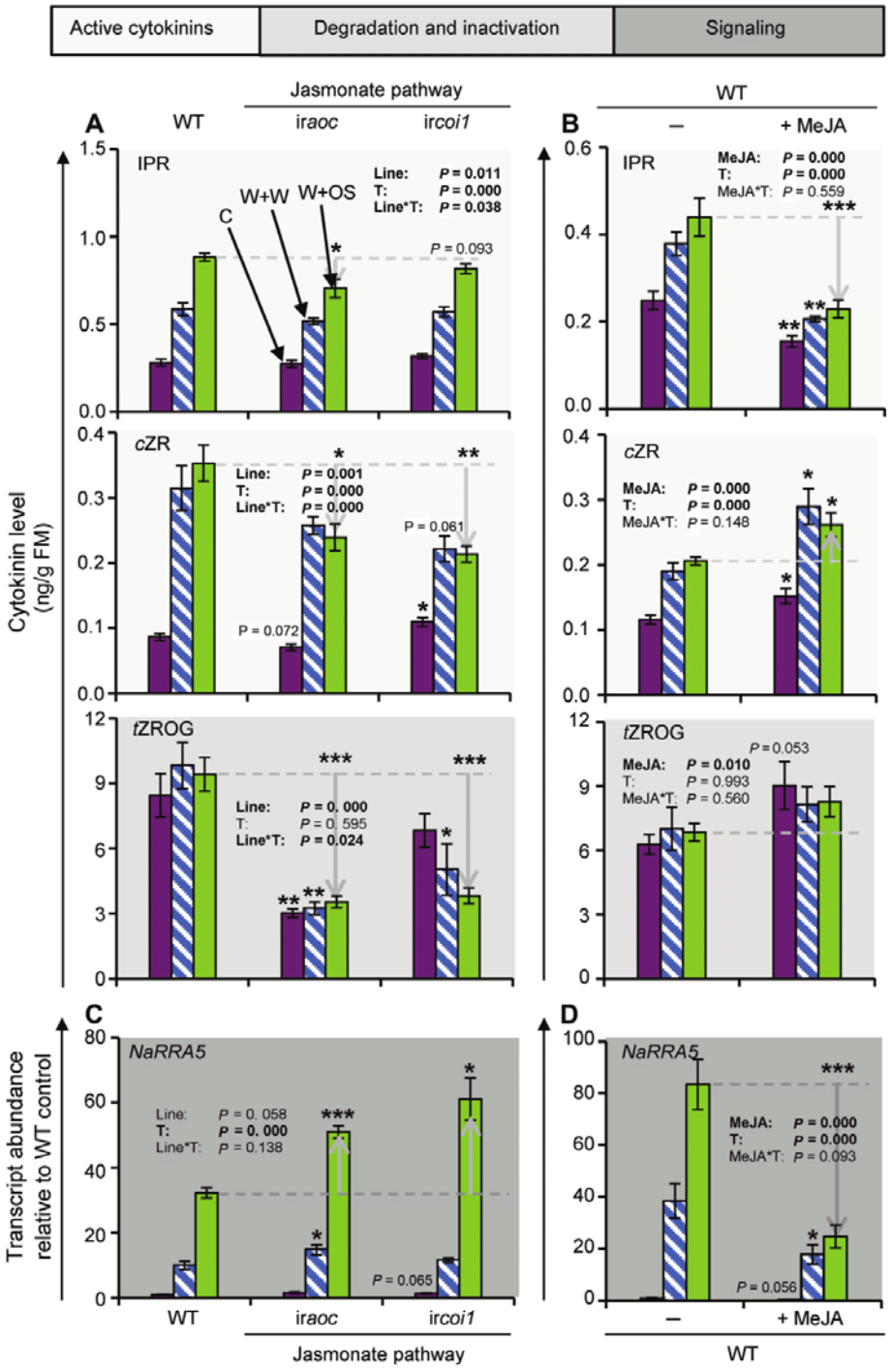

Figure 5. Continued.

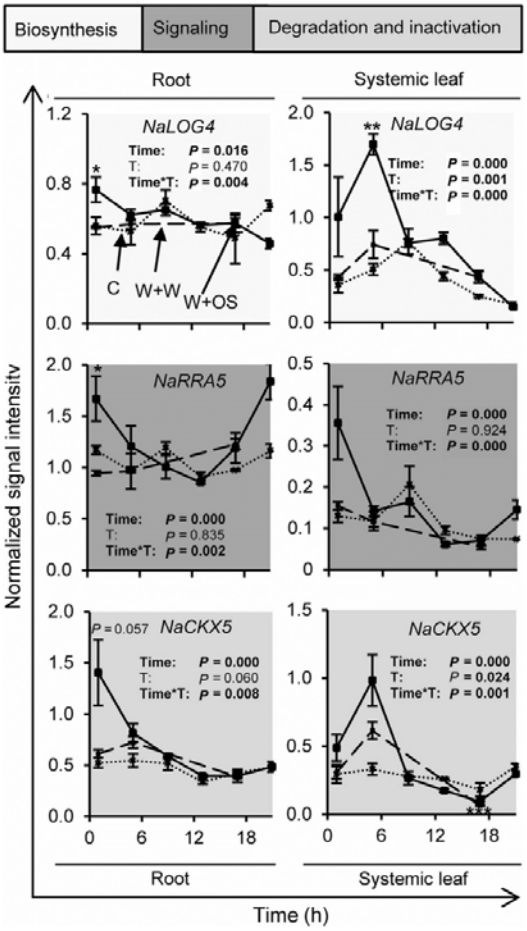

Figure 6. Wounding and herbivory regulate systemic transcript accumulation of cytokinin-related genes
Transcript accumulation was measured in roots and systemic leaves of *N. attenuata* at different time points after wounding and application of water (W + W; dashed line; 1, 5 and 17 h) or *M. sexta* 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 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: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$). Error bars are standard errors (N=3). For additional transcript information see Figure S13 and S14.

et al. 2012), future experiments should investigate a putative role of CKs in preventing pathogen infections after wounding and especially after attack by herbivores, which frequently are the vectors of pathogens (Sobek and Munkvold 1999; Frisinghelli et al. 2000). Regulation of CK metabolism and signaling might also play an important role for herbivory-induced changes in primary metabolism and source-sink regulation (Quilliam et al. 2006; Ferrieri et al. 2013; Machado et al. 2013). Additionally, CKs might be directly involved in assisting herbivore-induced defense responses as proposed, for example, by Dervinis et al. (2010) and Smigocki et al. (2000). Using transgenic plants with altered CK levels or signaling will allow these questions to be addressed.

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 5C, 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 5A, S15); therefore in addition to changes in CK metabolism, CK signaling might also be affected, as reported for the JA-interaction with other phytohormones

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Figure 5. Herbivory-induced cytokinin levels and signaling are regulated by jasmonates
(**A**) Isopentenyladenosine (IPR), *cis*-zeatin riboside (*c*ZR) 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, *c*ZR, 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 a 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: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$). Error bars are standard errors (**A, C** $n \geq 4$; **B, D** $n \geq 5$). FM, fresh mass.

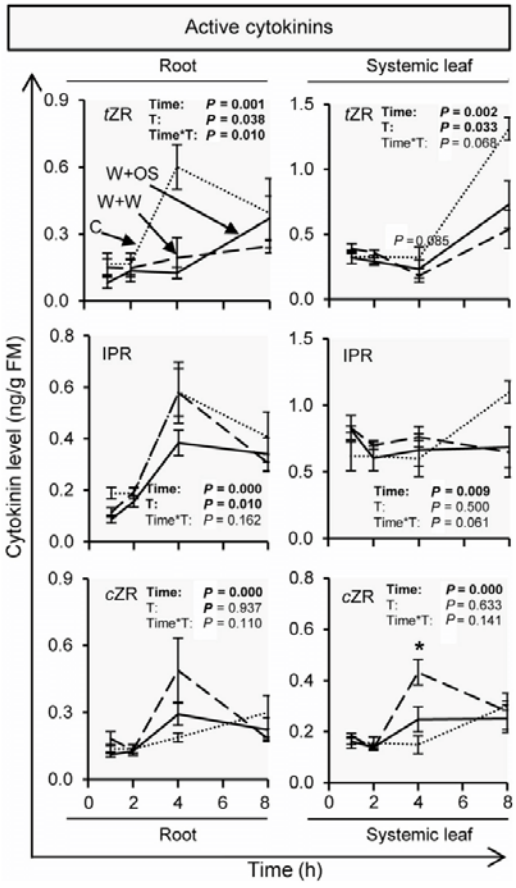

Figure 7. Wounding and herbivory regulate systemic changes in cytokinin levels

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: $*P \leq 0.05$). Error bars are standard errors ($n \geq 4$).

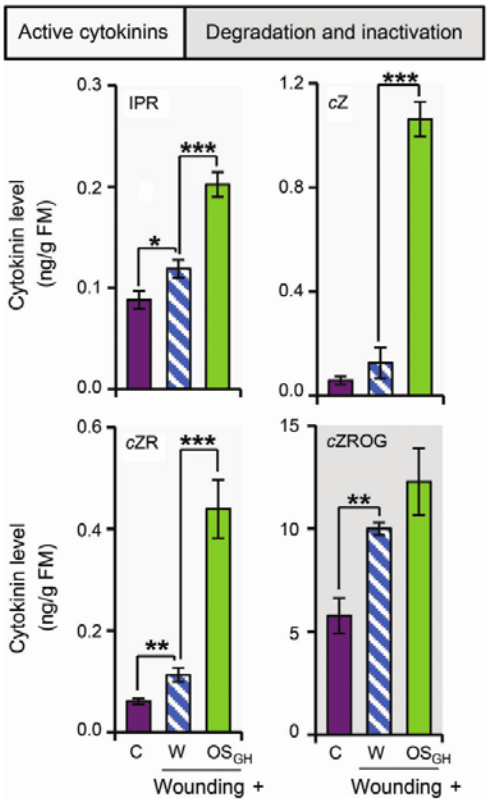

Figure 8. Wounding and herbivory induce changes in cytokinin levels in leaves of *Arabidopsis thaliana*

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 (OS_{GH}) to the puncture wounds, as well as in untreated control leaves (C). Asterisks indicate significant differences between W and OS_{GH} application to wounding sites or between wounding and W application compared to C, as indicated (independent samples *t*-test: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$). Error bars are standard errors ($n \geq 4$). 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.]

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 tZ. 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 *ircoi1* 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, see Figure 3; JA-Ile, 1 h, compare Meldau et al. (2011). We measured CKs only at a single time-point in JA-deficient

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 (*NaRRA5*). 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 (tZ-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 OS_{GH} (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 cZR 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 generation of the stable transformed *iraoc* (line number A-07-457) and *ircoi1* (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 glasshouse 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 glasshouse conditions with 26–28 °C under 16 h supplemental light from Master Sun-T PIA Agro 400 or Master Sun-T PIA Plus 600 W Na lights (Philips, Turnhout, Belgium). Fertilization was done by flood irrigation with additions of 240 g $Ca(NO_3)_2 \cdot 4H_2O$ (Merck, Schwalbach, Germany, http://www.merck-chemicals.com/) and 120 g Ferty B1 (Planta Düngemittel, Regenstauf, Germany, http://www.plantafert.com/) in a 400 L watering tank.

Arabidopsis thaliana plants (ecotype Col-0) were grown on a substrate consisting of 80% Fruhstorfer Nullerde, 10% vermiculite, and 10% sand, fertilized with Triabon (1 g/L) and Osmocote Exact Mini (1 g/L) in a growth chamber at 21 °C, 60% humidity with 10 h light/day with an intensity of 190–220 µmol/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 cZR were dissolved in 80% (v/v) EtOH (tZ, 1 mg/mL; cZR, 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, cZR 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 Gase 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-land 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, S1–S8) were confirmed by qPCR analysis of representative genes in samples from an independent experiment (Figures S9, S10).

qPCR analysis

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

using oligo(dT) primer and RevertAid reverse transcriptase (Invitrogen). qPCR was performed using actin as standard on a Stratagene Mx3005P 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 Kamı'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:H_2O: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 N NH_4OH) the CK-ribosides, free bases, and glucosides were eluted with 1 mL 0.35 N NH_4OH 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.arcticwhiteusa.com/) and Nunc 96-Well Deep Well Plates (Thermo Scientific, Waltham, MA USA, https://www.thermoscientific.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 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*, *tZ7G*, *cZ*, *cZR*, *cZROG*, *cZ9G*, 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 Haën (Seelze, D, http://www.riedeldehaen.de/), acetic acid by Carl Roth (Karlsruhe, D, http://www.carlroth.com/) and CK standards by Olchemim.

Statistical analysis

Data were analyzed by SPSS Statistics 17.0 (IBM, Ehningen, D, http://www.01.ibm.com/software/de/analytics/spss/) 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 HPT-family genes

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 *NaIPT5* 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

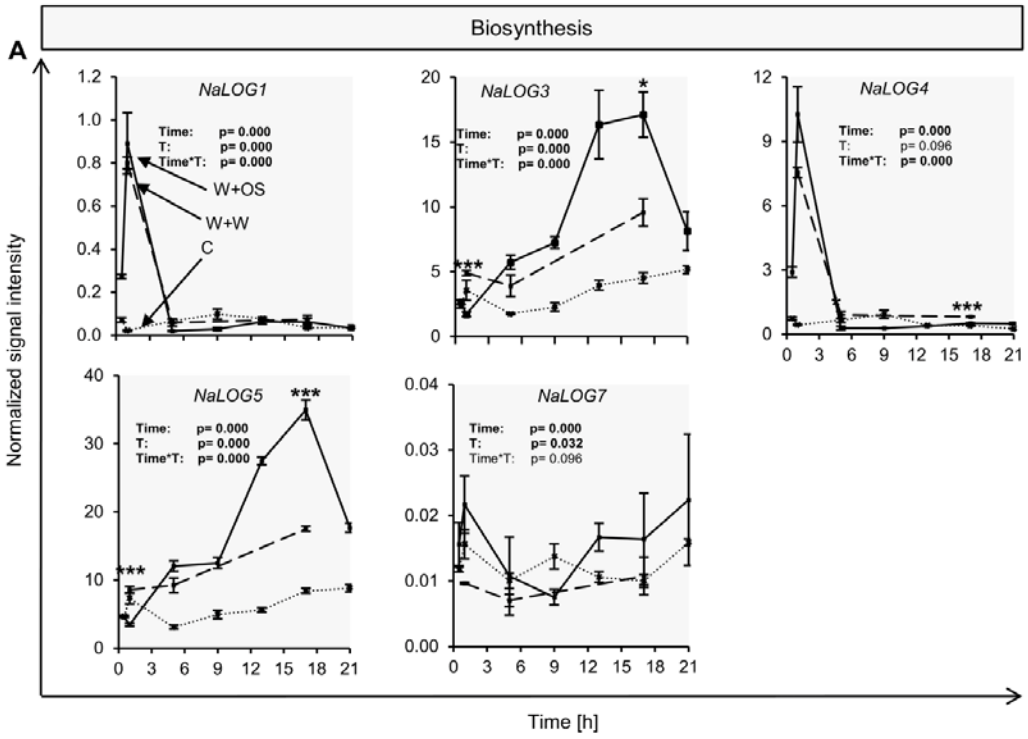
Figure S15. *Trans*-zeatin riboside levels are reduced in jasmonic acid pathway impaired transgenic plants

Table S1. Abbreviations

Table S2. Sequences of primers used for qPCR

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

Supplemental Figures


A
Biosynthesis
Normalized signal intensity
Time [h]
NaLOG1
Time: p= 0.000
T: p= 0.000
Time*T: p= 0.000
W+OS
W+W
C
NaLOG3
Time: p= 0.000
T: p= 0.000
Time*T: p= 0.000
NaLOG4
Time: p= 0.000
T: p= 0.096
Time*T: p= 0.000
NaLOG5
Time: p= 0.000
T: p= 0.000
Time*T: p= 0.000
NaLOG7
Time: p= 0.000
T: p= 0.032
Time*T: p= 0.096

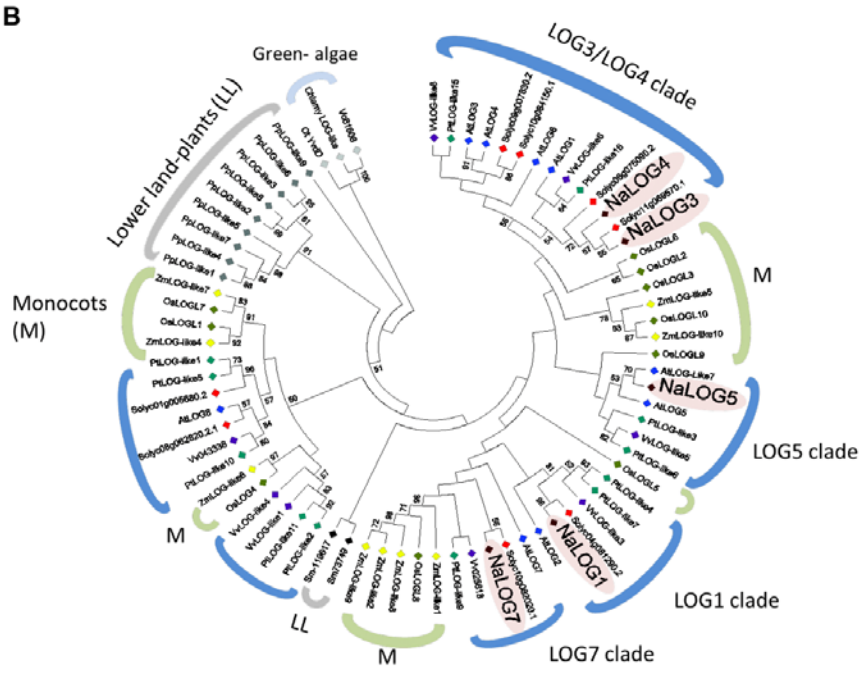

B
Green- algae
LOG3/LOG4 clade
Lower land-plants (LL)
Monocots
(M)
M
LOG5 clade
LOG1 clade
LOG7 clade
LL
M
NaLOG4
NaLOG3
NaLOG5
NaLOG1
NaLOG7

Figure S1. Wounding and herbivory regulate the expression of LOG-family genes.
(A) Relative transcript levels of cytokinin nucleoside 5' -monophosphate phosphoribohydrolases (LOG) were measured in 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; 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). Time and treatment (C and W+OS; T) effects and their interaction (Time*T) were analyzed by univariate ANOVA, except for *NaLOG3, NaLOG4* and *NaLOG7* data which 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≤0.001). Error bars are standard errors (N=3). Data are obtained from microarray kinetic analysis.
(B) Phylogenetic analysis of these cytokinin related genes and their homologs in the following species groups (in parentheses the abbreviations and label colors): green algae [*Volvox carteri* (Vc)*, Ostreococcus* tauri (Ot)*, Chlamydomonas reinhardtii* (Chlamy); all in grey]; lower-land plants [*Physcomitrella patens* (Pp, dark grey) and *Selaginella mollendorfii* (Sm, black)]; monocots [*Oryza sativa* cv. *Japonica* (Os, light green) and *Zea mays* (Zm, yellow)] and dicots [*Vitis vinifera* (Vv, purple)*, Solanum lycopersicum* (Solyc, red)*, Arabidopsis thaliana* (At, blue) and *Populus trichocarpa* (Pt, dark green)]. For *N. attenuata* we used the abbreviation Na the label color brown and light red shading.

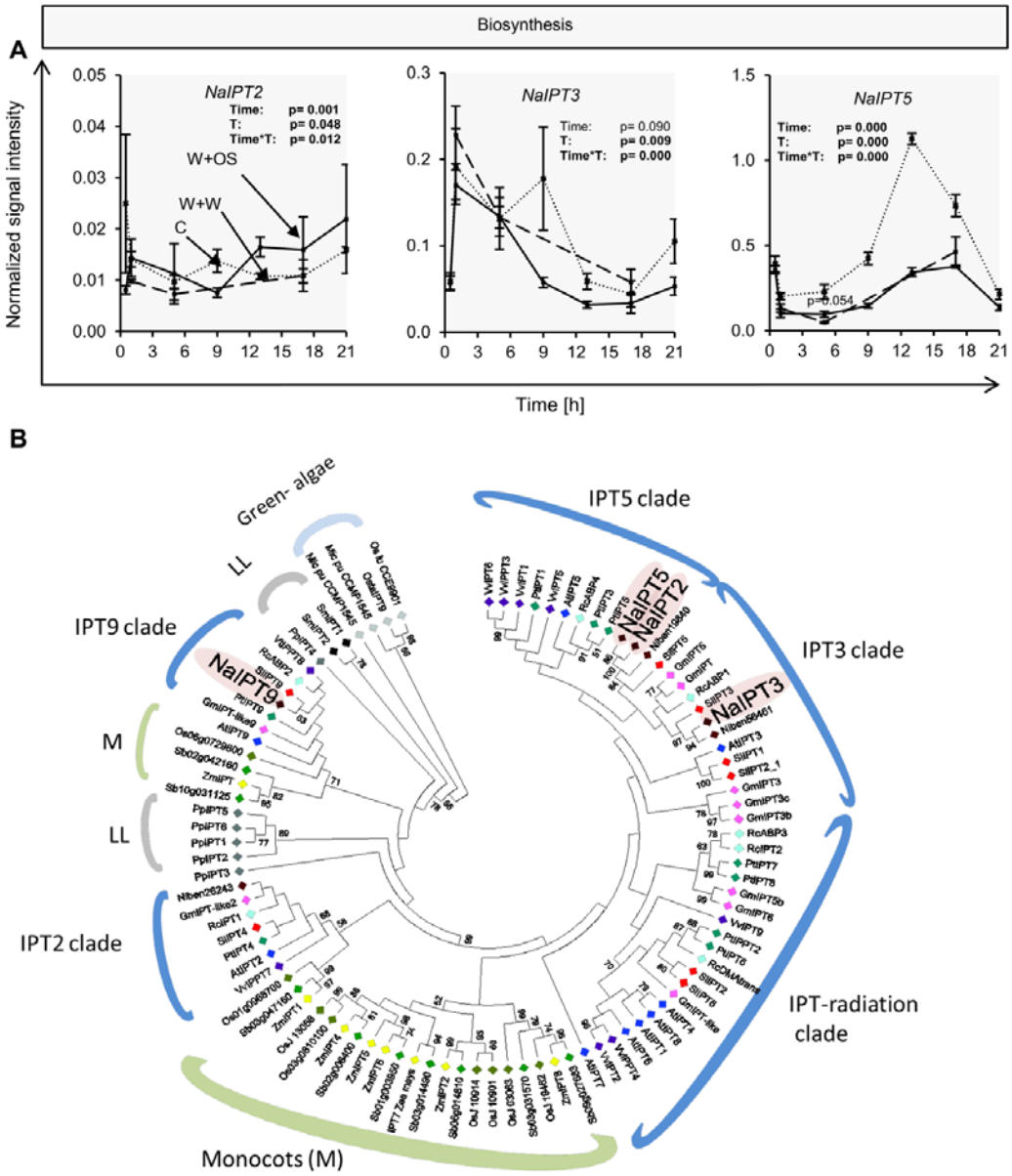


Figure S2. Wounding and herbivory regulate the expression of IPT-family genes. **(A)** Relative transcript levels of isopentenyltransferases (IPT) were measured in leaves of *N. attenuata* at different time points after wounding and application of water (W+W; dashed line; 1, 5 and 17 h) or *M. sexta* oral secretions (W+OS; solid line; 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). Time and treatment (C and W+OS; T) effects and their interaction (Time*T) were analyzed by univariate ANOVA, except for *NaIPT1* data which were analyzed by a generalized least squares model. Error bars are standard errors (N=3). Data are obtained from microarray kinetic analysis.
(B) Phylogenetic analysis of these cytokinin related genes and their homologs in the following species groups (in parentheses the abbreviations and label colors): green algae [*Micromonas pusilla* (MicPu), *Ostreococcus lucimarinus* (Oslu) and *Ostreococcus* tauri (Osta), all in grey]; lower-land plants [*Physcomitrella patens* (Pp, dark grey) and *Selaginella mollendorfii* (Sm, black)]; monocots [*Oryza sativa* cv. *Japonica* (Os, light green), *Sorghum bicolor* (Sb, bright green) and *Zea mays* (Zm, yellow)] and dicots [*Vitis vinifera* (Vv, purple), *Solanum lycopersicum* (Sl, red), *Arabidopsis thaliana* (At, blue), *Glycine max* (Gm, pink), *Ricinus communis* (Rc, light blue) and *Populus trichocarpa* (Pt, dark green)]. For *N. attenuata* we used the abbreviation Na(light brown, light red shading) and for *N. benthamiana* Niben(dark brown).

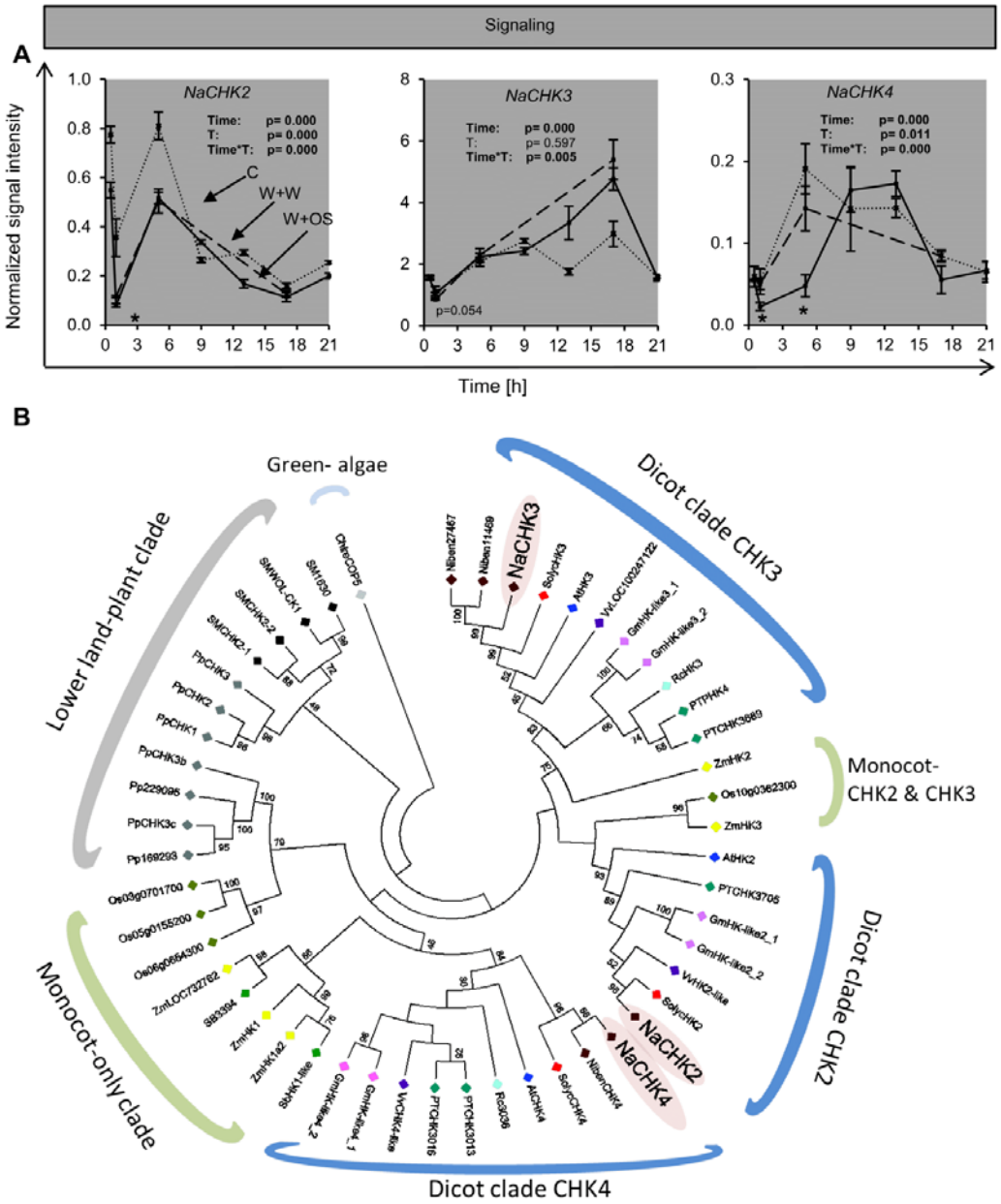


Figure S3. Wounding and herbivory regulate the expression of cytokinin receptor genes.

(A) Relative transcript levels of the corresponding histidine kinases were measured in 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; 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). Time and treatment (C and W+OS; T) effects and their interaction (Time*T) were analyzed by univariate ANOVA, except for *NaCHK2* and *NaCHK3* data which 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=3). Data are obtained from microarray kinetic analysis.

(B) Phylogenetic analysis of these cytokinin related genes and their homologs in the following species groups (in parentheses the abbreviations and label colors): the green alga *Chlamydomonas reinhardtii* (Chlre), (light grey); lower-land plants [*Physcomitrella patens* (Pp, dark grey) and *Selaginella mollendorfii* (Sm, black)]; monocots [*Oryza sativa* cv. *Japonica* (Os, light green), *Sorghum bicolor* (Sb, bright green) and *Zea mays* (Zm, yellow)] and dicots [*Vitis vinifera* (Vv, purple), *Solanum lycopersicum* (Solyc, red), *Arabidopsis thaliana* (At, blue), *Glycine max* (Gm, pink), *Ricinus communis* (Rc, light blue) and *Populus trichocarpa* (Pt, dark green)]. For *N. attenuata* we used the abbreviation Na(light brown, light red shading) and for *N. benthamiana* Niben (dark brown).

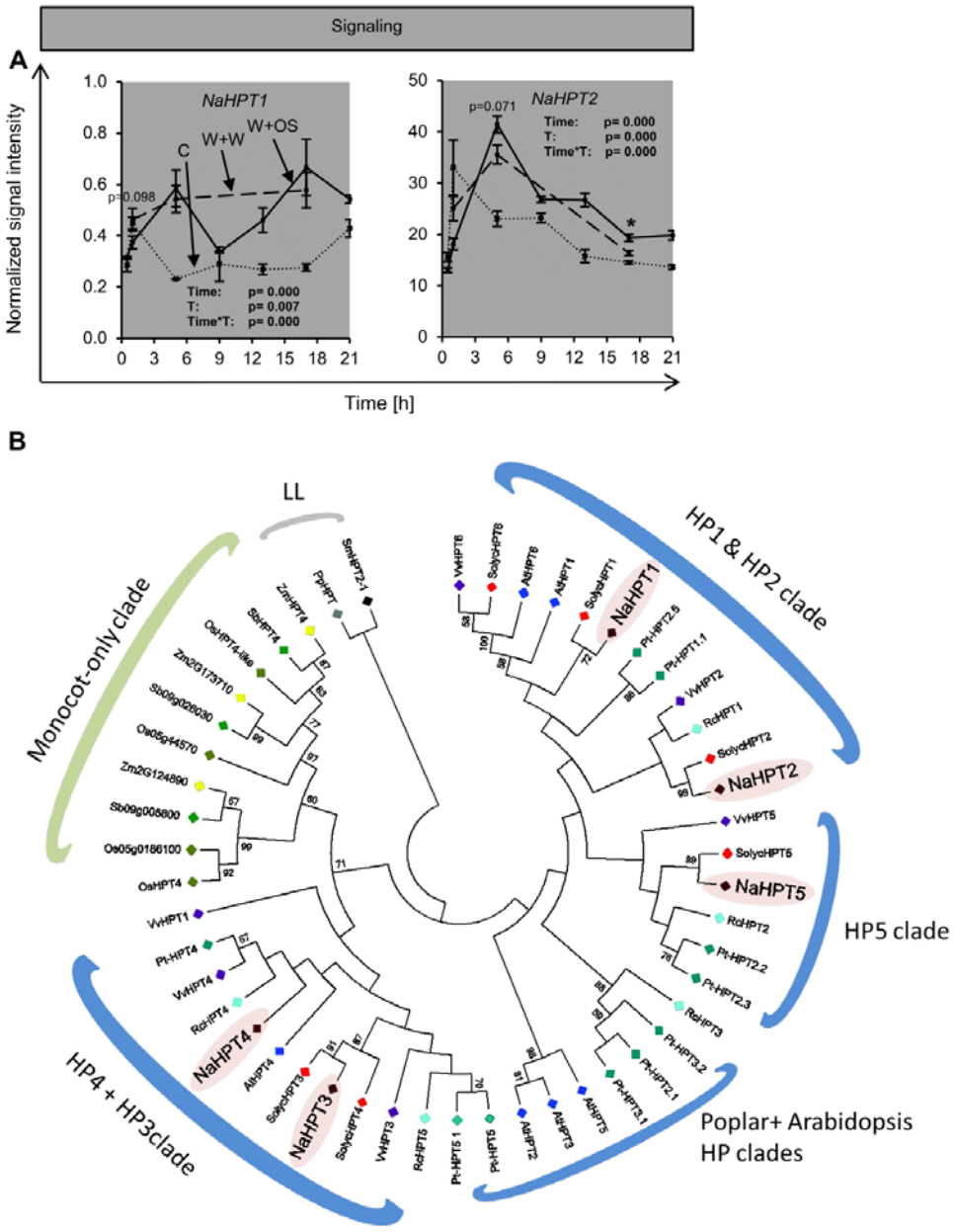
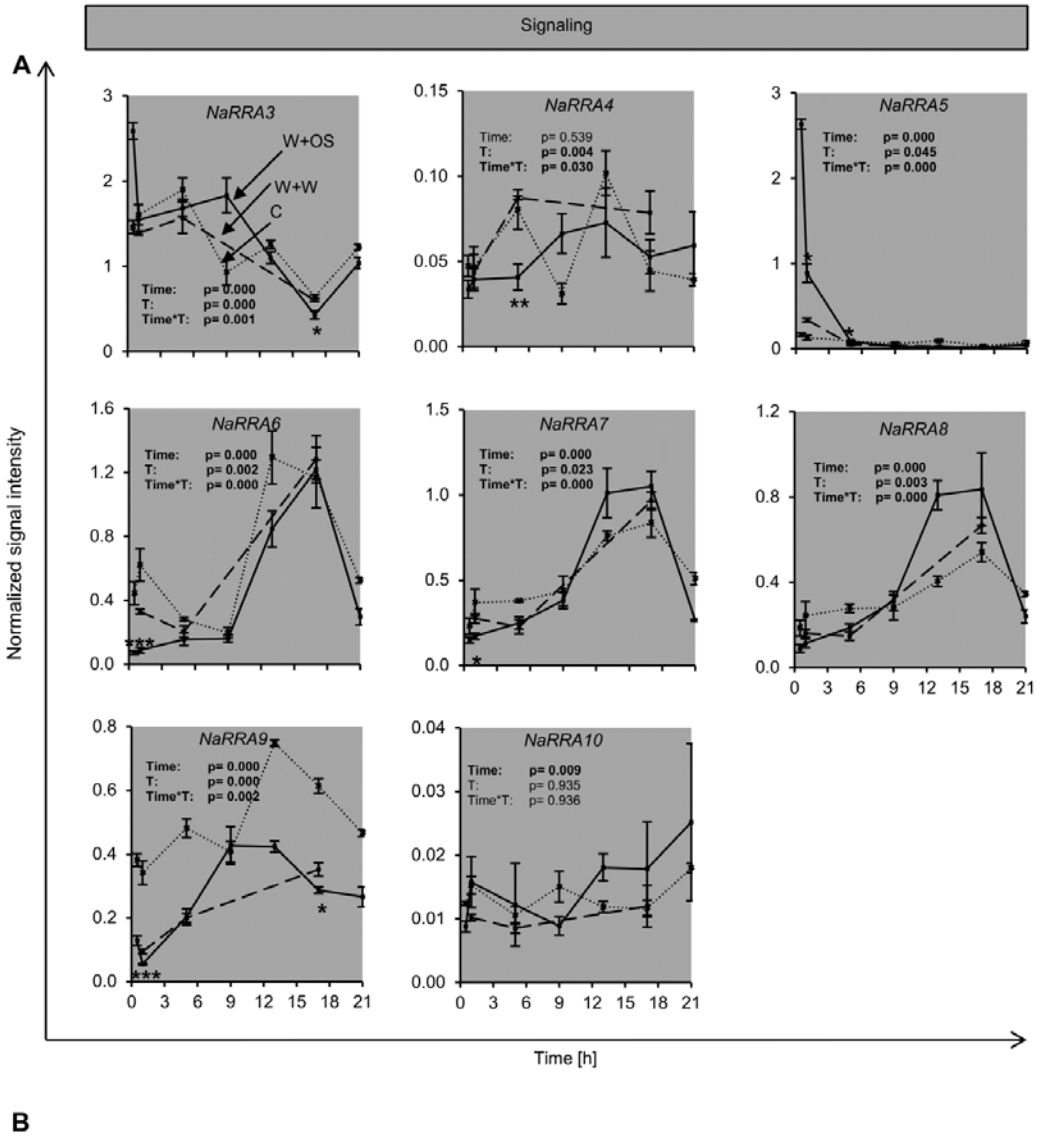


Figure S4. Wounding and herbivory regulate the expression of HPT-family genes. **(A)** Relative transcript levels of histidine phosphotranfer proteins (HPT) were measured in leaves of *N. attenuata* at different time points after wounding and application of water (W+W; dashed line; 1, 5 and 17 h) or *M. sexta* oral secretions (W+OS; solid line; 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). 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). Error bars are standard errors (N=3). Data are obtained from microarray kinetic analysis.
(B) Phylogenetic analysis of these cytokinin related genes and their homologs in the following groups of species (in parentheses the abbreviations and label colors): lower-land plants [*Physcomitrella patens* (Pp, dark grey) and *Selaginella mollendorfii* (Sm, black)]; monocots [*Oryza sativa* cv. *Japonica* (Os, light green), *Sorghum bicolor* (Sb, bright green) and *Zea mays* (Zm, yellow)] and dicots [*Vitis vinifera* (Vv, purple), *Solanum lycopersicum* (Solyc, red), *Arabidopsis thaliana* (At, blue), *Ricinus communis* (Rc, light blue) and *Populus trichocarpa* (Pt, dark green)]. For *N. attenuata* we used the abbreviation Na (light brown) and light red shading.


Signaling
A
Normalized signal intensity
NaRRA3
W+OS
W+W
C
Time: p= 0.000
T: p= 0.000
Time*T: p= 0.001
NaRRA4
Time: p= 0.539
T: p= 0.004
Time*T: p= 0.030
NaRRA5
Time: p= 0.000
T: p= 0.045
Time*T: p= 0.000
NaRRA6
Time: p= 0.000
T: p= 0.002
Time*T: p= 0.000
NaRRA7
Time: p= 0.000
T: p= 0.023
Time*T: p= 0.000
NaRRA8
Time: p= 0.000
T: p= 0.003
Time*T: p= 0.000
NaRRA9
Time: p= 0.000
T: p= 0.000
Time*T: p= 0.002
NaRRA10
Time: p= 0.009
T: p= 0.935
Time*T: p= 0.936
Time [h]
B

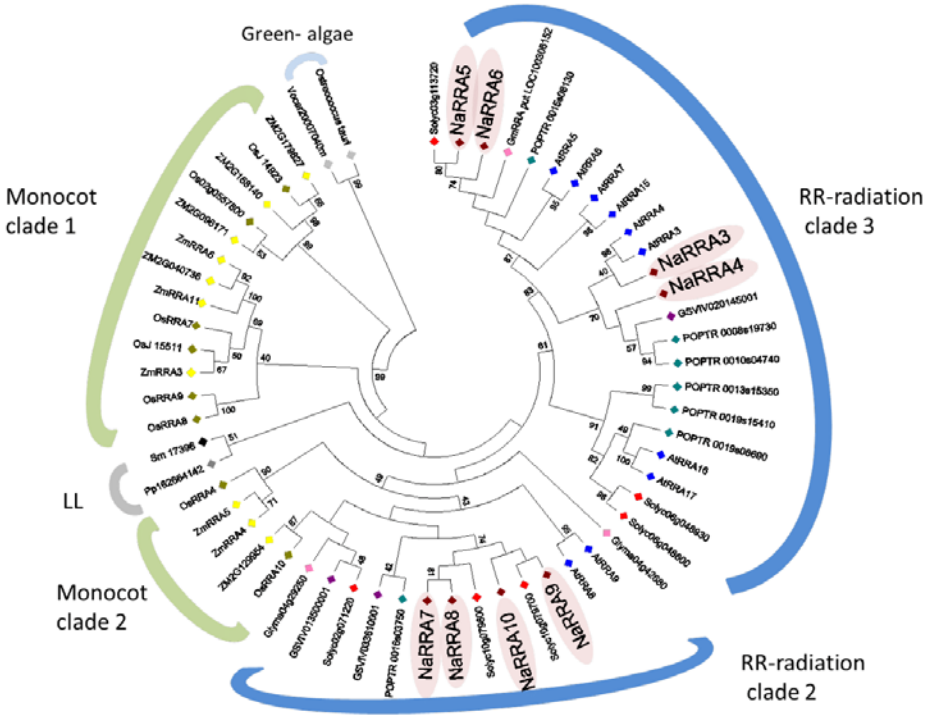
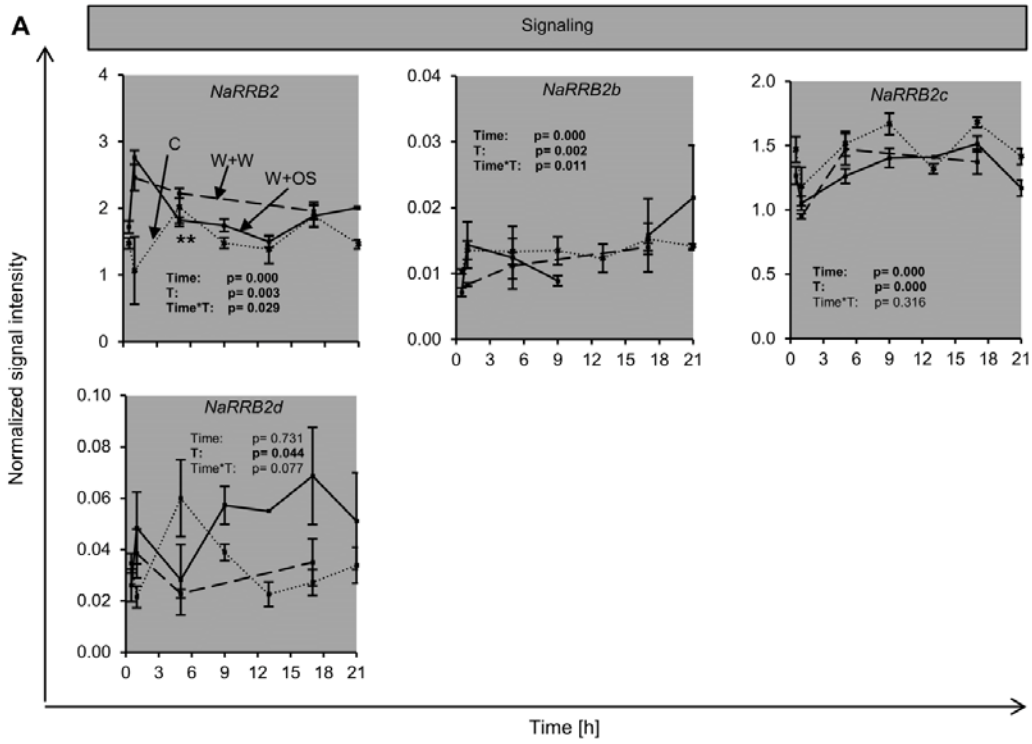


Figure S5. Wounding and herbivory regulate the expression of type-A RR-family genes.

(A) Relative transcript levels of type-A response regulators (RRA) were measured in 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; 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). Time and treatment (C and W+OS; T) effects and their interaction (Time*T) were analyzed by univariate ANOVA, except for *NaRRA3, NaRRA5, NaRRA6, NaRRA7, NaRRA8* and *NaRRA9* data which 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). Data are obtained from microarray kinetic analysis.

(B) Phylogenetic analysis of these cytokinin related genes and their homologs in the following species groups (in parentheses the abbreviations and label colors): green algae [*Volvox carteri* (Vocar) and *Ostreococcus* tauri; all in grey]; lower-land plants [*Physcomitrella patens* (Pp, dark grey) and *Selaginella mollendorfii* (Sm, black)]; monocots [*Oryza sativa* cv. *Japonica* (Os, light green) and *Zea mays* (Zm, yellow)] and dicots [*Vitis vinifera* (GSV, purple), *Solanum lycopersicum* (Solyc, red), *Arabidopsis thaliana* (At, blue), *Glycine max* (Gm/Glyma, pink) and *Populus trichocarpa* (POPTR, dark green)]. For *N. attenuata* we used the abbreviation Na (light brown) and light red shading.


A
Signaling
Normalized signal intensity
Time [h]
NaRRB2
C
W+W
W+OS
**
Time: p= 0.000
T: p= 0.003
Time*T: p= 0.029
NaRRB2b
Time: p= 0.000
T: p= 0.002
Time*T: p= 0.011
NaRRB2c
Time: p= 0.000
T: p= 0.000
Time*T: p= 0.316
NaRRB2d
Time: p= 0.731
T: p= 0.044
Time*T: p= 0.077

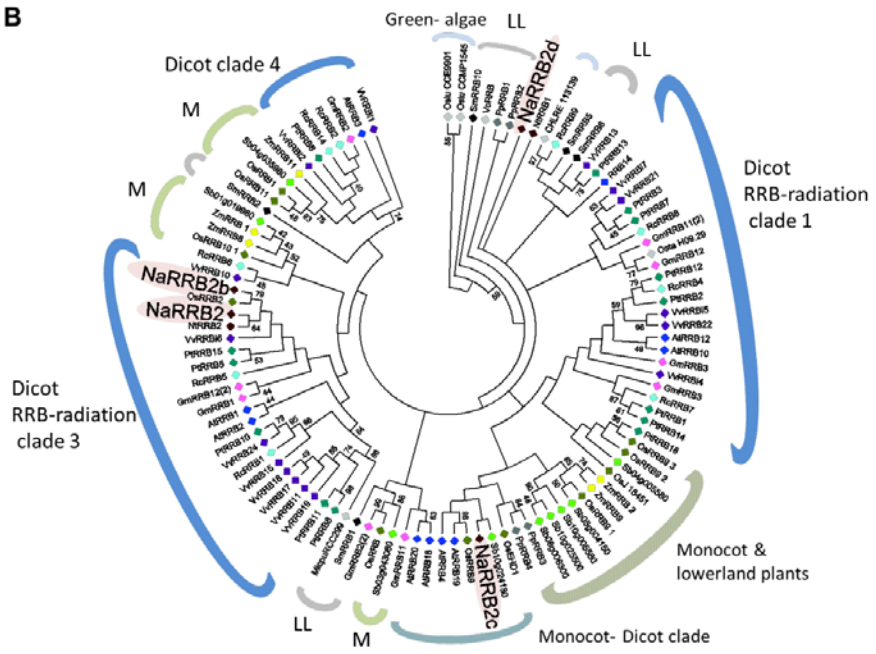

B
Green- algae
LL
Dicot clade 4
M
M
LL
Dicot
RRB-radiation
clade 1
NaRRB2a
NaRRB2b
NaRRB2
Dicot
RRB-radiation
clade 3
Monocot &
lowerland plants
LL
M
NaRRB2c
Monocot- Dicot clade

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

(A) Relative transcript levels of type-B response regulators (RRB) were measured in 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; 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). Time and treatment (C and W+OS; T) effects and their interaction (Time*T) were analyzed by univariate ANOVA, except for *NaRRB2* and *NaRRB2b* data which 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≤0.01). Error bars are standard errors (N=3). Data are obtained from microarray kinetic analysis.

(B) Phylogenetic analysis of these cytokinin related genes and their homologs in the following species (in parentheses the abbreviations and label colors): green algae [*Volvox carteri* (Vc), *Ostreococcus lucimarinus* (Oslu), *Ostreococcus tauri* (Osta), *Micromonas pusilla* (MicPu), all in grey]; lower-land plants [*Physcomitrella patens* (Pp, dark grey), *Selaginella mollendorfii* (Sm, black)]; monocots [*Oryza sativa* cv. *Japonica* (Os, light green), *Sorghum bicolor* (Sb, bright green) and *Zea mays* (Zm, yellow)] and dicots [*Vitis vinifera* (Vv, purple), *Arabidopsis thaliana* (At, blue), *Glycine max* (Gm, pink), *Ricinus communis* (Rc, light blue) and *Populus trichocarpa* (Pt, dark green)]. For *N. attenuata* we used the abbreviation Na(light brown) and for *N. tabacum* Nt (dark brown).

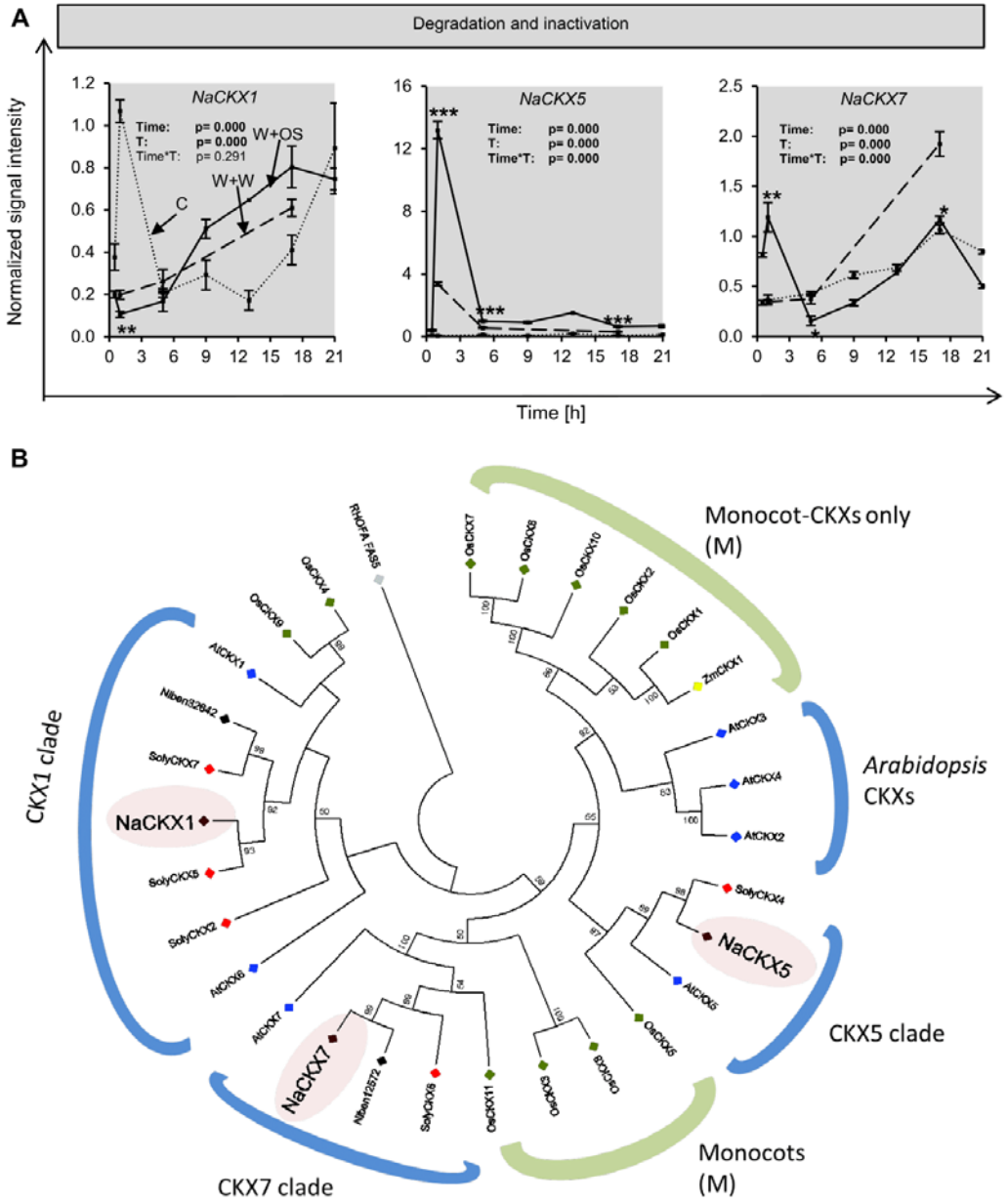
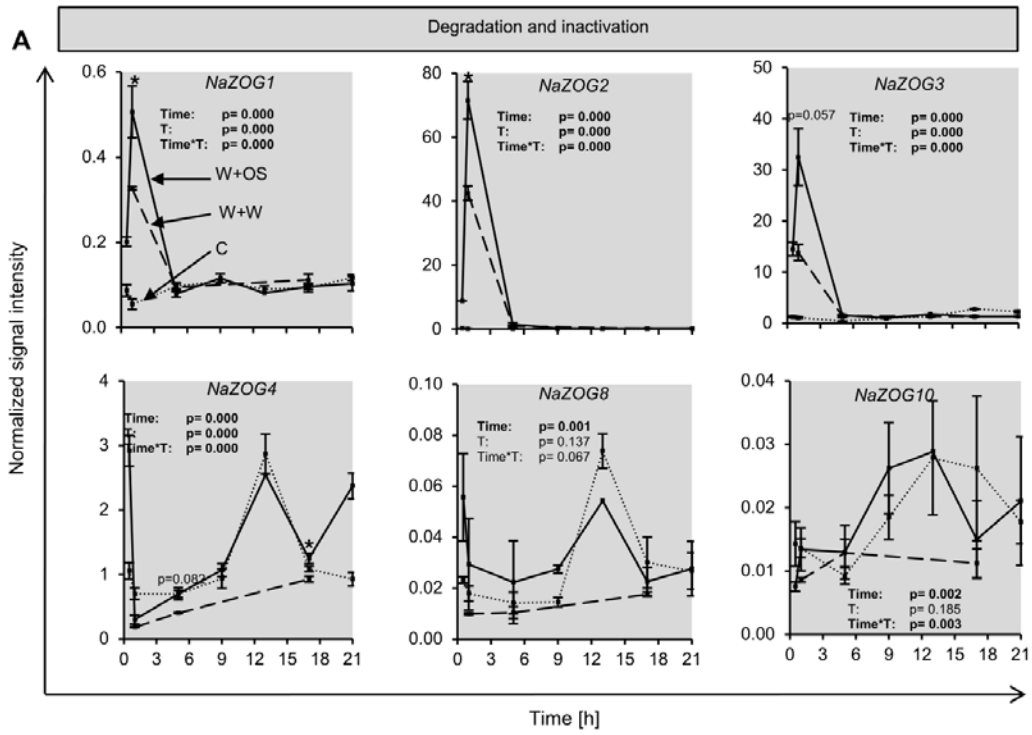


Figure S7. Wounding and herbivory regulate the expression of CKX-family genes. **(A)** Relative transcript levels of cytokinin oxidases/dehydrogenases (CKX) were measured in leaves of *N. attenuata* at different time points after wounding and

application of water (W+W; dashed line; 1, 5 and 17 h) or *M. sexta* oral secretions (W+OS; solid line; 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). Time and treatment (C and W+OS; T) effects and their interaction (Time*T) were analyzed by univariate ANOVA, except for *NaCKX5* and *NaCKX7* data which 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). Data are obtained from microarray kinetic analysis.

(B) Phylogenetic analysis of these cytokinin related genes and their homologs in the following species (in parentheses the abbreviations and label colors): monocots [*Oryza sativa* cv. *Japonica* (Os, light green) and *Zea mays* (Zm, yellow)] and dicots [*Solanum lycopersicum* (Solyc, red), *Arabidopsis thaliana* (At, blue), *N. attenuata* (Na, light brown) and *N. benthamiana* (Niben,dark brown)]. The outgroup is a bacterial species (*Rhodococcus fascians*), as no homolog was found in another green plant outside flowering plants.


A
Degradation and inactivation
Normalized signal intensity
NaZOG1
Time: p= 0.000
T: p= 0.000
Time*T: p= 0.000
W+OS
W+W
C
NaZOG2
Time: p= 0.000
T: p= 0.000
Time*T: p= 0.000
NaZOG3
p=0.057
Time: p= 0.000
T: p= 0.000
Time*T: p= 0.000
NaZOG4
Time: p= 0.000
T: p= 0.000
Time*T: p= 0.000
p=0.082
NaZOG8
Time: p= 0.001
T: p= 0.137
Time*T: p= 0.067
NaZOG10
Time: p= 0.002
T: p= 0.185
Time*T: p= 0.003
Time [h]

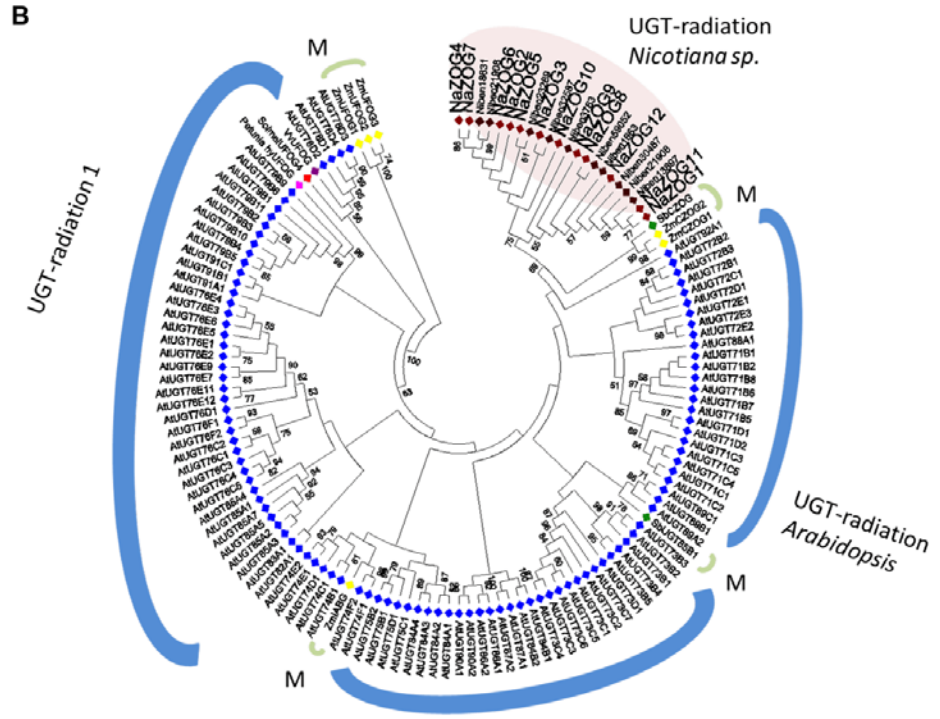

B
UGT-radiation
Nicotiana sp.
UGT-radiation 1
UGT-radiation
Arabidopsis
M

Figure S8. Wounding and herbivory regulate the expression of ZOG-family genes. **(A)** Relative transcript levels of zeatin *O*-glucosyltransferase (ZOG) were measured in 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; 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). Time and treatment (C and W+OS; T) effects and their interaction (Time*T) were analyzed by univariate ANOVA, except for *NaZOG2, NaZOG8* and *NaZOG10* data which 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=3). Data are obtained from microarray kinetic analysis. Only expression data of genes that are differentially regulated by treatments are presented. **(B)** Phylogenetic analysis of these cytokinin related genes and their homologs in the following species (in parentheses the abbreviations and label colors): Monocots [*Sorghum bicolor* (Sb, bright green) and *Zea mays* (Zm, yellow)], dicots [*Vitis vinifera* (Vv, purple), *Solanum spp.* (Sol, red), *Arabidopsis thaliana* (At, blue) and *Petunia spp*. (pink)]. For *N. attenuata* we used the abbreviation Na(light brown), for *N. benthamiana* Niben(dark brown). No ZOG genes were identified in either lower-land plants or green algae.

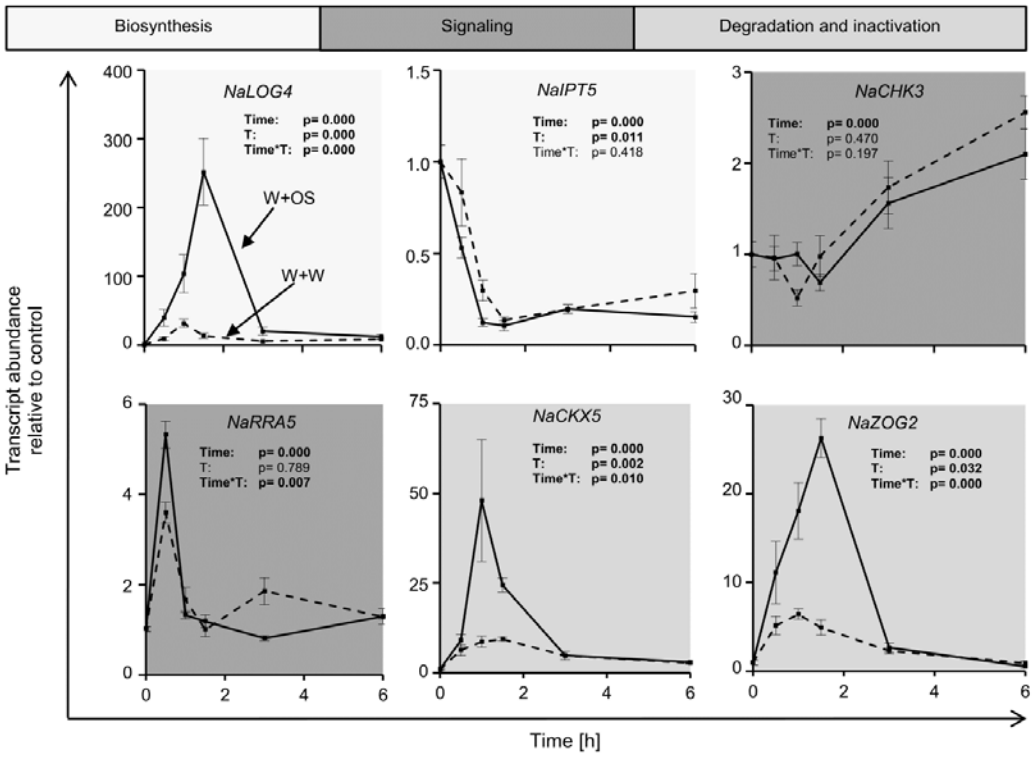


Figure S9. Confirmation of microarray expression data.

Relative transcript levels of cytokinin-related genes were measured in leaves of *N. attenuata* at different time points after wounding and application of water (W+W, dashed line) or *M. sexta* oral secretions (W+OS, solid line) to the puncture wounds by quantitative PCR. Time and treatment (C, W+W and W+OS; T) effects and their interaction (Time*T) were analyzed by univariate ANOVA, except for *NaRRA5* and *NaZOG2* data which were analyzed by a generalized least squares model. Error bars are standard errors (N≥3).

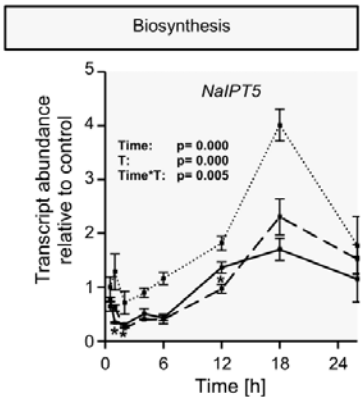


Figure S10. Confirmation of *NaIPT5* microarray data.

Relative transcript levels of isopentenyltransferases 5 (Na*IPT5*) were measured in leaves of *N. attenuata* at different time points (0.5, 1, 2, 4, 6, 12, 18 and 26 h) after wounding and application of water (W+W, dashed line) or *M. sexta* oral secretions (W+OS, solid line) to the puncture wounds, as well as in untreated control leaves (C; dotted line) by quantitative PCR. 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). Samples from five plants per time and treatment were pooled and tree technical replicates were measured. Error bars are standard errors (N=3).

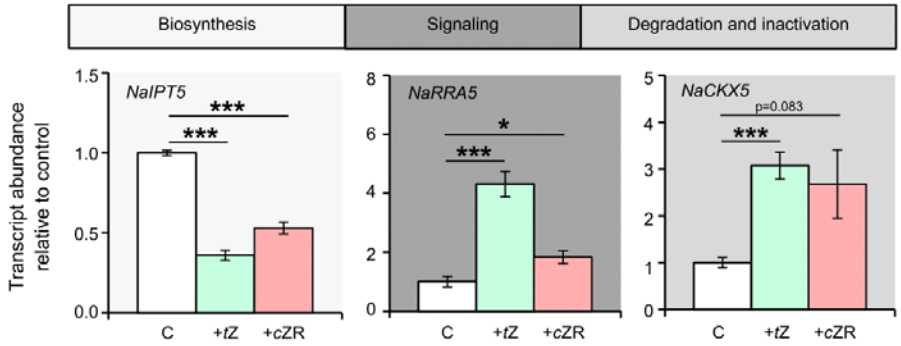


Figure S11. Cytokinin-spraying changes *NaIPT5, NaRRA5* and *NaCKX5* transcript accumulation.

Relative transcript accumulations of *NaIPT5, NaRRA5* and *NaCKX5* were measured in leaves of *N. attenuata* after three days of *trans*-zeatin (*tZ*; 1 µM) *or cis*-zeatin riboside (*c*ZR; 5 µM)-spraying or spraying of the buffer control (C). Spraying was performed three times per day.

Asterisks indicate significant differences between *tZ*/*c*ZR-sprayed samples compared to the control treatment (independent samples *t* test: * P≤0.05, *** P≤0.001). Error bars are standard errors (N=5).

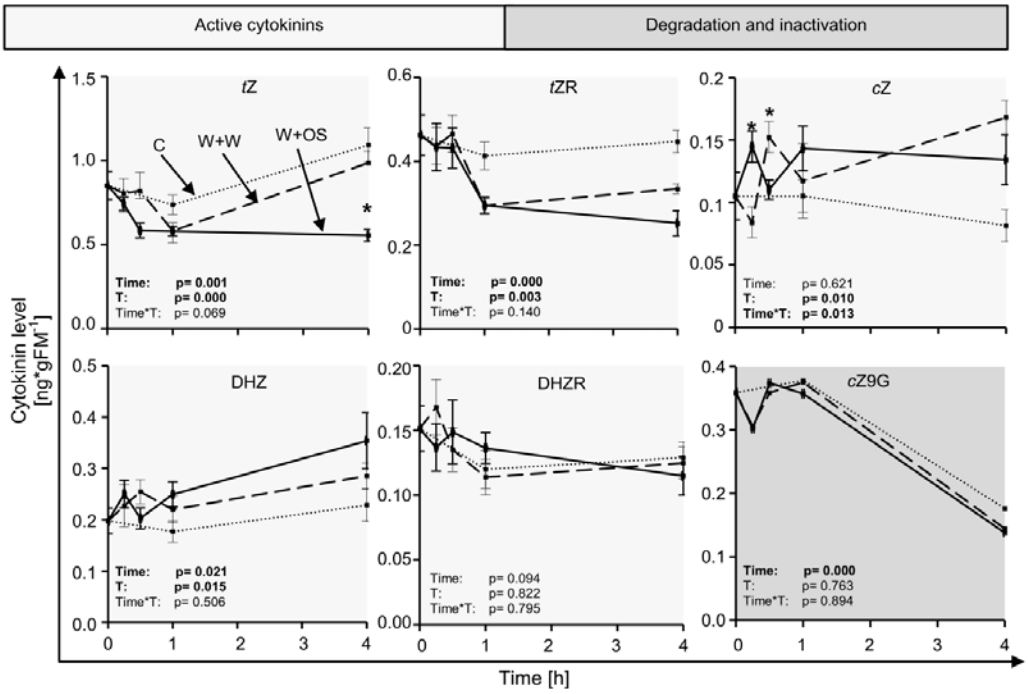


Figure S12. Wounding and herbivory-induced changes in cytokinin levels. *trans*-Zeatin (*t*Z), *trans*-zeatin riboside (*t*ZR), *cis*-Zeatin (*c*Z), dihydrozeatin (DHZ), dihydrozeatin riboside (DHZR) and *cis*-zeatin 9-glucoside (*c*Z9G) levels in leaves of *N. attenuata* at different time points after wounding and application of water (W+W, dashed line) or *M. 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, W+W and W+OS; T) effects and their interaction (Time*T) were analyzed by univariate ANOVA, except for *t*ZR and *c*Z9G data which 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=5). FM, fresh mass.

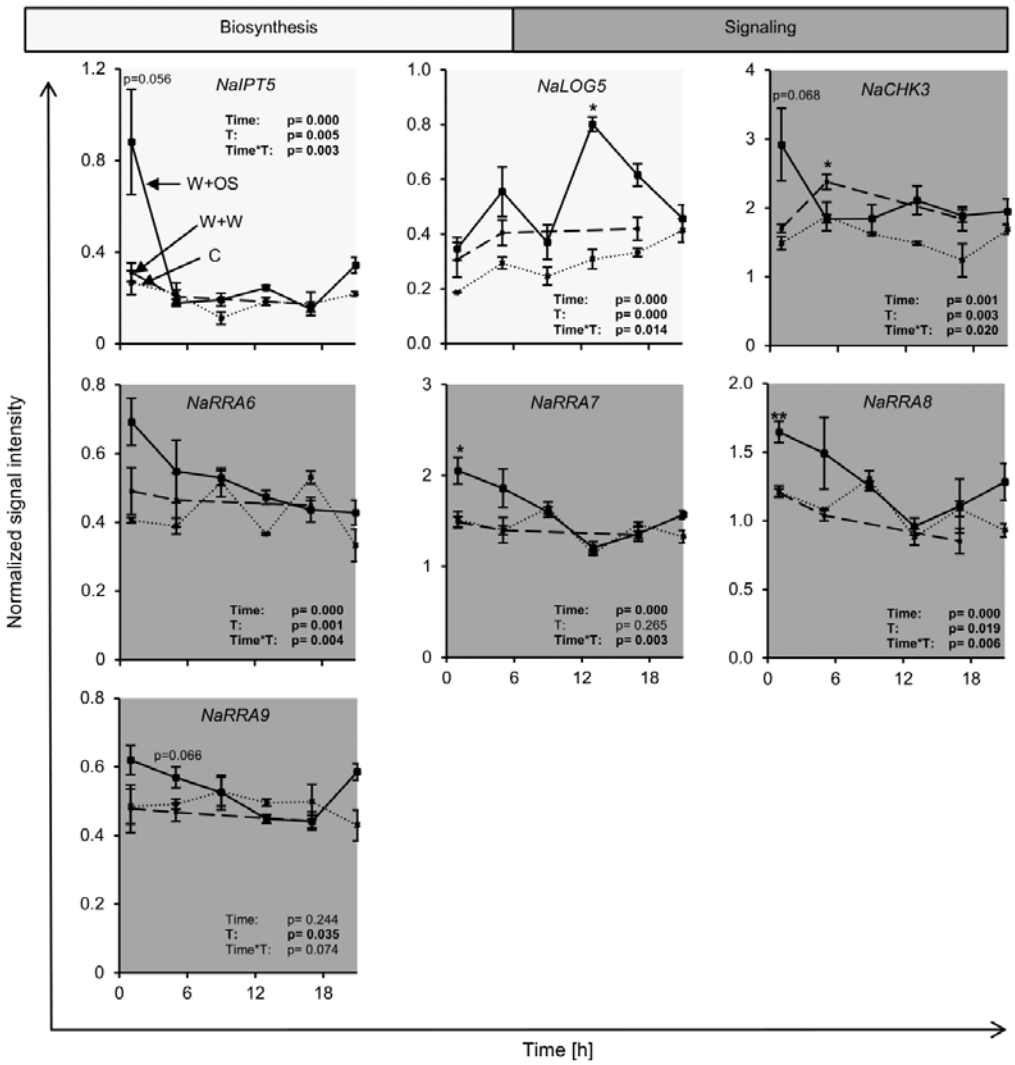


Figure S13. Wounding and herbivory regulate transcript accumulation of cytokinin-related genes in the root.

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 there interaction (Time*T) were analyzed for *NaRRA9* by univariate ANOVA; the other transcript data 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). Error bars are standard errors (N=3).

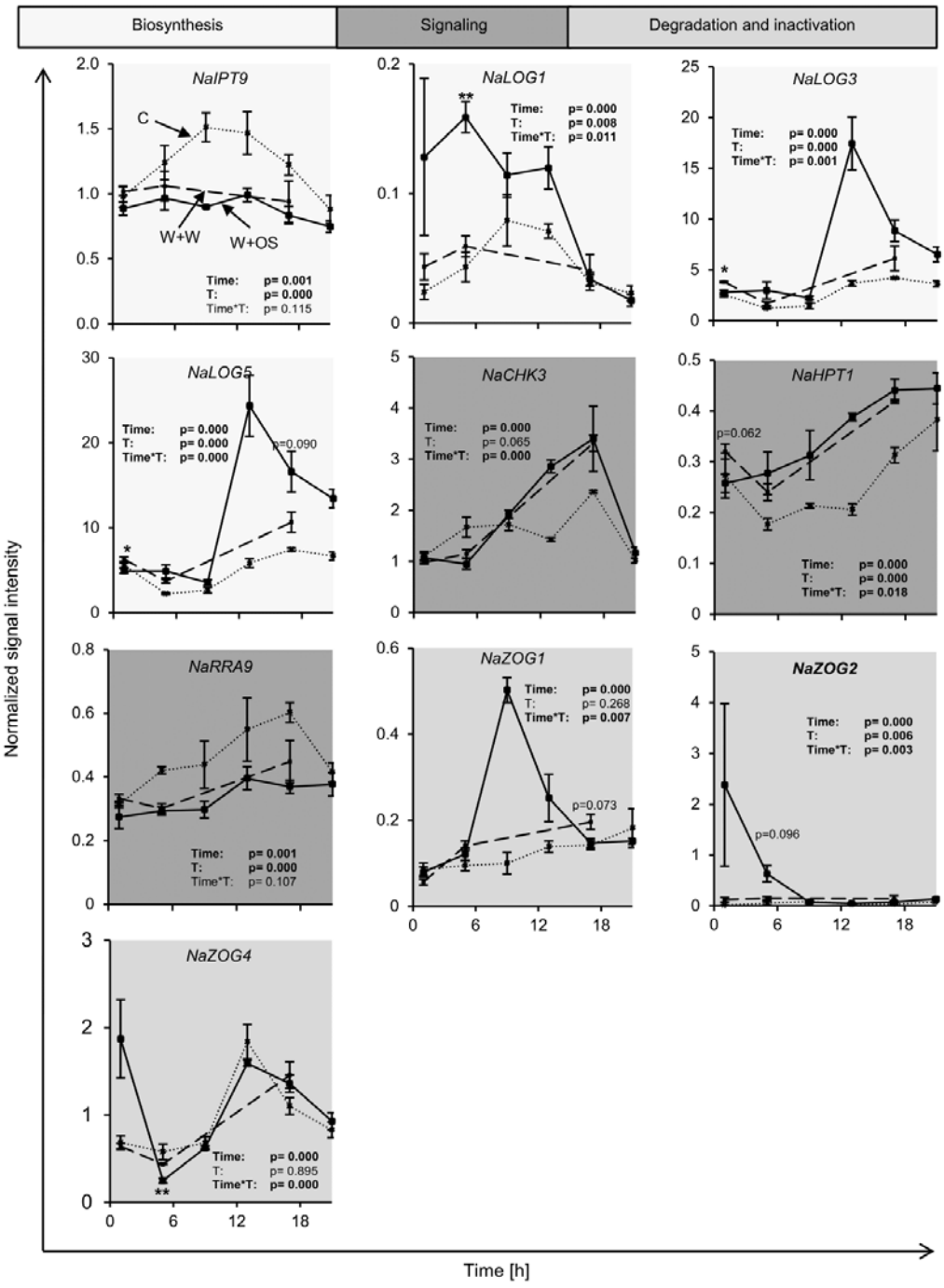

Biosynthesis
Signaling
Degradation and inactivation
NaIPT9
C
W+W
W+OS
Time: p= 0.001
T: p= 0.000
Time*T: p= 0.115
NaLOG1
**
Time: p= 0.000
T: p= 0.008
Time*T: p= 0.011
NaLOG3
Time: p= 0.000
T: p= 0.000
Time*T: p= 0.001
*
NaLOG5
Time: p= 0.000
T: p= 0.000
Time*T: p= 0.000
p=0.090
*
NaCHK3
Time: p= 0.000
T: p= 0.065
Time*T: p= 0.000
NaHPT1
p=0.062
Time: p= 0.000
T: p= 0.000
Time*T: p= 0.018
NaRRA9
Time: p= 0.001
T: p= 0.000
Time*T: p= 0.107
NaZOG1
Time: p= 0.000
T: p= 0.268
Time*T: p= 0.007
p=0.073
NaZOG2
Time: p= 0.000
T: p= 0.006
Time*T: p= 0.003
p=0.096
NaZOG4
Time: p= 0.000
T: p= 0.895
Time*T: p= 0.000
**
Normalized signal intensity
Time [h]

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

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 there interaction (Time*T) were analyzed for *NaIPT9* by univariate ANOVA; the other transcript data 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≤0.01). Error bars are standard errors (N=3).

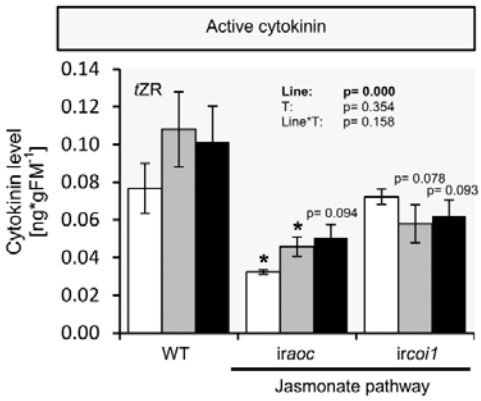


Figure S15. *Trans*-zeatin riboside levels in jasmonic acid pathway impaired transgenic plants.

Trans-zeatin riboside (*t*ZR) levels in leaves of *N. attenuata* 30 min after wounding and application of water (W+W, grey bars) or *M. sexta* oral secretions (W+OS, solid bars) to the puncture wounds and in untreated control leaves (C, open bars). Measurements were performed in leaves of wild-type (WT) plants and RNAi lines silenced in *AOC* or *COI1* expression.

Line and treatment (C, W+W and W+OS; T) effects and their interaction (Line*T) were analyzed by univariate ANOVA. Asterisks indicate significant differences between same treatments from RNAi lines and WT plants (independent samples *t* test: * P≤0.05). Error bars are standard errors (N≥3). FM, fresh mass.

Supplemental Tables

Table S1. Abbreviations

Abbr.	Definition	Abbr.	Definition
AOC	Allene oxide cyclase	IP	Isopentenyladenine
CHASE	Cyclase/histidine kinase extracellular associated sensing	IPR	Isopentenyladenosine
		IPT	Isopentenyltransferase
CHK	CHASE domain-containing histidine kinase	JA	Jasmonic acid
		JA-Ile	Jasmonic acid-isoleucine conjugate
CIG2	Cytokinin-induced gene 2	JAZ	Jasmonate zim-domain
CK	Cytokinin	MeJA	Methyl-jasmonate
CKX	Cytokinin oxidase/dehydrogenase	OS	Oral secretion
COI1	Coronatine insensitive 1	OS_{GH}	Grasshopper oral secretion
CRK1	Cytokinin-regulated kinase 1	RR	Response regulator
*c*Z	*Cis*-zeatin	RRA	Type-A response regulator
*c*Z9G	*Cis*-zeatin N^9-glucoside	RRB	Type-B response regulator
*c*ZR	*Cis*-zeatin riboside	*t*Z	*Trans*-zeatin
*c*ZROG	*Cis*-zeatin riboside *O*-glucoside	*t*Z7G	*Trans*-zeatin N^7-glucoside
DHZ	Dihydrozeatin	*t*ZR	*Trans*-zeatin riboside
DHZR	Dihydrozeatin riboside	*t*ZROG	*Trans*-zeatin riboside *O*-glucoside
FAC	Fatty acid-amino acid conjugate	W+OS	Wounding and application of oral secretion
HAMP	Herbivore-associated molecular pattern	W+W	Wounding and water
HPT	Histidine-containing phosphotransfer protein	ZOG	Zeatin *O*-glucosyltransferase

Table S2. Sequences of primers used for qPCR.

Gene	forward primer	reverse primer
NaActin	5‘ggtcgtaccaccggtattgtg3’	5‘gtcaagacggagaatggcatg3’
NaLOG4	5‘ctcagctcacaaagtcttcacg3’	5‘ccattaagccaacacttccacc3’
NaIPT5	5‘tcagccacttattaatttccgagag3’	5‘ttggctagatcaatggatagtctag3’
NaRRA5	5‘agatgagttgcatgttcttgctgt3’	5‘tcaatccccacagaggtcttct3’
NaCKX5	5‘ttgtcggcttattgtaaccgtcg3’	5‘gttaagaactgccatcggctc3’
NaZOG2	5‘agtcatgcaagtcaatttaagagctc3’	5‘aggaaatttgggaagaaggtgtaag3’
NaCHK3	5‘tgctctccggagaggaagatc3’	5‘ttagaaggaagatcggttttgtaaact3’

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

Analyte	Q1 [m/z] →	Q3 [m/z]	DP	CE	CXP	Internal Standard
*t*Z	220.2	136.3	26	25	16	[^{2}H5]*t*Z
*t*ZR	352.2	220.3	76	25	30	[^{2}H5]tZR
*t*ZROG	514.1	382.1	96	25	16	[^{2}H5]*t*ZROG
*t*Z7G	382.1	220.2	71	31	16	[^{2}H5]*t*Z7G
*c*Z	220.2	136.3	26	25	16	[^{2}H5]*t*Z
*c*ZR	352.2	220.3	76	25	30	[^{2}H5]*t*ZR
*c*ZROG	514.1	382.1	96	25	16	[^{2}H5]*t*ZROG
*c*Z9G	382.1	220.0	71	31	16	[^{2}H5]*t*Z9G
IP	204.1	136.0	81	23	16	[^{2}H6]IPR[a]
IPR	336.1	204.3	61	23	26	[^{2}H6]IPR
DHZ	222.0	136.0	76	27	20	[^{2}H5]*t*Z[b]
DHZR	354.2	220.1	101	29	18	[^{2}H5]tZR[c]
[^{2}H5]*t*Z	225.2	136.3	26	25	16	
[^{2}H5]*t*ZR	357.2	225.3	76	25	30	
[^{2}H5]*t*ZROG	519.1	387.1	96	25	16	
[^{2}H5]*t*Z7G	387.1	225.0	71	31	16	
[^{2}H5]*t*Z97G	387.1	225.0	71	31	16	
[^{2}H6]IPR	342.0	210.1	61	23	26	

[a] Reference factor -> $m_{IP} = 8.772 \times m_{D6\text{-}IPR}$

[b] Reference factor -> $m_{DHZ} = 0.6029 \times m_{D5\text{-}tZ}$

[c] Reference factor -> $m_{DHZR} = 0.5525 \times m_{D5\text{-}tZR}$

3.2 Manuscript II

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*

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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,

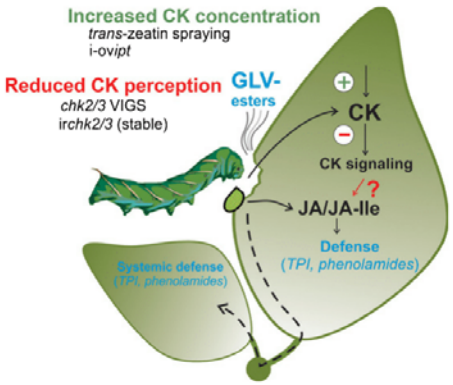

Fig. 1 Experimental logic used to analyze cytokinin (CK)-mediated effects on the herbivory-inducible defense response. Herbivore-derived elicitors in the oral secretions of *Manduca sexta* are well known to induce rapid increases in the concentrations of jasmonic acid (JA) and the active JA–isoleucine 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

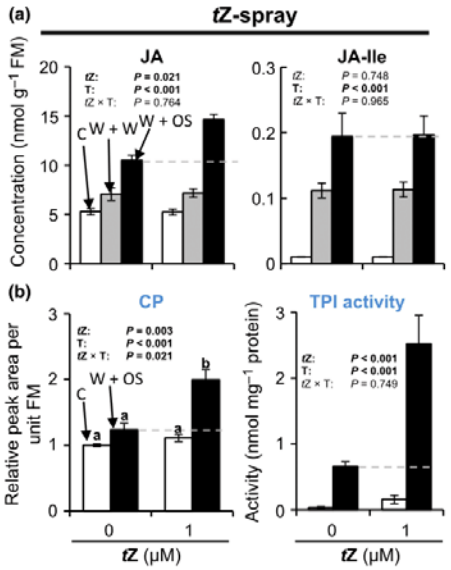

Fig. 2 Cytokinin application increases the herbivory-inducible defense response. (a) Jasmonic acid (JA) and JA–isoleucine conjugate (JA-Ile) accumulation in leaves of *Nicotiana attenuata* 1 h after wounding and application of water (W + W; gray bars) or *Manduca sexta* oral secretions (W + OS; black 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).

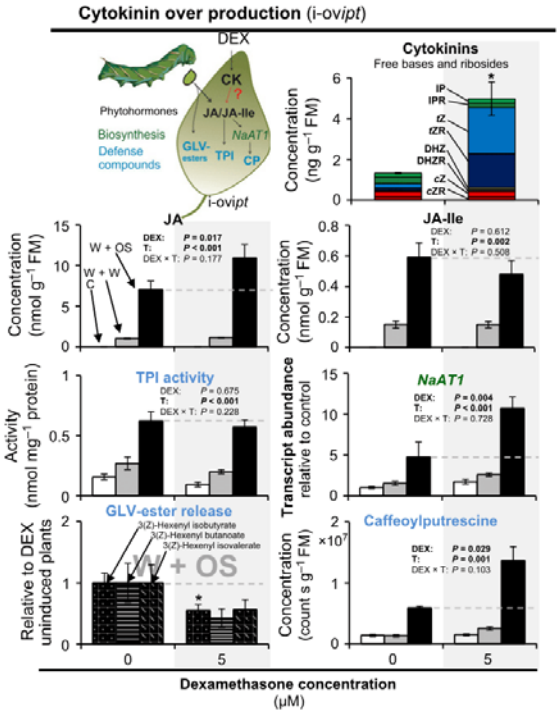

Fig. 3 Cytokinin concentrations regulate herbivory-inducible defense responses. Isopentenyladenine (IP), isopentenyladenosine (IPR), *trans*-zeatin (*t*Z), *trans*-zeatin riboside (*t*ZR), dihydrozeatin (DHZ), dihydrozeatin riboside (DHZR), *cis*-zeatin (cZ) and *cis*-zeatin riboside (cZR) concentrations 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,

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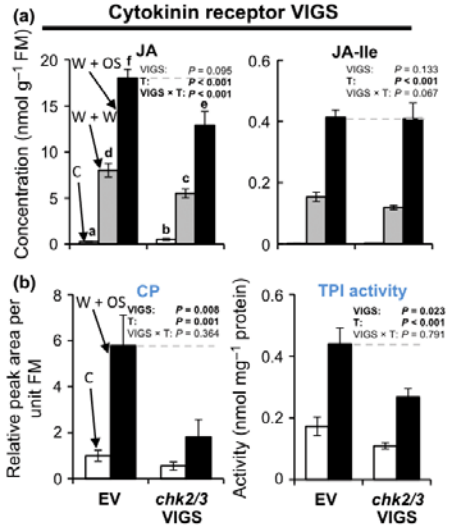

Fig. 4 Impaired cytokinin perception decreases herbivory-inducible defense responses. (a) Jasmonic acid (JA) and JA–isoleucine conjugate (JA-Ile) concentrations in leaves of *Nicotiana attenuata* 90 min after wounding and application of water (W + W; gray bars) or *Manduca sexta* oral secretions (W + 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.

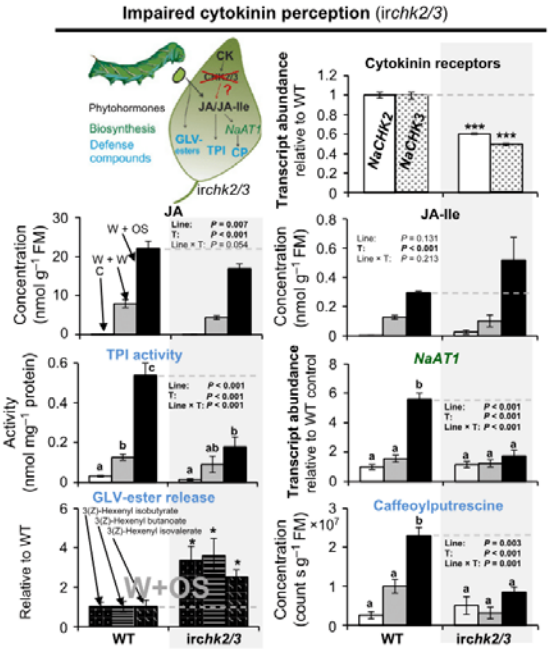

Fig. 5 Cytokinin perception regulates herbivory-induced defense responses. Transcript abundance of *CHASE-DOMAIN CONTAINING HIS KINASE 2* (*NaCHK2*) and *NaCHK3* in the shoots of 6-d-old *Nicotiana attenuata* seedlings was measured. Jasmonic acid (JA) and JA–isoleucine conjugate (JA-Ile) accumulation 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

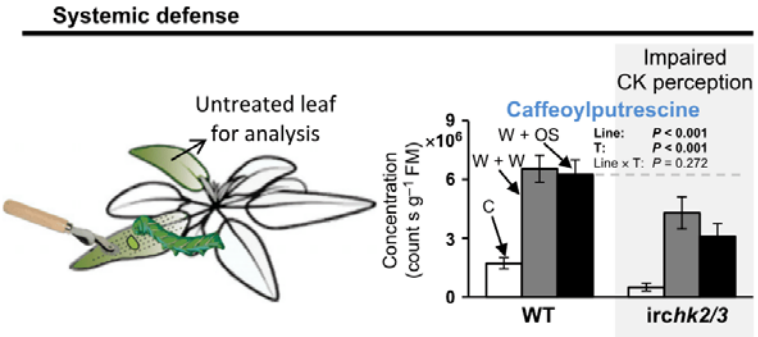

Fig. 6 Cytokinin (CK) perception regulates systemic caffeoylputrescine concentrations. Caffeoylputrescine accumulation was measured in untreated systemic leaves of *Nicotiana attenuata* 2 d after wounding and treatment with water (W + W; gray bars) or *Manduca sexta* oral secretions (W + OS; black bars) 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

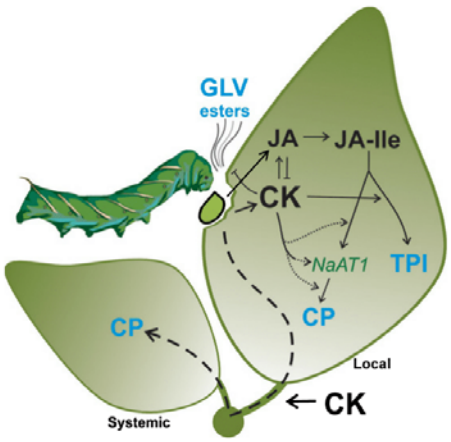

Fig. 7 The roles of cytokinins (CKs) in the herbivore defense responses of *Nicotiana attenuata*. CK metabolism and signaling are regulated by wounding and perception of herbivore-derived elicitors from the oral secretions of *Manduca sexta*. Jasmonic acid (JA) suppresses the herbivory-induced CK signaling 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.

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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.

Supporting Information

Figs S1-S17 and Tables S1–S4

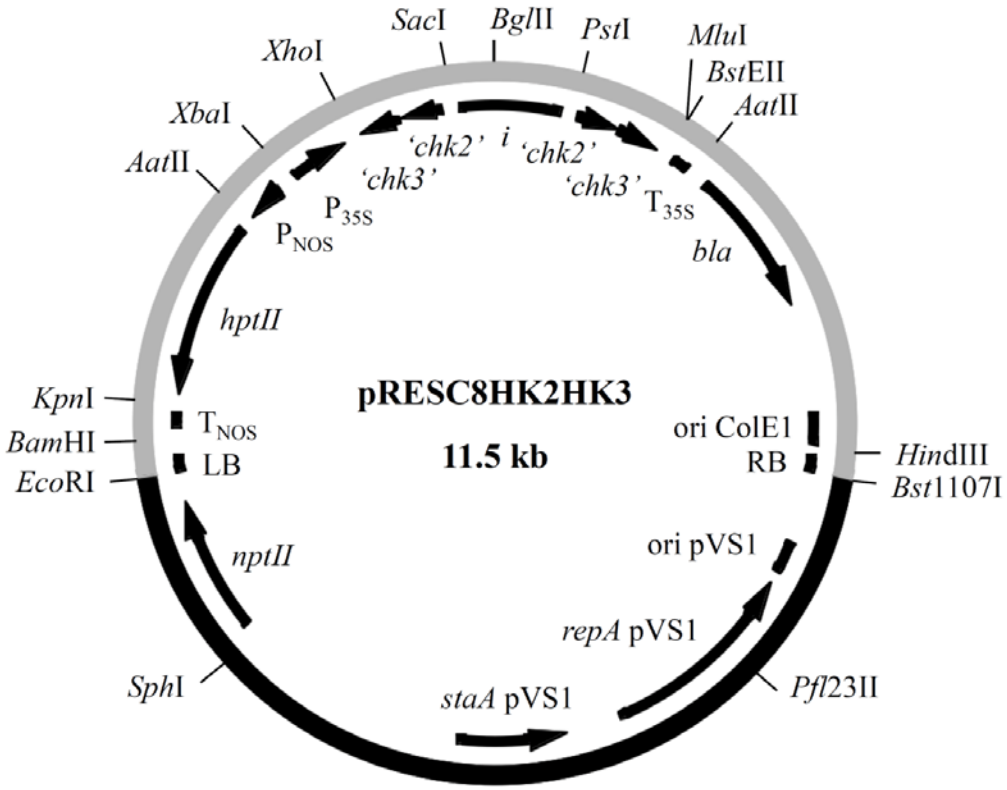

Fig. S1 Plasmid vector for cytokinin receptor silencing.

Plasmid design of the pRESC8HK2HK3 vector for *CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2)* and *NaCHK*3 silencing.

LB/RB, left/right border of T-DNA; P_{NOS}/T_{NOS}, promoter/terminator of the nopaline synthase gene from the Ti plasmid of *Agrobacterium tumefaciens*; P_{35S}/T_{35S}, 35S promoter/terminator from cauliflower mosaic virus; *hptII*, hygromycin phosphotransferase gene from pCAMBIA-1301 (AF234297); *chk2/chk3,* fragments of the *N. attenuata* cytokinin receptors *NaCHK2*/*NaCHK3* (see Supplemental Table 4 online)*;* i, intron 3 of *Flaveria trinervia* pdk gene for pyruvate, orthophosphate dikinase; nptII, aminoglycoside phosphotransferase class II; ori, origin of replication.

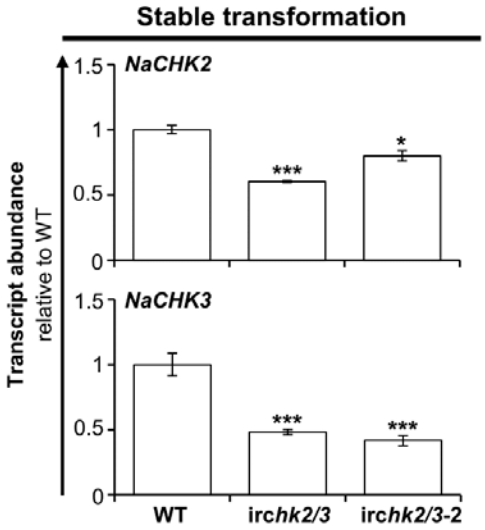

Fig. S2 ir*chk2/3* and ir*chk2/3*-2 plants are silenced in *CHASE-DOMAIN CONTAINING HIS KINASE 2* (*NaCHK2*) and *NaCHK3* expression.
Relative *NaCHK2* and *NaCHK3* transcript abundance in wild-type plants (WT) and two independent *NaCHK2*/*NaCHK3*-silenced lines (ir*chk2/3*, ir*chk2/3*-2). Measurements were performed in pooled shoot samples of 6 d old *N. attenuata* seedlings.
Asterisks indicate significant differences between WT and the transgenic lines (independent samples *t* test: * $P \leq 0.05$, *** $P \leq 0.001$). Error bars are standard errors (N=3).

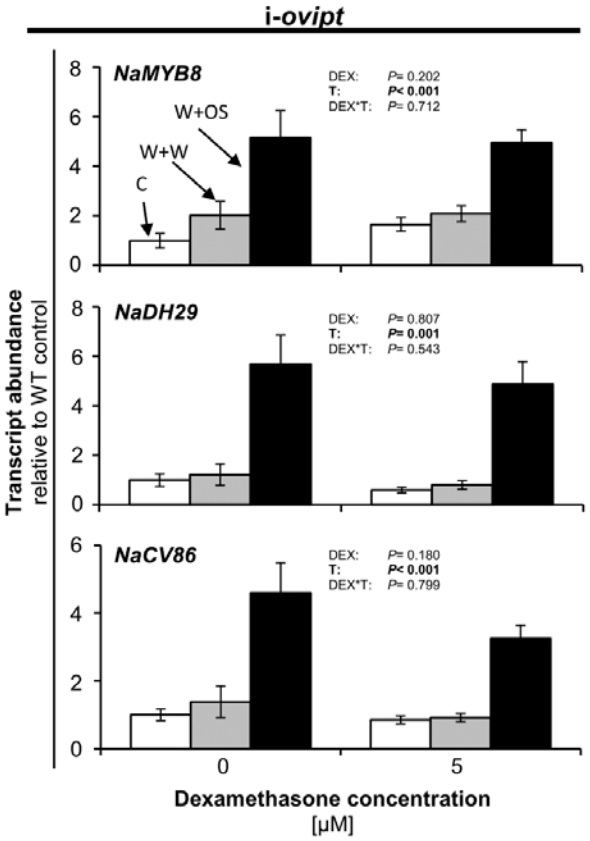

Fig. S3 Increased cytokinin concentrations are not sufficient to upregulate the herbivory-induced transcript accumulation of *NaMYB8*, *NaDH29* and *NaCV86.*

Transcript abundance of *NaMYB8*, *NaDH29* and *NaCV86* were measured in leaves of *Nicotiana attenuata* two days after wounding and treatment with water (W+W, grey bars) or *Manduca sexta* oral secretions (W+OS, black bars) and in untreated control leaves (C, white bars). Measurements were performed in leaves of dexamethasone (DEX)-inducible isopentenyltransferase-overexpressing plants (i-ov*ipt*) treated with 0 and 5 µM DEX-containing lanolin paste 1 d prior to the experiment. DEX and treatment (C, W+W and W+OS; T) effects and their interactions (DEX*T) were analyzed by two-way ANOVA. Error bars are standard errors (N≥4).

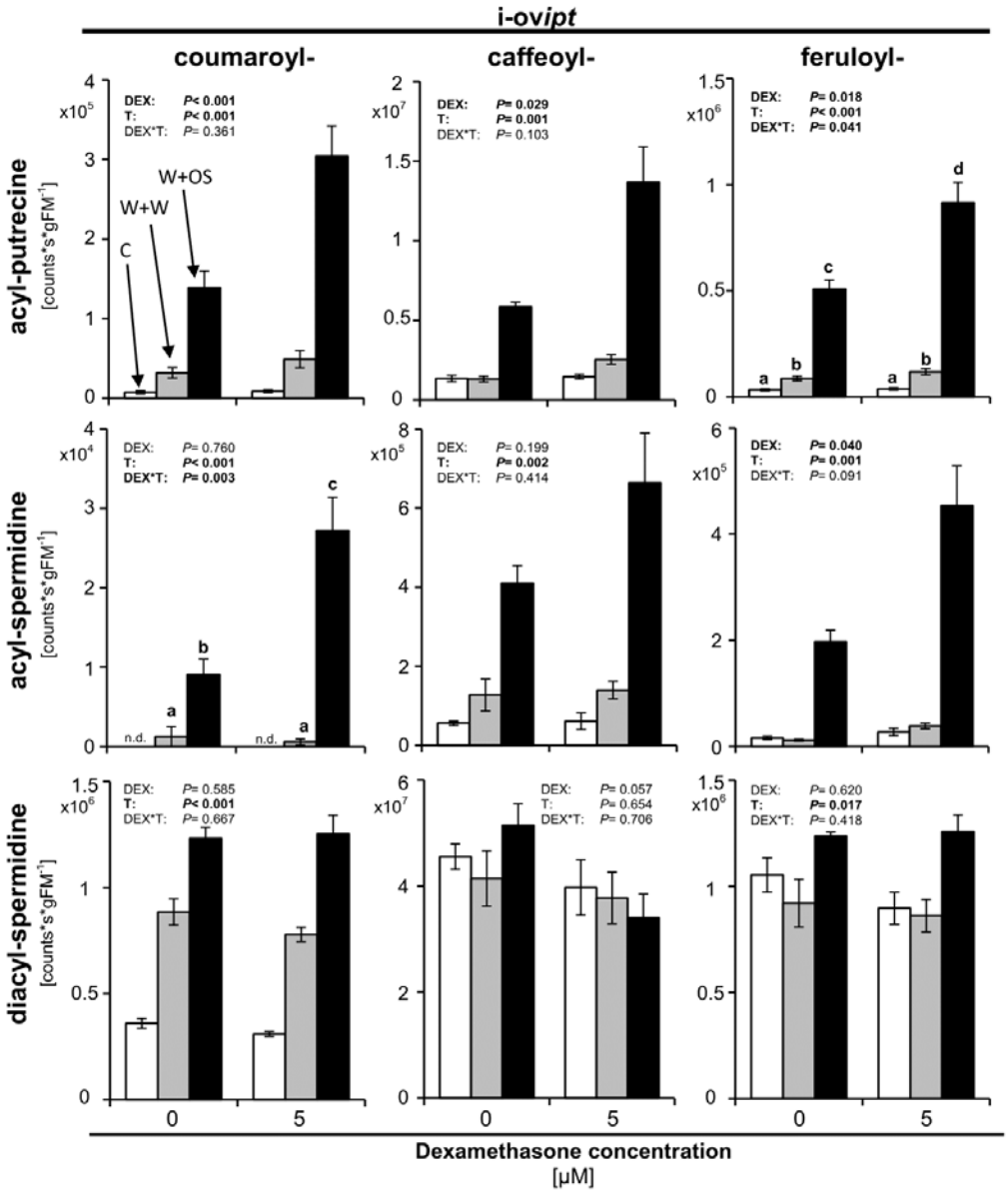

Fig. S4 Increased cytokinin concentrations amplify the accumulation of monoacyl-putrescines and -spermidines.

Amounts of monoacylated putrescines and monoacylated, as well as diacylated spermidines were determined in leaves of *Nicotiana attenuata* two days after wounding and treatment with water (W+W, grey bars) or *Manduca sexta* oral secretions (W+OS,

black bars) and in untreated control leaves (C, white bars). Measurements were performed in leaves of dexamethasone (DEX)-inducible isopentenyltransferase-overexpressing plants (i-ov*ipt*) treated with 0 and 5 µM DEX-containing lanolin paste 1 d prior to the experiment.
DEX and treatment (C, W+W and W+OS; T) effects and their interactions (DEX*T) were analyzed by two-way ANOVA, except for coumaroylspermidin data, which were analyzed by a generalized least squares model instead. Different letters indicate significant differences (feruloylspermidine, Tukey's HSD test: $P \leq 0.05$; coumaroylspermidine, factor level reduction: $P \leq 0.05$). Error bars are standard errors ($N \geq 4$). Caffeoylputrescine data are identical to Fig. 3. FM, fresh mass; n.d., not detected; first row (left to right): coumaroylputrescine, caffeoylputrescine and feruloylputrescine; second row (left to right): coumaroylspermidine, caffeoylspermidine and feruloylspermidine; third row (left to right): coumaroylcaffeoylspermidine, dicaffeoylspermidine and diferuloylspermidine.

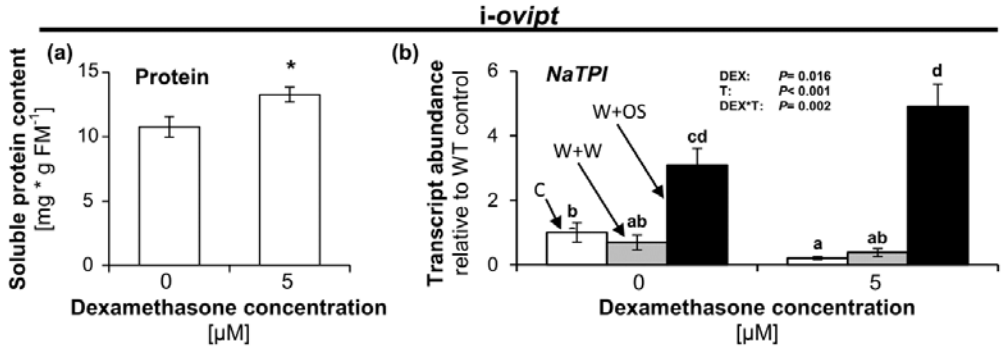

Fig. S5 Increased cytokinin concentrations affect the content of soluble proteins and the transcript accumulation of *TRYPSIN PROTEASE INHIBITOR* (*NaTPI)*.

(a) Soluble proteins were measured in leaves of dexamethasone (DEX)-inducible isopentenyltransferase-overexpressing *Nicotiana attenuata* plants (i-ov*ipt*) treated with 0 and 5 µM DEX-containing lanolin paste for 3 d.

(b) Transcript abundance of *NaTPI* were measured in leaves of *N. attenuata* two days after wounding and treatment with water (W+W, grey bars) or *Manduca sexta* oral secretions (W+OS, black bars) and in untreated control leaves (C, white bars). Measurements were performed in i-ov*ipt* plants treated with 0 and 5 µM DEX-containing lanolin paste 1 d prior to the experiment. DEX and treatment (C, W+W and W+OS; T) effects on the *NaTPI* transcript abundance and their interactions (DEX*T) were analyzed by two-way ANOVA (b). Different letters indicate significant differences (Tukey's HSD test: $P \leq 0.05$). Protein concentrations were analyzed by *t* test. Asterisks indicate significant differences between DEX-induced and non-induced i-ov*ipt* plants (independent samples *t* test: * $P \leq 0.05$). Error bars are standard errors (N≥4).

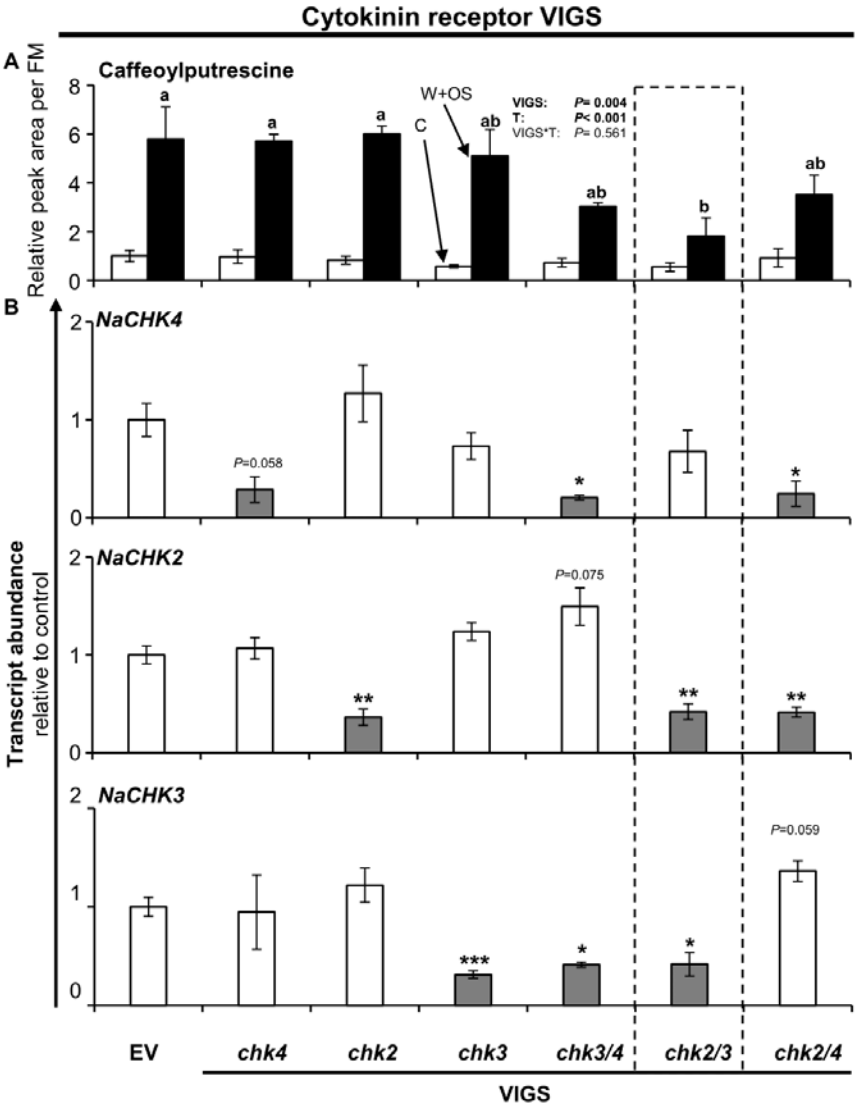

Fig. S6 Impaired cytokinin perception by CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2) and NaCHK3 attenuates the caffeoylputrescine accumulation.
(A) Caffeoylputrescine accumulation in leaves of *Nicotiana attenuata* two days after wounding and application of *Manduca sexta* oral secretions to the puncture wounds (W+OS, black bars) and in untreated control leaves (C, white bars). Measurements were performed with leaves of plants silenced in single or multiple cytokinin (CK) receptors

(*CHK*s) by virus-induced gene silencing (VIGS) and in plants injected with the empty vector control (EV), as indicated.
(B) Relative transcript accumulation of CK receptors in leaves of the plants mentioned in (A). Silencing is highlighted as grey bar.
VIGS and treatment (C and W+OS; T) effects and their interactions (VIGS*T) were analyzed by two-way ANOVA. Different letters indicate significant differences between W+OS-treated samples (Tukey's HSD test: $P \leq 0.05$). Asterisks indicate significant differences between same treatments in EV and CK receptor-silenced plants (independent samples *t* test: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$). Error bars are standard errors (N≥3). FM, fresh mass.

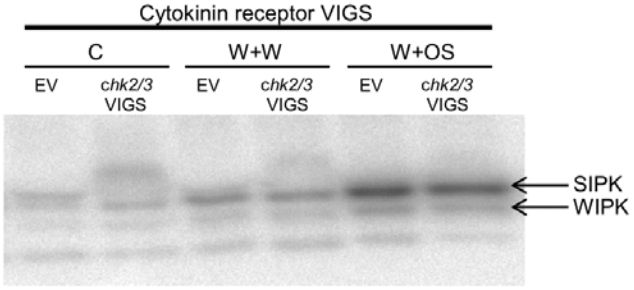

Fig. S7 CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2) and NaCHK3-mediated cytokinin perception is not necessary for SIPK and WIPK activation after wounding and herbivory.

SIPK and WIPK activity in leaves of *Nicotiana attenuata* 90 min after wounding and application of water (W+W) or *Manduca sexta* oral secretions (W+OS) to the puncture wounds and in untreated control leaves (C). Measurements were performed with leaves of plants silenced in *NaCHK2*/*NaCHK3* expression by virus-induced gene silencing (*chk2/3* VIGS) and empty vector control (EV) plants. Kinase activity was determined by in-gel kinase assay.

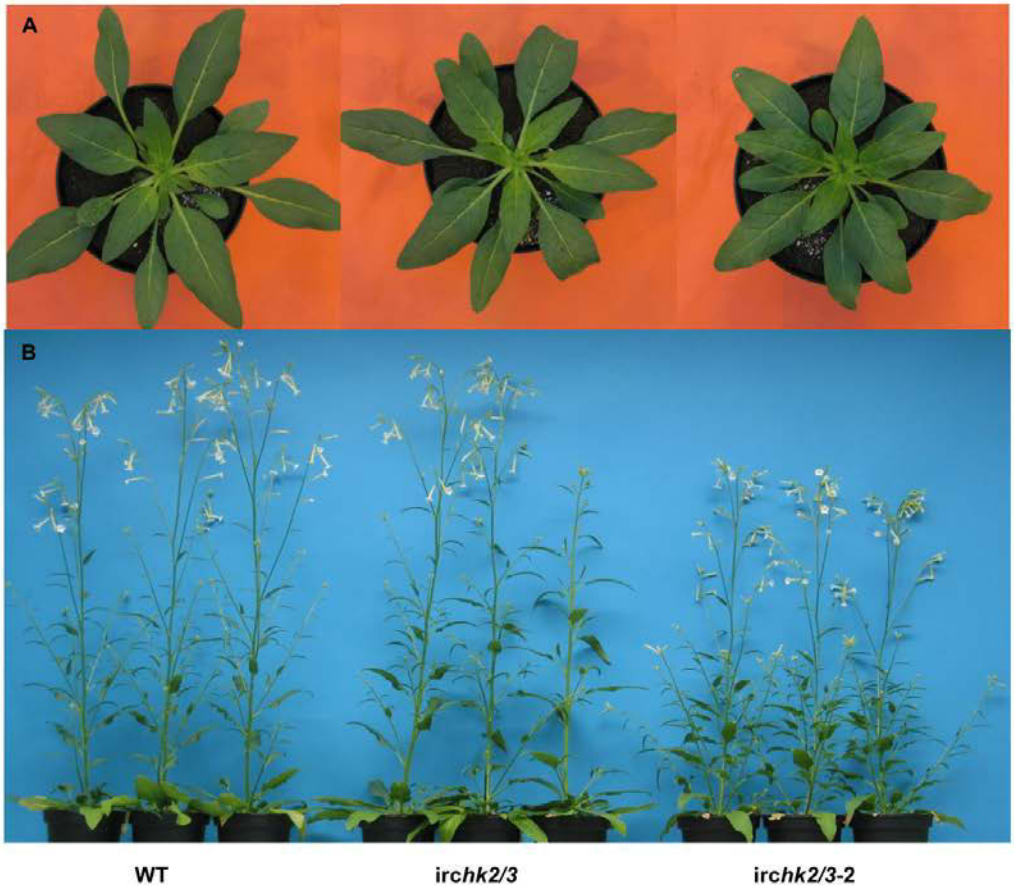

Fig. S8 *CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2)* and *NaCHK3*-silenced plants show only minor phenotypic differences compared to wild type plants. Rosette stage (A) and flowering stage (B) *Nicotiana attenuata* plants from the constitutive RNAi-mediated *NaCHK2/NaCHK3*-silenced lines ir*chk2/3* and ir*chk2/3*-2, as well as wild type (WT) plants. Both lines originate from independent transformation events.

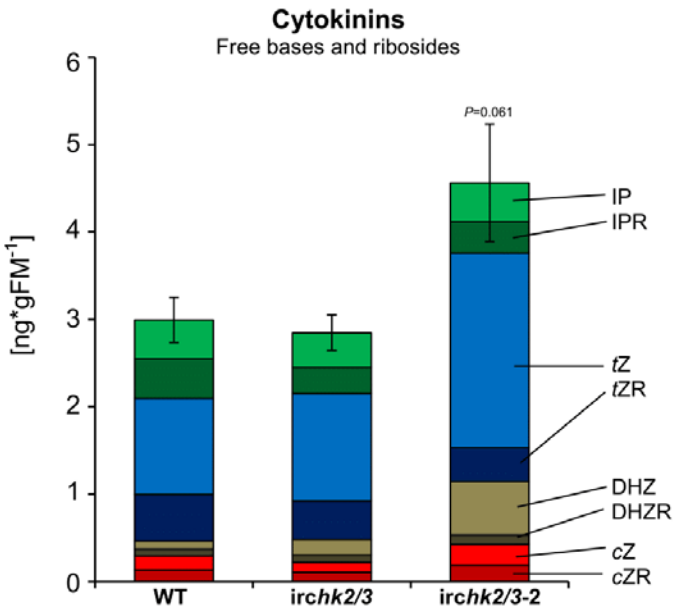

Fig. S9 *CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2)* and *NaCHK3*-silenced plants show only minor changes in the concentrations of active cytokinins compared to wild type plants.

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*. Measurements were performed in leaves of wild-type plants (WT) and *NaCHK2/NaCHK3*-silenced plants (irc*hk2/3,* irc*hk2/3*-2). Differences between WT and irc*hk2/3* or irc*hk2/3*-2 plants were analyzed by independent samples *t* test. Error bars are standard errors (N≥5).FM, fresh mass.

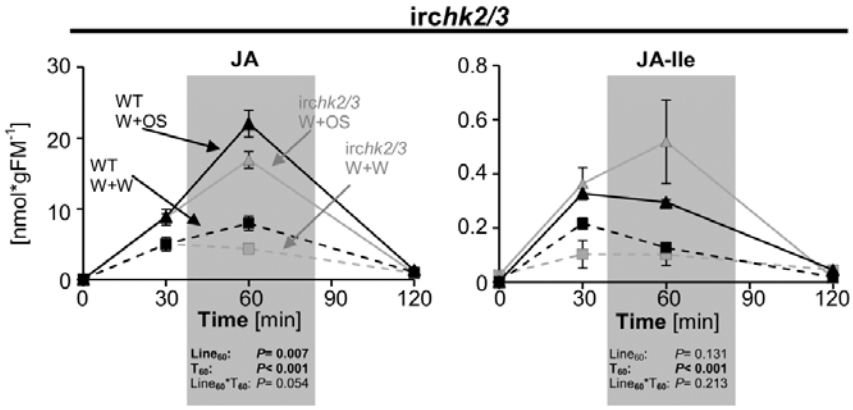

Fig. S10 Impaired cytokinin perception reduces the jasmonic acid, but not jasmonic acid-isoleucine conjugate accumulation.

Jasmonic acid (JA) and JA-isoleucine conjugate (JA-Ile) accumulation in 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). Measurements were performed in leaves of wild-type (WT, black lines) and in *CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2)* and *NaCHK3*-silenced plants (ir*chk2/3*, grey lines).

$Line_{60}$ and treatment (C, W+W and W+OS; T_{60}) effects and their interactions ($Line_{60}$*T_{60}) at time point 60 min after induction were analyzed by two-way ANOVA, except for JA-Ile data, which were analyzed by a generalized least squares model instead. Error bars are standard errors (N≥4). FM, fresh mass.

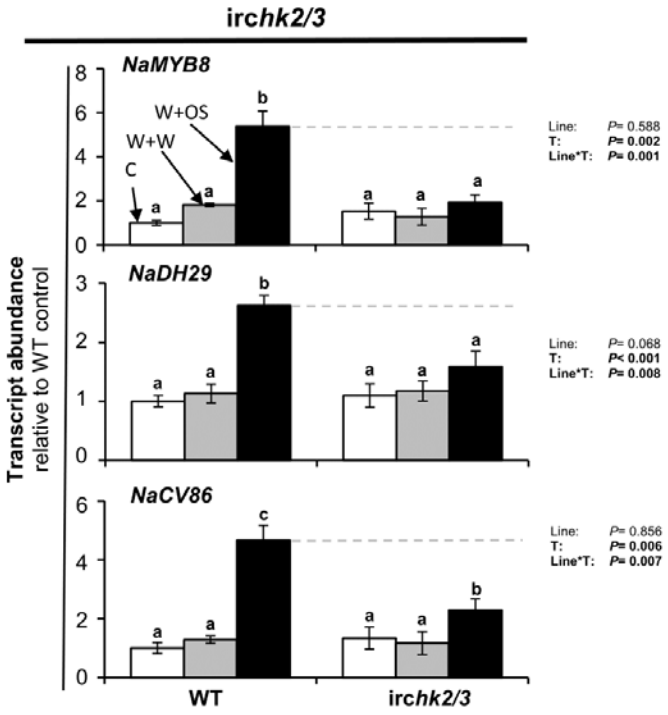

Fig. S11 Impaired cytokinin perception reduces the herbivory-induced accumulation of *NaMYB8*, *NaDH29* and *NaCV86* transcripts.

Transcript abundance of *NaMYB8*, *NaDH29* and *NaCV86* were measured in leaves of *Nicotiana attenuata* two days after wounding and treatment with water (W+W, grey bars) or *Manduca sexta* oral secretions (W+OS, black bars) and in untreated control leaves (C, white bars). Measurements were performed in leaves of wild-type plants (WT) and in *CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2) and NaCHK3*-silenced plants (ir*chk2/3*).

Line and treatment (C, W+W and W+OS; T) effects and their interactions (Line*T) were analyzed by two-way ANOVA, except for *NaMYB8* and *NaCV86* data, which were analyzed by a generalized least squares model instead. Different letters indicate significant differences (*NaDH29*, Tukey's HSD test: $P \leq 0.05$; *NaMYB8* and *NaCV86,* factor level reduction: $P \leq 0.05$). Error bars are standard errors (N≥3).

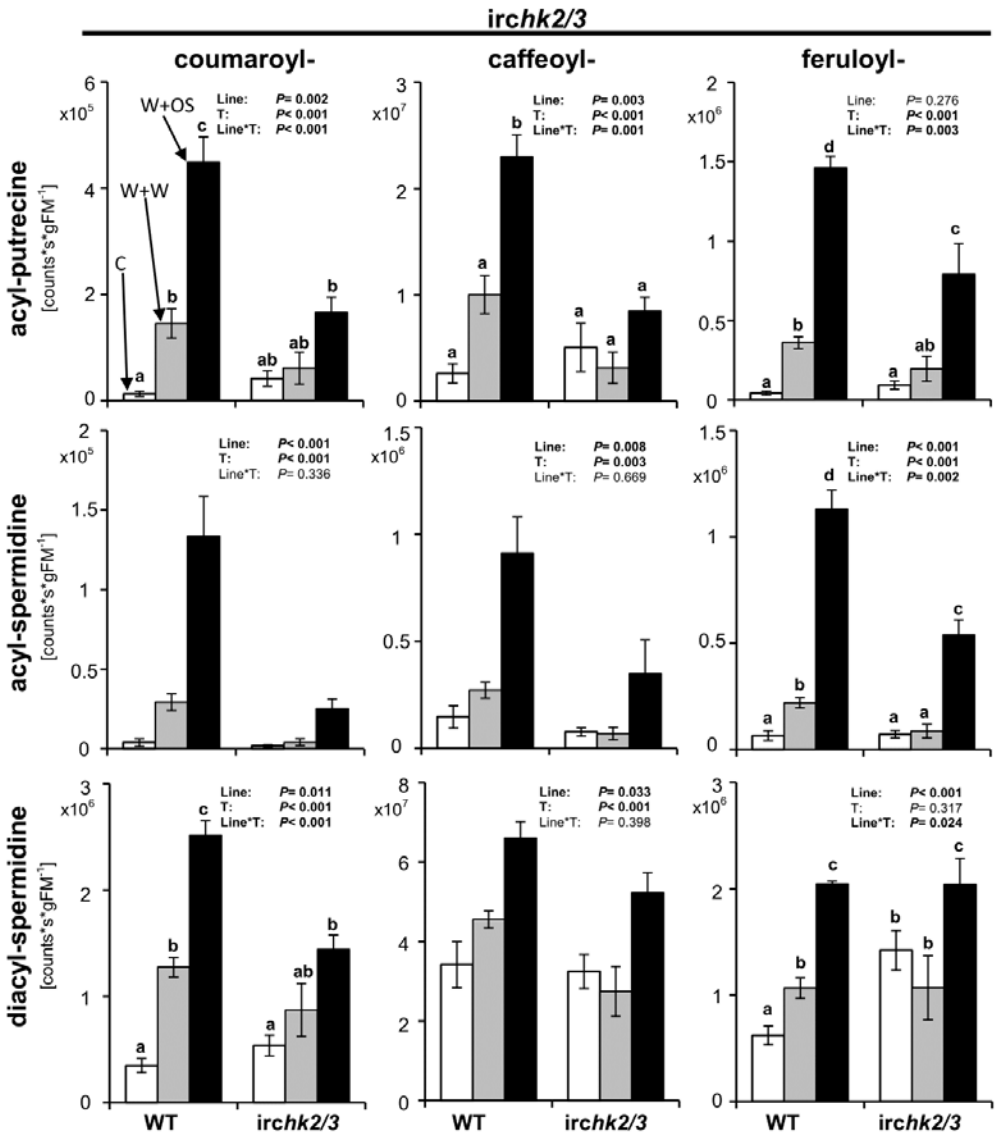

Fig. S12 Impaired cytokinin perception reduces the herbivory-induced accumulation of phenolamides.

Amounts of monoacylated putrescines and monoacylated, as well as diacylated spermidines were determined in leaves of *Nicotiana attenuata* two days after wounding and treatment with water (W+W, grey bars) or *Manduca sexta* oral secretions (W+OS,

black bars) and in untreated control leaves (C, white bars). Measurements were performed in leaves of wild-type plants (WT) and in *CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2)* and *NaCHK3*-silenced plants (irc*hk2/3*).
Line and treatment (C, W+W and W+OS; T) effects and their interactions (Line*T) were analyzed by two-way ANOVA, except for feruloylputrescine and diferuloylspermidine data, which were analyzed by a generalized least squares model instead. Different letters indicate significant differences (coumaroylputrescine, caffeoylputrescine and coumaroylcaffeoylspermidine, Tukey's HSD test: $P \leq 0.05$; feruloylputrescine and diferuloylspermidine, factor level reduction: $P \leq 0.05$). Error bars are standard errors ($N \geq 4$). Caffeoylputrescine data are identical to Fig. 7. FM, fresh mass; first row (left to right): coumaroylputrescine, caffeoylputrescine and feruloylputrescine; second row (left to right): coumaroylspermidine, caffeoylspermidine and feruloylspermidine; third row (left to right): coumaroylcaffeoylspermidine, dicaffeoylspermidine and diferuloylspermidine.

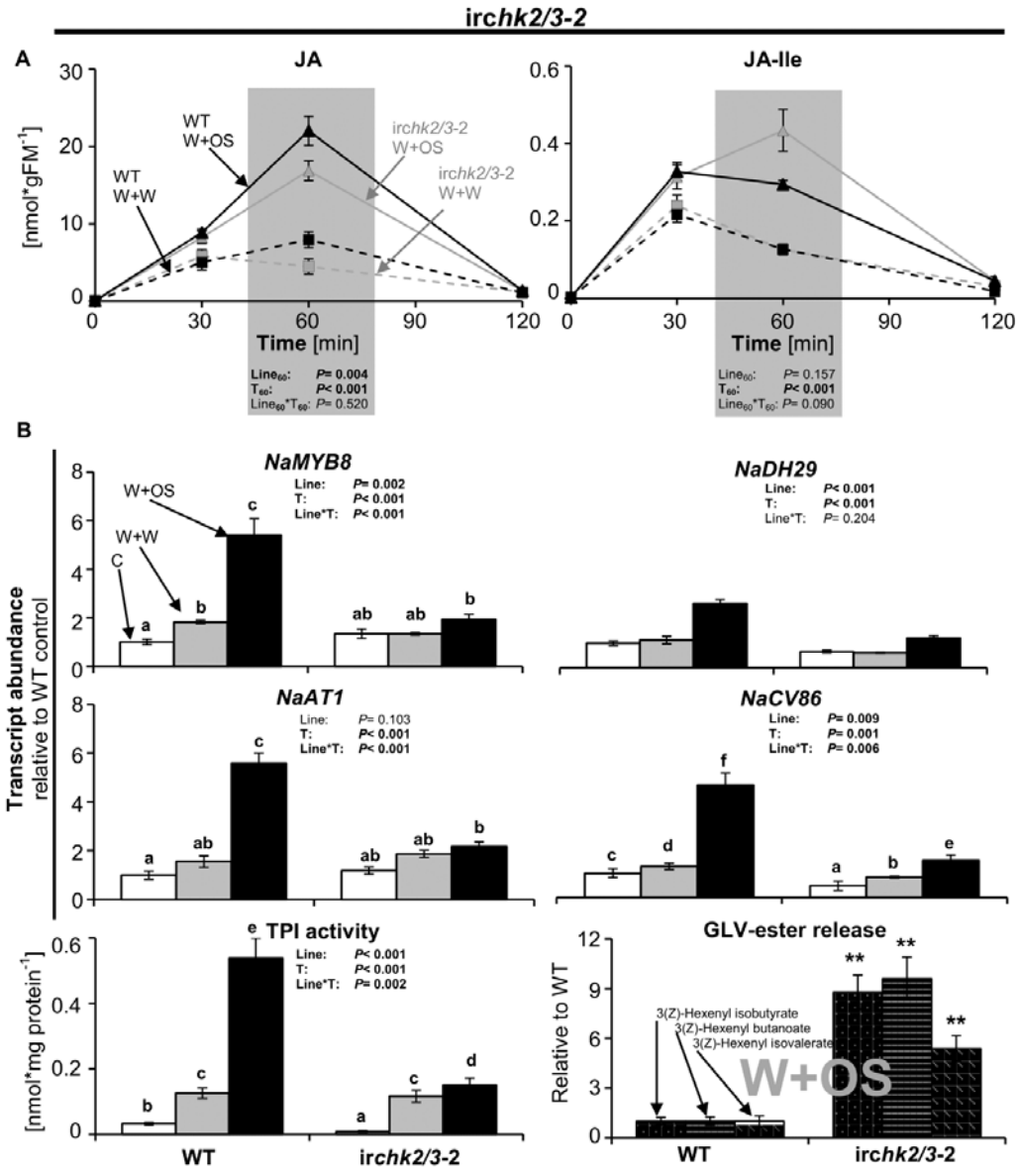

Fig. S13 The independently transformed *CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2)* and *NaCHK3*-silenced plants (ir*chk2/3*-2) confirm cytokinin signaling-mediated effects on the herbivory-induced defense responses.

(A) Jasmonic acid (JA) and JA-isoleucine conjugate (JA-Ile) abundance in 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 and in untreated control leaves (C, dotted line). Measurements were performed in leaves of wild-type plants (WT, black lines) and *NaCHK2/NaCHK3*-silenced plants (ir*chk2/3*-2, grey lines).

(B) Trypsin proteinase inhibitor (TPI) activity, as well as transcript abundance of *NaMYB8*, *NaAT1*, *NaDH29* and *NaCV86* in leaves two days after W+W (grey bars) or W+OS (black bars) treatment and in untreated control leaves (C, white bars). Green leaf volatile (GLV)-ester release of *N. attenuata* leaves was measured in the night and the next 12 h of the following photoperiod after a twice repeated W+OS treatment. Measurements were performed in leaves of WT plants and ir*chk2/3*-2 plants, as indicated.

Line and treatment (C, W+W and W+OS; T) effects and their interactions (Line*T) were analyzed by two-way ANOVA, except for JA-Ile, *NaCV86* and TPI activity data, which were analyzed by a generalized least squares model instead. JA and JA-Ile data were analyzed at time point 60 min after induction (indicated as $Line_{60,}$ T_{60} and $Line_{60}$*T_{60} respectively). Different letters indicate significant differences (*NaMYB8* and *NaAT1*, Tukey's HSD test: $P \leq 0.05$; *NaCV86* and TPI activity, factor level reduction: $P \leq 0.05$). GLV-esters were analyzed by *t*-test. Asterisks indicate significant differences between WT and ir*chk2/3*-2 plants (independent samples *t*-test: ** $P \leq 0.01$). Error bars are standard errors (N≥4). For the corresponding phenolamide data see Fig. S14. FM, fresh mass.

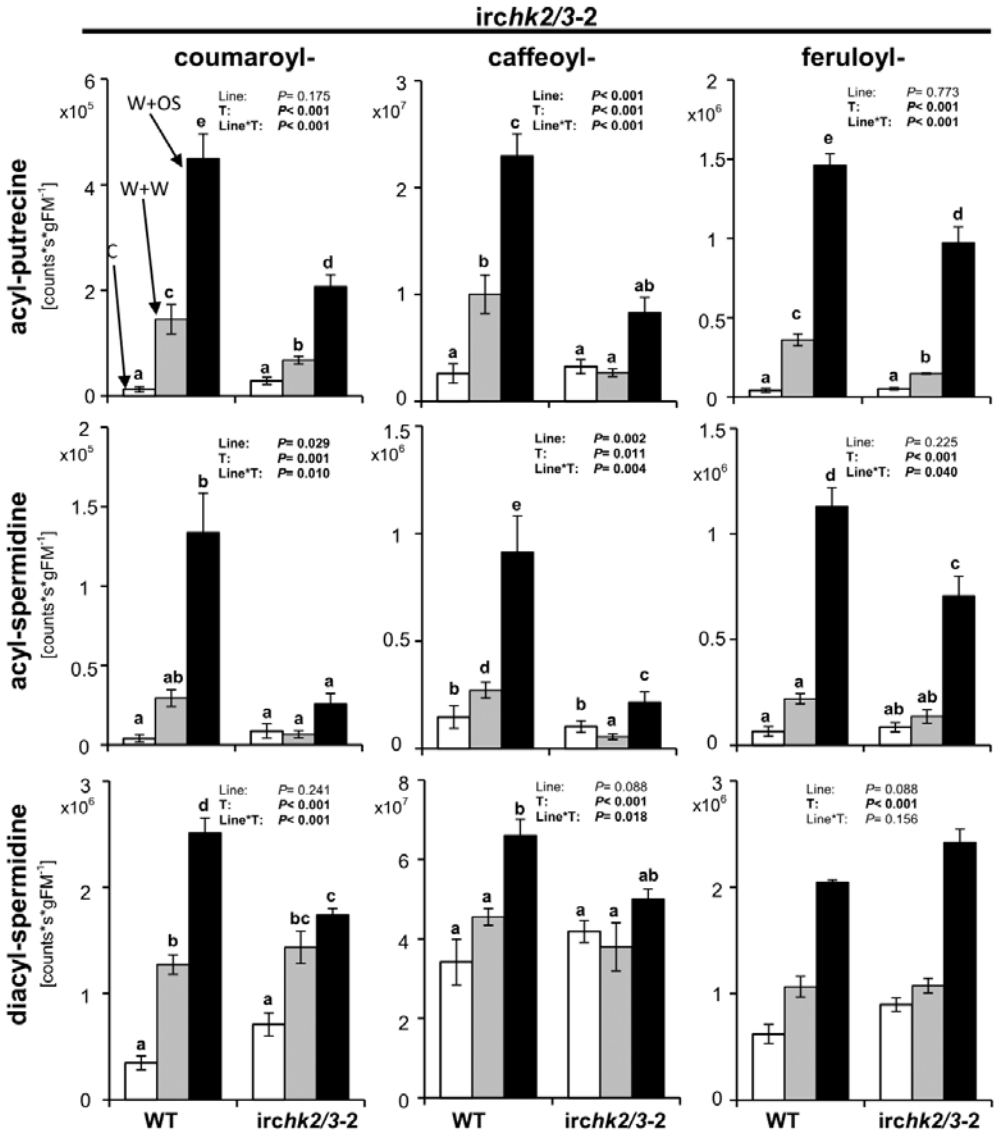

Fig. S14 The independently transformed *CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2)* and *NaCHK3*-silenced plants (ir*chk2/3*-2) confirm cytokinin signaling-mediated effects on the herbivory-induced accumulation of phenolamides. Amounts of monoacylated putrescines and monoacylated, as well as diacylated spermidines were determined in leaves of *Nicotiana attenuata* two days after wounding

and treatment with water (W+W, grey bars) or *Manduca sexta* oral secretions (W+OS, black bars) and in untreated control leaves (C, white bars). Measurements were performed in leaves of wild-type plants (WT) 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 with two-way ANOVA, except for coumaroylputrescine, feruloylputrescine and caffeoylspermidine data, which were analyzed a by a generalized least squares model instead. Different letters indicate significant differences (coumaroylputrescine, caffeoylputrescine and coumaroylcaffeoylspermidine, Tukey's HSD test: $P \leq 0.05$; feruloylputrescine and diferuloylspermidine, factor level reduction: $P \leq 0.05$). Error bars are standard errors (N≥4). FM, fresh mass; first row (left to right): coumaroylputrescine, caffeoylputrescine and feruloylputrescine; second row (left to right): coumaroylspermidine, caffeoylspermidine and feruloylspermidine; third row (left to right): coumaroylcaffeoylspermidine, dicaffeoylspermidine and diferuloylspermidine.

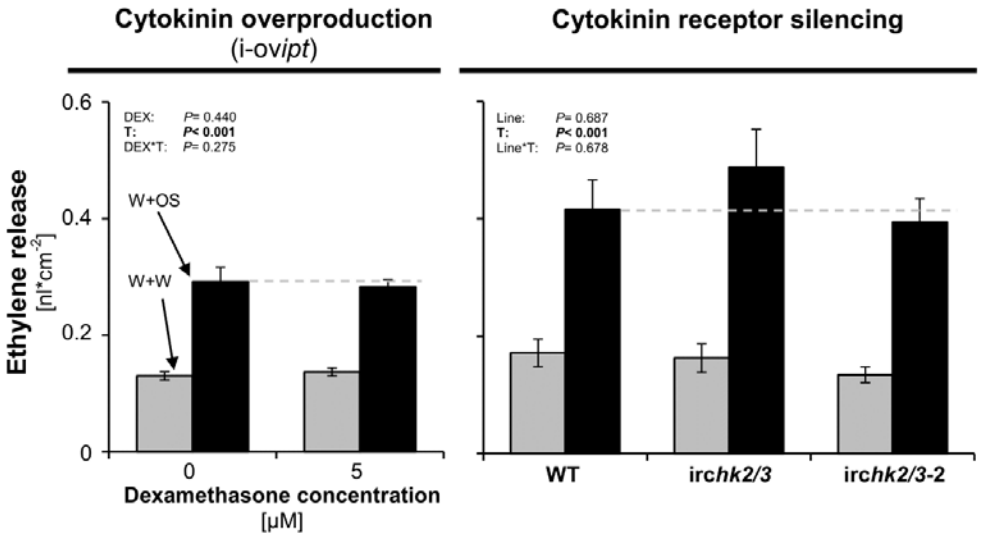

Fig. S15 Cytokinin concentrations and their perception by CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2) and NaCHK3 do not regulate the herbivory-induced ethylene release.

Ethylene release was determined from leaf-discs of *Nicotiana attenuata* after wounding and treatment with water (W+W, grey bars) or *Manduca sexta* oral secretions (W+OS, black bars). Ethylene was accumulated for 5 h before measurement. Ethylene release was measured in dexamethasone (DEX)-inducible isopentenyltransferase-overexpressing plants (i-ov*ipt*) treated with 0 and 5 µM DEX-containing lanolin paste 1 d prior to the experiment, as well as in wild-type plants (WT) and two independent-transformed *NaCHK2/NaCHK3*-silenced lines (irc*hk2/3,* irc*hk2/3*-2). DEX/Line and treatment (C, W+W and W+OS; T) effects and their interactions (DEX*T and Line*T respectively) were analyzed with two-way ANOVA. Error bars are standard errors (N=5).

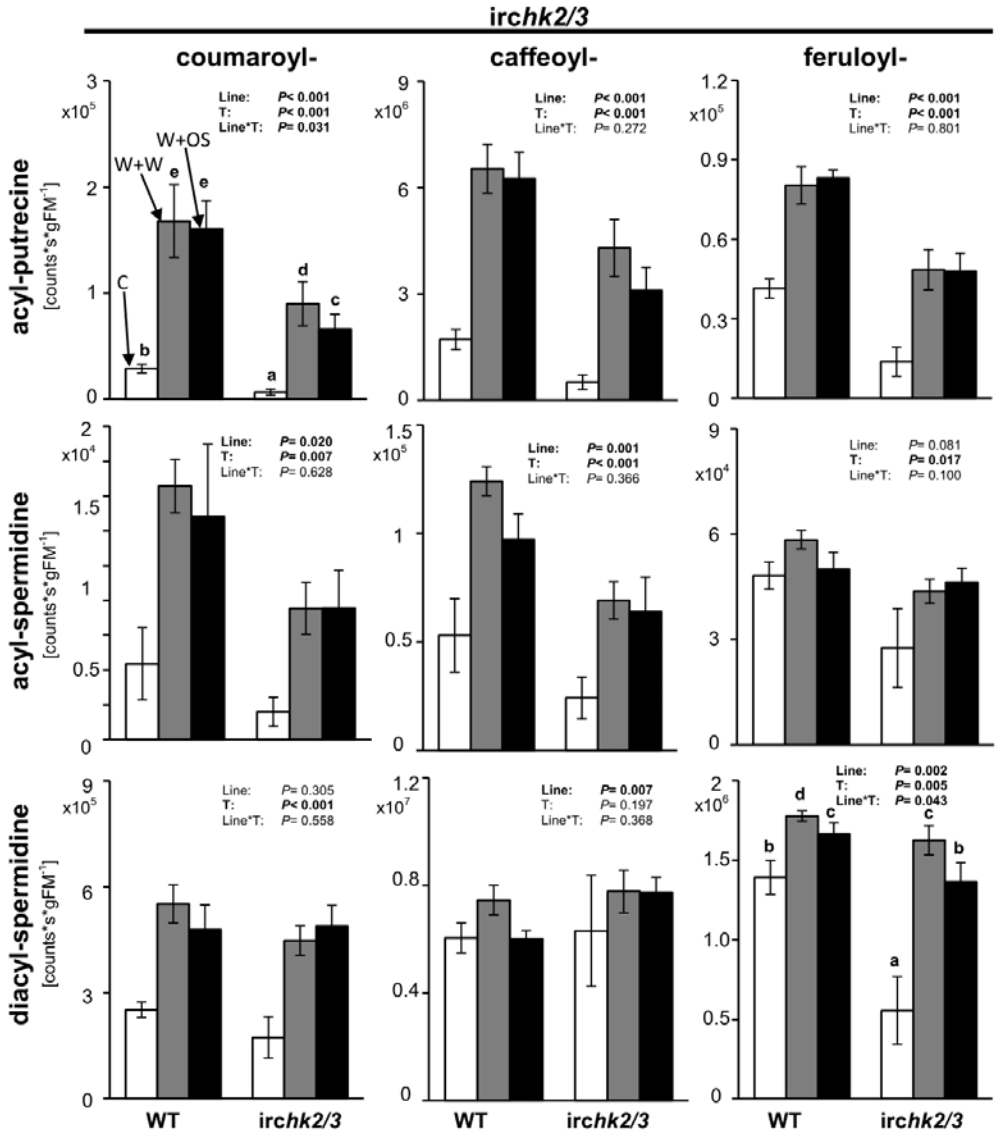

Fig. S16 Impaired cytokinin perception reduces the systemic accumulation of monoacyl-putrescines and -spermidines.

Amounts of monoacylated putrescines and monoacylated, as well as diacylated spermidines were determined in untreated systemic leaves of *Nicotiana attenuata* two days after wounding and treatment with water (W+W, grey 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 plants (WT) and in *CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2)* and *NaCHK3*-silenced plants (ir*chk2/3*).
Line and treatment (C, W+W and W+OS; T) effects and their interactions (Line*T) were analyzed with two-way ANOVA, except for coumaroylputrescine, feruloylspermidine, diferuloylspermidine and dicaffeoylspermidine data, which were analyzed a by a generalized least squares model instead. Different letters indicate significant differences (coumaroylputrescine, diferuloylspermidine, factor level reduction: $P \leq 0.05$). Error bars are standard errors (N=5). FM, fresh mass; first row (left to right): coumaroylputrescine, caffeoylputrescine and feruloylputrescine; second row (left to right): coumaroylspermidine, caffeoylspermidine and feruloylspermidine; third row (left to right): coumaroylcaffeoylspermidine, dicaffeoylspermidine and diferuloylspermidine.

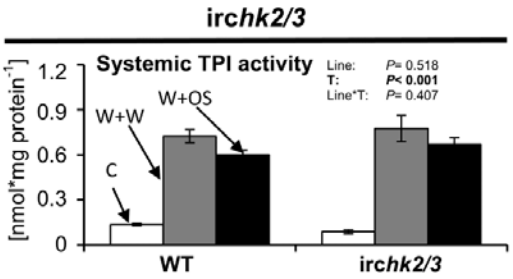

Fig. S17 Cytokinin perception by CHASE-DOMAIN CONTAINING HIS KINASE 2 (NaCHK2) and NaCHK3 does not regulate the systemic increase of trypsin proteinase inhibitor (TPI) activity.

TPI activity in untreated systemic leaves of *Nicotiana attenuata* two days after wounding and treatment with water (W+W, grey 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 plants (WT) plants and *NaCHK2/NaCHK3*-silenced plants (irc*hk2/3*).

Line and treatment (C, W+W and W+OS; T) effects and their interactions (Line*T) were analyzed by two-way ANOVA. Error bars are standard errors (N=5). FM, fresh mass.

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

Gene	sequence
NaCHK2	5'ttggtcacatcttccatgctgccatcaaccgaatcgtcgaagttgagggtcagtatcaagaaatgatg gaactcaaacatcgtgctgaggctgccgatatagcaaaatctcagtttcttgcaacggtttctcatgaaa tcaggactccgatgaatggtgttttaggcatgcttcaaatgctcatggacacaaaccttgaccctacgca actggattatgcacaaaccgctcatgctagtg3'
NaCHK3	5'tcattctgaagtggtttcagatttgaatcactgtttgtcttacttaagaactgacaatagggtcacaaata tgatcctcattgaacaggaggtctgggatactgatttgggaatgtcaattctctttgtcaaaaacttaagaa atatcgatgctaatgaccctcctaaactatttatattagctaatgctataaattctaaccgagttggtgtagc caatggtctttcttctccatttgtca3'
NaCHK4	5'tcttcctcttaattggctacacgggttacaaatccgcaagccacattaataaagtagaggatgattttc ataaaatgcaggaactaaaggttcaagctgaagcagctgatgttgccaaatcccagttcttggctactg tttcacatgaaataagaactcctatgaatgggatcctaggaatgcttgctctgctcctagatacaaatctg aattcaactcaaagagattatgctcaaactgctcaggcttgtgg3'

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

Gene	forward primer	reverse primer
NaActin	5'ggtcgtaccaccggtattgtg3'	5'gtcaagacggagaatggcatg3'
NaGAPDH	5'tcactgataaagacaaggctgct3'	5'tcataaggccctcaacaattcca3'
NaCHK4	5'gaatgagcaatttgactcaaagag3'	5'ctccttctgattagcatccatag3'
NaCHK2	5'ccttggtgttgttcttacatttgc3'	5'acaatagtttgcttgcttgcaagc3'
NaCHK3	5'tgctctccggagaggaagatc3'	5'ttagaaggaagatcggttttgtaaact3'
NaMYB8	5'aacctcaagaaactcaggacatacaa3'	5'gatgaatgtgtgaccaaattttcc3'
NaAT1	5'tcacaaggttcacttgtggctctg 3'	5'gcatttgccttgagtttgcctagg 3'
NaDH29	5'atcaactagccattagaatg3'	5'ccaaaaatgatttgcaaggtc3'
NaCV86	5'atcaaatagctgaagatgtc3'	5'ccaacaaagtagtgctgtact3'
NaTPI	5'ctcaggagatagtaaatatggctg3'	5'gcatctgcatgttccacattgct3'

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

Analyte		[m/z][1]	Retention time[2] [min]	
Acyl-putrescine	Coumaroyl-	235.14	2.4	2.6
	Caffeoyl-	251.13	1.9	2.3
	Feruloyl-	265.15	2.5	2.7
Acyl-spermidine	Coumaroyl-	292.20	1.9	
	Caffeoyl-	308.20	1.5	
	Feruloyl-	322.20	1.8	2.2
Diacyl-spermidine	Coumaroyl-[3]	454.23	3.2	
	Caffeoyl-	470.23	3.0	
	Feruloyl-	498.26	3.5	

[1] Width: ±0.05

[2] Retention times of all analyzed isomers

[3] Coumaroylcaffeoylspermidine

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

Analyte	**Target ion** [m/z]	**Reference ion** [m/z] / [%][1]	**Retention time** [min]
3(*Z*)-hexenyl isobutyrate	67	71/51; 82/87	17.76
3(*Z*)-hexenyl butyrate	67	71/65; 82/82	19.07
3(*Z*)-hexenyl isovalerate	82	67/92; 57/67	20.46

[1]relative intensity to target ion

3.3 Manuscript III

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Changes in cytokinins are sufficient to alter developmental patterns of defense metabolites in *Nicotiana attenuata*

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SUMMARY

Plant defense metabolites are well known to be regulated developmentally. The optimal defense (OD) theory posits that a t
ssue's fitness values and probability of attack should determine defense metabolite allocations. Young leaves are expected to provide a larger fitness value to the plant, and therefore their defense allocations should be higher when compared with older leaves. The mechanisms that coordinate development with defense remain unknown and frequently confound tests of the OD theory predictions. Here we demonstrate that cytokinins (CKs) modulate ontogeny-dependent defenses in *Nicotiana attenuata*. We found that leaf CK levels highly correlate with inducible defense expressions with high levels in young and low levels in older leaves. We genetically manipulated the developmental patterns of two different CK classes by using senescence- and chemically inducible expression of CK biosynthesis genes. Genetically modifying the levels of different CKs in leaves was sufficient to alter ontogenic patterns of defense metabolites. We conclude that the developmental regulation of growth hormones that include CKs plays central roles in connecting development with defense and therefore in establishing optimal patterns of defense allocation in plants.

Keywords: cytokinins, optimal defense, herbivores, inducible defense, *Nicotiana attenuata*, *Manduca sexta*, plant development, immunosenescence, phytohormones.

INTRODUCTION

The fitness of plants in natural environments and the performance of crops depend on an optimal allocation of resources towards: (i) growth and reproduction; and (ii) resistance against biotic and abiotic stress. The production of defenses that function in resistance against herbivores, for example, often impose a fitness cost and reduce plant productivity (Herms and Mattson, 1992). Plant defenses are dependent on their developmental regulation (Meldau *et al.*, 2012). In addition, these distribution patterns have been interpreted as being consistent with various defense theories formulated to describe the regulation of a plant's investment in defenses (Stamp, 2003). These theories include the growth-differentiation balance hypothesis (Herms and Mattson, 1992) and the optimal defense (OD) theory (McKey, 1974). The OD theory has enjoyed the most experimental support and is arguably the most influential theory describing plant defense syndromes (Rhoades, 1976, 1979; Barto and Cipollini, 2005). The main observation of the OD theory is that the distribution of defenses is unequal amongst different plant parts, and predicts that plants optimize their fitness by using their limited resources to protect those tissues that contribute most to fitness and are most likely to be attacked. Consistent with these predictions are the observations that young leaves frequently harbor higher concentrations of defense metabolites, are more frequently attacked, and are more valuable for a plant's future fitness than older leaves, as they will contribute more to the net carbon fixation of the plant (Coley *et al.*, 1985; Harper, 1989).

Developmentally regulated patterns of defense metabolites as they are predicted by the OD theory have been reported in many plant species (James, 1950; Mothes, 1955; Bowers and Stamp, 1992; Zangerl and Rutledge, 1996; Ohnmeiss *et al.*, 1997; Agostini *et al.*, 1998; Gleadow

and Woodrow, 2000; Ohnmeiss and Baldwin, 2000; Voelckel *et al.*, 2001; Brown *et al.*, 2003; Anderson and Agrell, 2005; Radhika *et al.*, 2008; Gutbrodt *et al.*, 2011; Heath *et al.*, 2014; Massad *et al.*, 2014; Kariñho-Betancourt *et al.*, 2015). However, little is known about the responsible molecular mechanisms (Meldau *et al.*, 2012). Other plant defense hypotheses propose general physiological processes that could account for why plants coordinate growth and development with defense expression. The growth rate/resource availability theory (Coley *et al.*, 1985) states that inherent growth rates might account for the investment in plant defenses, with lowest investment at highest growth rates and highest investment at intermediate growth rates.

One way to understand the mechanisms responsible for developmental patterns of within-plant defense distribution, as predicted for example by the OD theory, is to scrutinize the physiological differences between tissues with contrasting defense patterns. Developmental patterns may be established by the availability of resources in different tissues (Arnold *et al.*, 2004) or by changes in the responsiveness of defense pathways to environmental cues (Diezel *et al.*, 2011). An increasing number of publications demonstrate that growth hormones regulate both the growth and differentiation of plant tissues, as well as the pathways that regulate defense metabolites (for review, see Robert-Seilaniantz *et al.*, 2011; Erb *et al.*, 2012). One class of growth hormones that regulate plant development and defense responses are the cytokinins (CKs). CKs are adenine derivatives with a side-chain on the *N6* position. The most frequently reported CKs have a side-chain that consists of an isoprene moiety, while other types of CKs, for example, with an aromatic side-chain are described (Sakakibara, 2006). Commonly found CKs are *trans*-zeatin (*tZ*), isopentenyladenine (IP), *cis*-zeatin (*cZ*) and dihydrozeatin (DHZ), as well as their ribosides, phosphates and glucosides. Based on receptor affinity assays, the free bases are expected to represent the bioactive form of CKs, but their ribosides are also frequently reported to have high affinities for CK receptors (Yonekura-Sakakibara *et al.*, 2004; Stolz *et al.*, 2011). In contrast, the results of the recently developed plant membrane-based receptor affinity assay (instead of microorganism-based systems) by Lomin *et al.* (2015) and the crystal structure of a CK receptor sensor domain (Hothorn *et al.*, 2011) indicate that only the free bases bind to the receptors, whereas the ribosides possess no or only a low affinity. Because only a subset of CK receptors, namely AHK2, AHK3, AHK4 and ZmHK1, were analyzed with these methods and because other receptors with higher relative affinity to ribosides were reported (e.g. ZmHK3a by Yonekura-Sakakibara *et al.*, 2004), it remains an open question if other CK receptors might use the ribosides as a ligand. Based on their activity in classical bioassays, such as the cucumber cotyledon greening assay, the oat leaf senescence assay and tobacco callus growth assay (Fletcher *et al.*, 1982; Gajdosova *et al.*, 2011), CK-ribosides should be considered as biologically relevant, although their effects might require their rapid conversion to the free bases.

Cytokinin levels are highest in young developing tissues, whereas senescent leaves often have reduced levels (Hewett and Wareing, 1973; Ori *et al.*, 1999). CKs are also known to regulate defense responses against pathogens (Choi *et al.*, 2010; Grosskinsky *et al.*, 2011; Argueso *et al.*, 2012) and herbivores (Smigocki *et al.*, 1993, 2000; Dervinis *et al.*, 2010; Schäfer *et al.*, 2015a). Increasing CK levels amplify the accumulation of secondary metabolites in several plant species (Hino *et al.*, 1982; Grosskinsky *et al.*, 2011; Schäfer *et al.*, 2015a). However, these studies have not considered the action of CKs in the context of the OD theory and their influence on the developmental regulation of defense patterns.

Here we analyzed the role of CKs in the control of developmental patterns in herbivory-induced chemical defenses following predictions of the OD theory of a native tobacco, *Nicotiana attenuata*.

This species has been intensively studied as an ecological model for plant–herbivore interactions and their molecular mechanisms (Baldwin, 1998, 1999; Ohnmeiss and Baldwin, 2000; Baldwin *et al.*, 2001; Halitschke *et al.*, 2001; Kessler and Baldwin, 2001, 2002; Wu and Baldwin, 2010). Several anti-herbivory defense metabolites, including nicotine (Steppuhn *et al.*, 2004), trypsin protease inhibitors (TPI; Zavala and Baldwin, 2004) and *N*-acetylated polyamines (phenolamides; PAs; Kaur *et al.*, 2010) have been characterized in *N. attenuata* and were shown to increase plant fitness in environments with herbivores (Baldwin, 1998). One of the most abundant PAs, caffeoylputrescine (CP), whose biosynthesis is very nitrogen demanding (Ullmann-Zeunert *et al.*, 2013), is highly inducible by herbivore attack and accumulates in developmental patterns consistent with the predictions of the OD theory with higher levels in younger leaves (Kaur *et al.*, 2010). CP accumulation in young, rosette-stage plants is also regulated by CK levels and signaling (Schäfer *et al.*, 2015a). Here we use the accumulation of CP in leaves as a reliable marker to investigate mechanisms responsible for developmental patterns of herbivory-inducible defenses. We analyzed if CK levels correlate with developmental gradients of herbivory-induced defense metabolites, such as CP in *N. attenuate*, and if altering CK levels within physiologically realistic ranges is sufficient to change their ontogenic patterns.

RESULTS

Distribution patterns of inducible defense metabolites in *Nicotiana attenuata* are developmentally regulated

To evaluate if the herbivory-induced defense metabolites in *N. attenuata* follow developmental patterns predicted by

the OD theory, we analyzed CP accumulations in two developmental gradients: (i) in a standardized set of leaves growing at eight sequential nodes from flowering plants (Figure 1a); and (ii) in a developmentally standardized leaf position from plants at two different growth stages (Figure 2a). In the first, whole plants were sprayed with methyl jasmonate (MJ; Figure 1a), a defense elicitor (Keinanen *et al.*, 2001), to uniformly activate defense responses (including CP) in all tissues. In the second, CP accumulation was induced by the feeding of neonate larvae of the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae; Figure 2b). We also measured the accumulation of

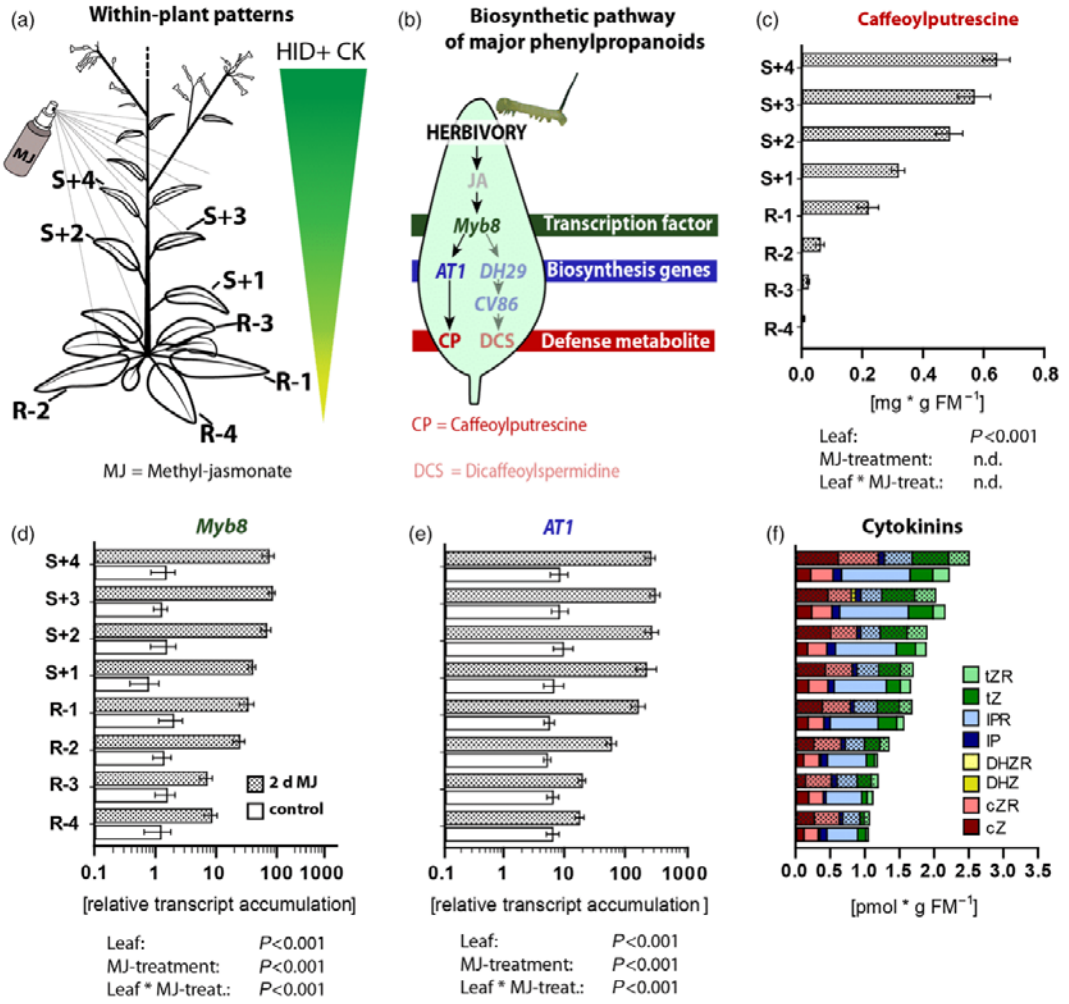

Figure 1. Herbivory-induced defense metabolites (HIDs) and cytokinins (CKs) follow the same within-plant distributions in *Nicotiana attenuata*.
(a) Experimental design and distribution of HID and CKs within a plant.
(b) Scheme of biosynthetic pathway of major phenolamides (PAs).
(c) Caffeoylputrescine (CP); not detectable (n.d.) in control leaves.
(d) Relative transcript abundance of transcription factor *NaMYB8* and (e) *NaAT1* as well as (f) CKs (*cis*-zeatin, *cZ*; *cis*-zeatin riboside, *cZR*; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, IP; isopentenyladenosine, IPR; trans-zeatin, *tZ*; *trans*-zeatin riboside, *tZR*; other CKs in Table S1), in different leaf classes of flowering plants: rosette leaves R−1 (youngest) to R−4 (oldest) and stem-leaves S + 1 (oldest) to S + 4 (youngest). Plants were sprayed for 2 days with 1 mM methyl jasmonate (2 days MJ; dotted bars) or water as control (open bars). Data were analyzed by two-way ANOVAS (d, e) or one-way ANOVAS (c), *P*-values indicate influence of the single factors leaf and MJ-treatment or the interaction of both (Leaf * MJ-treat.). Statistics for CKs can be found in Table S2. Error bars depict standard errors ($N \geq 5$). FM, fresh mass.

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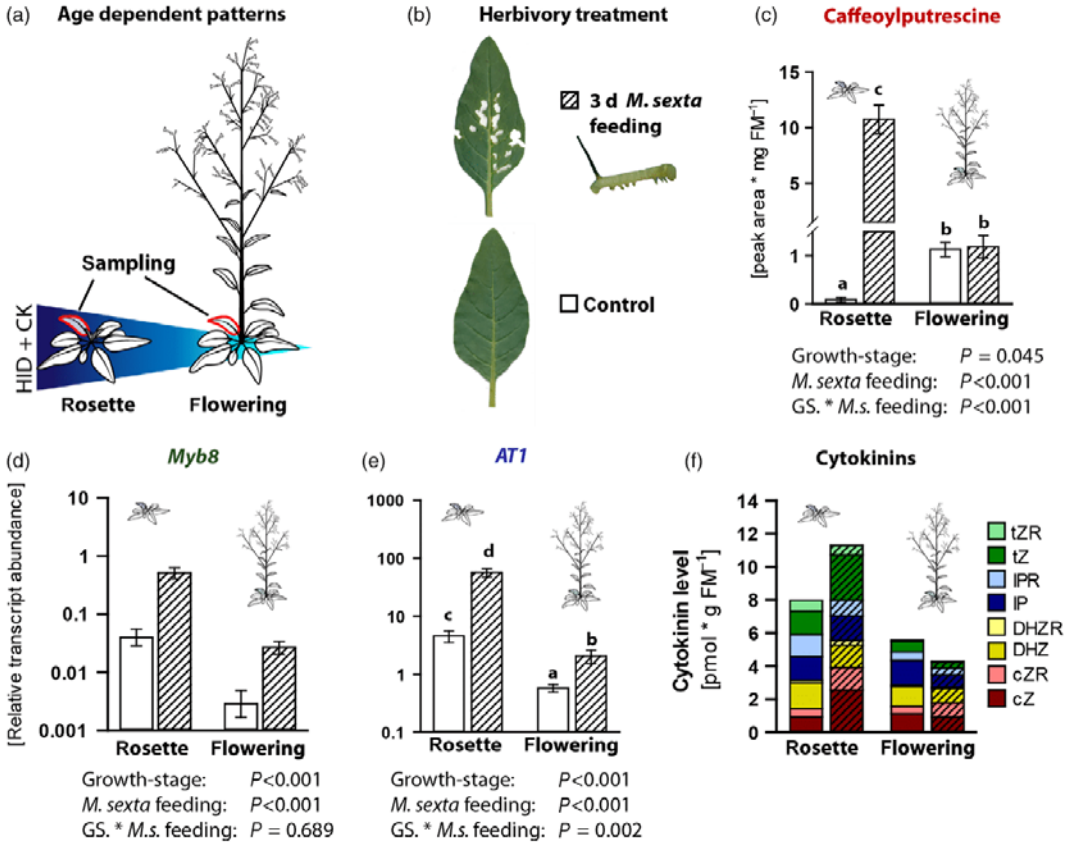

Figure 2. Herbivory-induced defense metabolites (HIDs) and cytokinins (CKs) follow similar developmental patterns in *Nicotiana attenuata*.
(a) Experimental design and the distribution of HIDs and CKs during plant development.
(b) Typical damage after 3 days *Manduca sexta* feeding and control leaf.
(c) Caffeoylputrescine (CP), (d) relative transcript abundance of transcription factor *NaMYB8* and (e) *NaAT1*, as well as (f) CKs (*cis*-zeatin, *cZ*; *cis*-zeatin riboside, *cZR*; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, IP; isopentenyladenosine, IPR; trans-zeatin, *tZ*; *trans*-zeatin riboside, *tZR*; other CKs in Table S5) in the same leaf position (young rosette leaf) in two growth stages: vegetative rosette plants and reproductive flowering plants. Open bars: control levels, diagonally striped bars: levels after 3 days *M. sexta* feeding. Two-way ANOVAs, *P*-values indicate influence of the single factors growth stage (GS) and *M. sexta* (*M.s.*) feeding or the interaction of both (GS * *M.s.* feeding). Statistics for CKs can be found in Table S6. Different letters indicate significant differences (if interaction was significant: Tukey HSD *post hoc* test: $P < 0.05$). Error bars depict standard errors ($N \geq 9$). FM, fresh mass.

transcripts of *MYB8* (*NaMYB8*, which regulates PA biosynthesis; Onkokesung *et al.*, 2012) and *AT1* (*NaAT1*, an enzyme involved in the final step of CP biosynthesis; Onkokesung *et al.*, 2012), both are regulated by herbivory and MJ in *N. attenuata* (Figure 1b).

Real and simulated *M. sexta* feeding strongly increased CP accumulations in a pattern consistent with the predictions of the OD theory, with the highest levels found in young leaves when comparing different leaves within a plant as well as leaves of plants in different developmental stages (Figures 1c, 2c and S3c). MJ-induced CP levels in older leaves of flowering plants are marginally detectable, whereas they were highly induced in young stem-leaves (leaf class, one-way ANOVA: $P < 0.001$; Figure 1c). These results were confirmed in a second experiment using pooled samples of leaf classes growing at three consecutive nodes (Figure S3a): old rosette leaves, young rosette leaves, first three stem-leaves and stem-leaves 4–6. The ninefold induction of CP after MJ application in stem-leaves 4–6 was completely lost in old rosette leaves [Figure S3c; two-way ANOVA (TWA): $P < 0.001$]. In young rosette stage plants, CP levels were approximately 120-fold induced after herbivore feeding, whereas induction was completely lost in similar leaves of flowering plants (treatment * growth stage TWA: $P < 0.001$; Figure 2c), confirming previous results (Kaur *et al.*, 2010; Diezel *et al.*, 2011). Consistent with the patterns of CP accumulation, the accumulation of transcripts of *MYB8* and *AT1* followed a

gradient decreasing from young to old leaves in both treatments (Figures 1d and e, and 2d and e). However, the induction of these genes was not abolished in flowering-stage plants, suggesting that CP accumulation is at least partially controlled by other mechanisms. The induced levels of other defenses, including dicaffeoylspermidine (DCS), and the transcript accumulation of its biosynthetic genes, as well as TPI activities similarly follow OD predictions (Figures S1, S2a, S3d and e, S4 and S5a). In contrast, nicotine concentrations were not induced by MJ in flowering plants and by *M. sexta* herbivory in rosette leaves, and its distribution did not follow OD predictions; instead nicotine levels remained unaffected or were slightly lower in younger leaf classes ($P < 0.001$; Figure S2b; $P = 0.004$; Figure S3f) or younger growth stages ($P < 0.001$; Figure S5b). This result, which is inconsistent with results from field-grown plants (Baldwin and Ohnmeiss, 1993; Baldwin, 1999), is likely due to the plants becoming pot-bound during their growth in the glasshouse (Baldwin, 1988), as nicotine biosynthesis is located in roots (Iljin, 1958).

Developmental distributions of CKs follow the same gradients as inducible defenses

Developmental transitions in plants are known to be regulated by growth hormones like CKs (Werner and Schmülling, 2009; Durbak *et al.*, 2012). It has been hypothesized that CKs might also play a role in the developmental control of defense responses (Meldau *et al.*, 2012; Giron *et al.*, 2013; Schäfer *et al.*, 2015b). We analyzed the concentrations of the bioactive CK free bases *t*Z, *c*Z, DHZ and IP as well as their corresponding ribosides. The CK levels were measured in the same tissues that were used for the quantification of defense metabolite levels. Given that the activity of different CK-types can differ greatly among the receptors used to perceive them within and between plants (Lomin *et al.*, 2012), it is important to note that the sum of CK free bases and ribosides may not necessarily precisely reflect their biological activity. However, the summed CK values provide an overview about the changes in the abundance of compounds with presumably high direct (CK-receptor binding) or indirect (e.g. rapid conversion to active form) biological activity. Consistent with the literature (Hewett and Wareing, 1973), the levels of CK free bases and ribosides in *N. attenuata* were highest in young leaves (Figures 1f, 2f and S3b). The highest levels were found in rosette plants and young stem-leaves of flowering plants, whereas the lowest levels were in old rosette leaves of flowering plants (Figures 1f, 2f and S3b; Tables S1–S6). Importantly, MJ-induced defense compounds highly correlated with these CK levels (i.e. with *t*Z, *t*ZR, IP, IPR; Figures 3 and S6). Mean values of *t*Z, *t*ZR, IP and IPR at a given leaf position were positively correlated with levels of CP, DCS and *NaTPI* transcripts that were induced by MJ application to the same positions. Nicotine showed weaker correlations (Figure 3). The highest R^2-values were found between IPR and *t*ZR levels and defense markers. IPR correlated with CP ($R^2 = 0.9215$), DCS ($R^2 = 0.9195$), *NaTPI* ($R^2 = 0.905$) as well as nicotine ($R^2 = 0.7195$). Also tZR levels correlated highly with CP ($R^2 = 0.9377$), DCS ($R^2 = 0.9297$) and *NaTPI* ($R^2 = 0.8721$).

The levels of CKs in leaves after induction with MJ also correlated with the induced levels of defenses (single samples; Figure S6). Correlations of *t*Z, *t*ZR, IP and IPR with all defense markers except nicotine were significant (Pearson product moment correlation; PPMC). We found strongest correlations between *t*ZR and CP ($R^2 = 0.504$, PPMC: $P < 0.001$), DCS ($R^2 = 0.462$, $P < 0.001$) and transcripts of *NaTPI* ($R^2 = 0.445$, $P < 0.001$). These results suggest that basal levels of CKs in leaves might be involved in regulating induced levels of defenses, except in the case of nicotine, which was induced to uniformly high levels across all leaf positions by the MJ spray, likely a reflection of the separation of site of production (roots) and accumulation (shoots) for this defense metabolite and the uniform mode of elicitation.

Manipulating developmental patterns of CKs alters the normal distribution of defense metabolites

To evaluate a possible causal relationship behind these correlations, we manipulated the naturally occurring developmental CK gradients. We developed transgenic *N. attenuata* plants, which allowed us to modify the developmentally defined levels of CKs in leaves. To manipulate the within-plant distribution of CKs, we used transgenic plants (i-ov*IPT*) containing the pOp6/LhGR expression system for chemically inducible expression of the *Agrobacterium tumefaciens* isopentenyltransferase *Tumor morphology root* after treating leaves with dexamethasone (DEX; Schäfer *et al.*, 2013). These plants allowed us to increase levels of *t*Z-type active CKs in a spatially, quantitatively and temporally restricted manner in single leaves (Schäfer *et al.*, 2013, 2015a).

Treating single rosette leaves of a flowering i-ov*IPT* plant with DEX for 2 days (Figure 4a) increased active CK levels (Figure 4b; Table S7) and altered the normal distribution of MJ-induced CP (Figure 4e). The induced CP levels increased fourfold ($P < 0.001$) compared with mock-treated leaves, resulting in levels typically found in the first three stem-leaves (Figure 4e). Also the transcripts of the biosynthesis gene *AT1,* but not the transcription factor *MYB8*, were affected by the DEX treatment (Figure 4c and d). Similar results were found for plants with CK levels manipulated in multiple leaf positions (Figure S9; Tables S8 and S9). Leaves with higher CK levels had higher MJ-induced levels of CP compared with corresponding mock-treated leaves. DCS responded similarly to CP, while nicotine was again not affected (Figures S7–S9). These temporally and quantitatively restricted CK changes did not influence the plant's

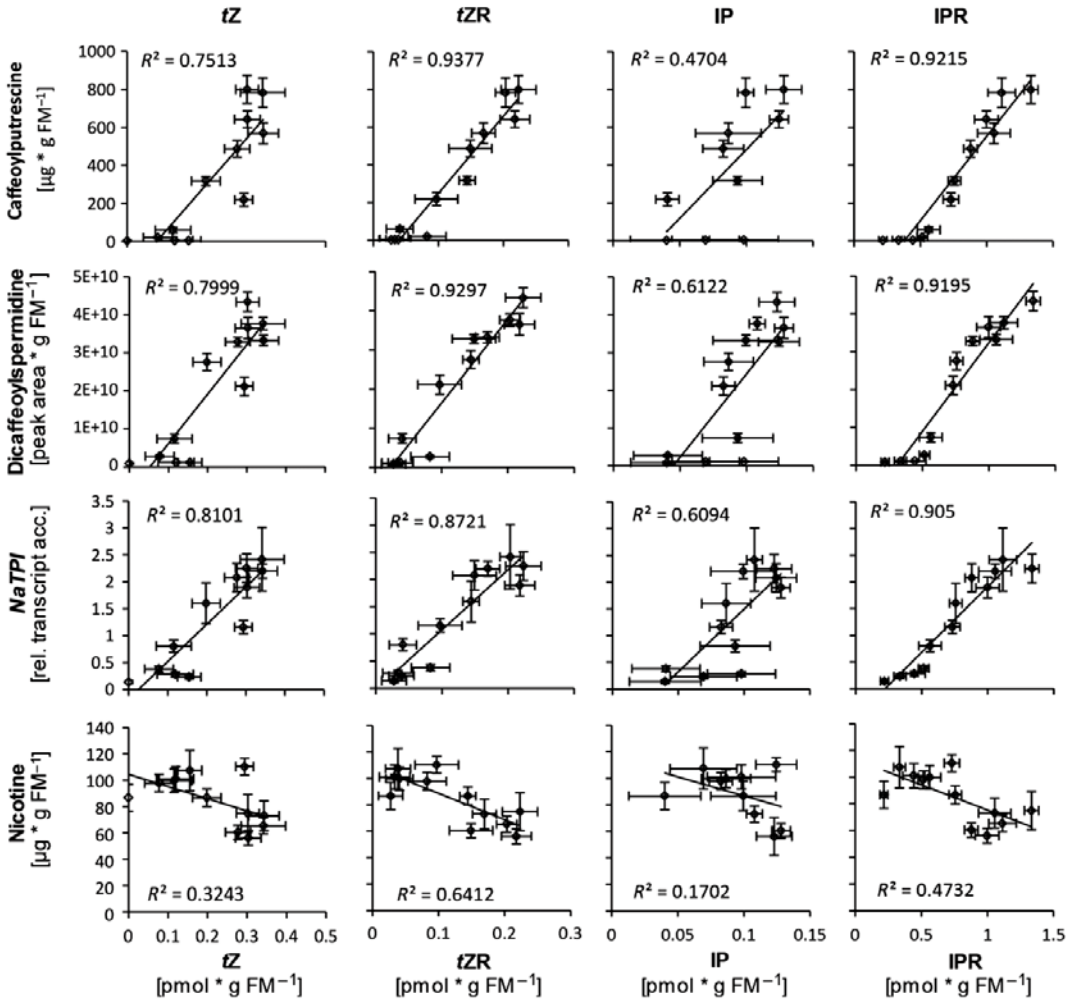

Figure 3. Correlations of cytokinin (CK) levels in untreated leaves with the accumulations of different defense compounds after methyl jasmonate (MJ)-induction in *Nicotiana attenuata*.

Average levels of caffeoylputrescine (CP), dicaffeoylspermidine (DCS), *NaTPI* transcript levels and nicotine in different leaf types of a flowering plant after induction with MJ plotted against levels of CKs (isopentenyladenine, IP; isopentenyladenosine, IPR; *trans*-zeatin, *tZ*; *trans*-zeatin riboside, *tZR*) in uninduced leaves at the same leaf position. Error bars depict standard errors ($N \geq 5$). FM, fresh mass.

morphology (no obvious visual changes observed) as observed for other CK pathway-manipulated plants (Smigocki, 1995; Riefler *et al.*, 2006).

To manipulate age-dependent CK levels, we used *N. attenuata* plants expressing the isopentenyltransferase 4 (*IPT4*) from *Arabidopsis thaliana*, which catalyzes a rate-limiting step in the biosynthesis of IP-type CKs, driven by the promotor of the *A. thaliana* senescence-associated gene 12 (*SAG*). We used two independently transformed transgenic lines (*SAG-IPT4*-1 and *SAG-IPT4*-2) for all experiments (Figure S10). Results from line *SAG-IPT4*-1 are presented in Figures 5, S11 and S12, and results of *SAG-IPT4*-2 are provided in the supplemental tables (Tables S10–S15). The SAG promotor activity correlates with leaf age, but is also induced by *M. sexta* feeding in flowering plants (Figure S10). Because CKs inhibit the senescence processes, the construct is auto-regulated, allowing for changes in CK levels well within the normal physiological range of a plant (Figure 5a; compare Gan and Amasino, 1995).

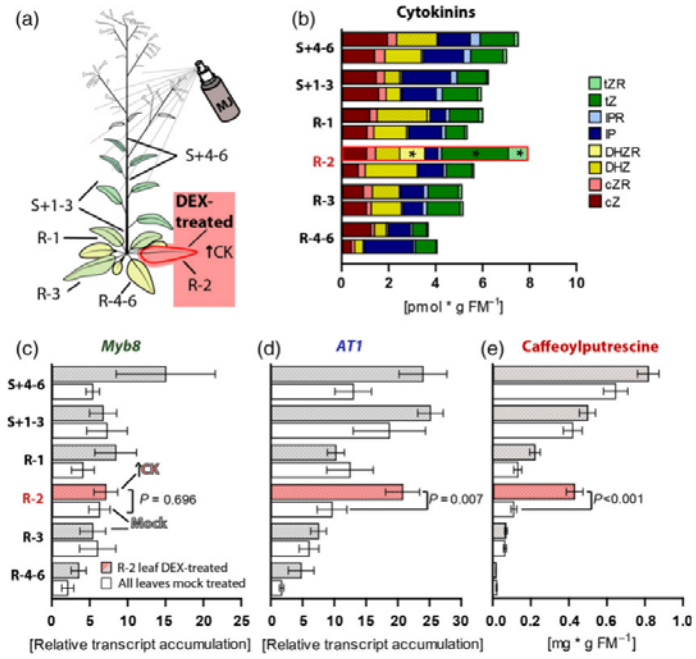

Figure 4. Manipulation of the within-plant cytokinin (CK) gradient alters the distribution of herbivory-inducible defenses in *Nicotiana attenuata*.
(a) Experimental design.
(b) CKs: *cis*-zeatin, *cZ*; *cis*-zeatin riboside, *cZR*; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, IP; isopentenyladenosine, IPR; *trans*-zeatin, *tZ*; *trans*-zeatin riboside, *tZR*; other CKs in Table S7.
(c) Relative transcript abundance of transcription factor *NaMYB8* and (d) *NaAT1* and (e) caffeoylputrescine (CP) in different leaf classes [rosette leaves 4–6, R−4−6, rosette leaf 3, 2 and 1 with R−1 being the youngest and R−6 being the oldest, first three stem-leaves 1–3 (S + 1−3) and stem-leaves 4–6 (S + 4−6)] of flowering plants transformed with a construct for dexamethasone (DEX)-inducible expression of the CK biosynthesis enzyme isopentenyltransferase (*i-ovIPT*). R−2 was treated with 5 μM DEX and 1% DMSO in lanolin paste (DEX; red color; ↑CK) to increase levels of *tZ*-type CKs in the leaves or with 1% DMSO in lanolin as control (mock, white color). All other leaves were mock-treated. Gray bars indicate levels from plants in which one leaf was DEX-treated. Plants were sprayed for 2 days with 1 mM methyl jasmonate (MJ). *P*-values above brackets over R−2 leaves represent results of a *t*-test between DEX- and mock-treated R−2 leaves. Asterisks in different sections of CK-bars represent statistically significant differences ($P < 0.05$) from *t*-tests between single CKs. Error bars depict standard errors ($N \geq 4$); FM, fresh mass.

Rosette leaves of flowering *SAG-IPT4* plants contained higher levels of CK free bases and ribosides (i.e. *tZ*, *tZR*, IPR) than did those of wild-type (WT) plants (Figure 5a; Tables S10 and S11). These CK levels in flowering plants were comparable to the levels found in younger developmental stages of WT plants. Defense metabolites in rosette leaves of flowering-stage WT plants are no longer inducible. Both the inducibility of the defense, CP (Figure 5d; Tables S12 and S13), as well as the transcripts of its regulators *MYB8* and *AT1* (Figure 5b and c; Tables S14 and S15) were fully restored in *SAG-IPT4* plants, likely a result of the increase in CKs or respective downstream events in flowering *SAG-IPT4* plants. Other inducible defense metabolites such as DCS and TPI were affected in a similar way, whereas nicotine, which did not exhibit a developmental OD pattern in our experiments, was not (Figures S11 and S12; Tables S12–S15). We conclude that restoring CKs in leaves of flowering plants to the levels found in earlier developmental stages is sufficient to alter the developmentally dependent patterns of defenses.

DISCUSSION

Inducible defense metabolites such as CP in *N. attenuata* clearly follow developmental gradients. We found the highest levels of defenses in young leaves of flowering plants and in leaves of plants in vegetative stages. Our results are consistent with previous studies showing higher levels of defenses in vegetative growth stages or in younger leaves within a plant (Agostini *et al.*, 1998; Ohnmeiss and Baldwin, 2000; Brown *et al.*, 2003; Zavala *et al.*, 2004a; Anderson and Agrell, 2005). The investment in defense metabolites is often costly for the plant and therefore needs to be tightly regulated (Zangerl and Rutledge, 1996; Karban and Baldwin, 1997; Ullmann-Zeunert *et al.*, 2013). Costly defenses are often only produced on demand after induction by damage and perception of specific elicitors from herbivores as it

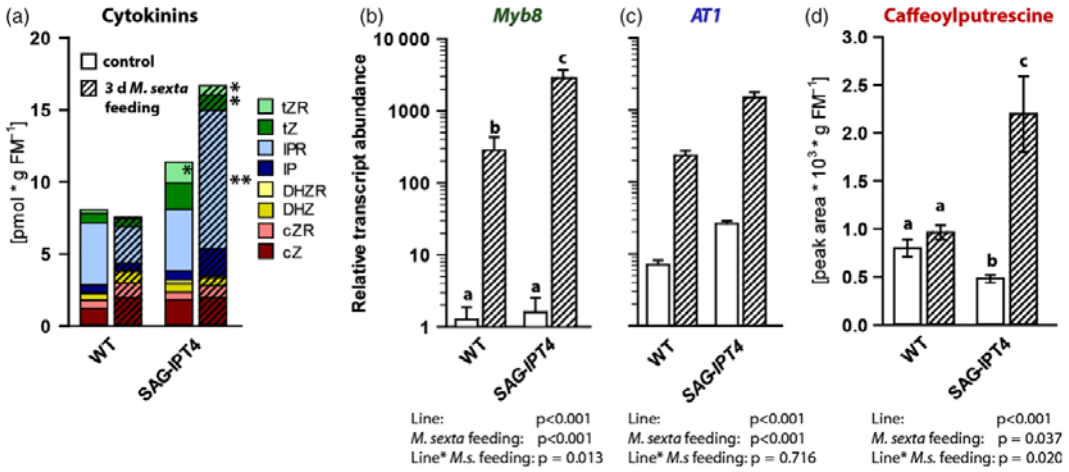

Figure 5. Restoring cytokinin (CK) levels to an earlier developmental stage increases defense gene expression and recovers inducibility of defenses in flowering *Nicotiana attenuata* plants.
Flowering wildtype (WT) and *SAG-IPT4* plants.
(a) CKs (*cis*-zeatin, *cZ*; *cis*-zeatin riboside, *cZR*; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, IP; isopentenyladenosine, IPR; trans-zeatin, *tZ*; *trans*-zeatin riboside, *tZR*; other CKs in table S10).
(b) Relative transcript abundance of transcription factor *NaMYB8* and (c) *NaAT1* and (d) caffeoylputrescine (CP). Levels were measured in the youngest rosette leaf after 3 days of *Manduca sexta* feeding (3 days *M. sexta* feeding, diagonal striped bars) and in control leaves of unattacked plants (control; open bars). Data were analyzed by two-way ANOVAS (c) or generalized least-squares models. (b, d) *P*-values indicate influence of the single factors genotype (line) and *M. sexta* (*M.s.*) feeding or the interaction of both (Line * *M.s.* feeding). Different letters indicate significant differences [if interaction was significant: pairwise Wilcoxon rank-sum test with Bonferroni correction (b, d): $P < 0.05$]. Asterisks in different sections of active CK-bars indicate significant differences ($P < 0.05$) in *t*-tests with single CKs between control and induced levels of different genotypes, respectively (*t*-tests; $*P < 0.05$, $**P < 0.01$). Results of two-way ANOVAS of CKs can be found in Table S11. Results for line *SAG-IPT4-2* can be found in Tables S10–S15. Error bars show standard errors ($N \geq 5$). FM, fresh mass.

is for example with CP in *N. attenuata* (Keinanen *et al.*, 2001). The second way of regulation, which is described by the OD theory, is the investment in defense only in tissues where benefits of high levels of defense metabolites outweigh their costs (see overview in Figure 6). Often, these are the tissues with a high fitness value for the plant. Regarding leaves, this usually means that young leaves should be better defended compared with older leaves as they have a greater value for the plant (Harper, 1989), which has been confirmed experimentally (Ohnmeiss and Baldwin, 2000; Barto and Cipollini, 2005). The production of defense metabolites typically decreases as annual plants reach reproductive maturity and produce seeds (Baldwin, 1998; Zavala *et al.*, 2004b), a result consistent with the fitness costs of defenses. Therefore, the developmental regulation of defenses according to the OD theory is consistent with evolutionary expectations.

We found that CK levels showed similar within-plant and developmental patterns such as inducible defenses. In an ecological perspective this co-regulation of defense inducibility and CK levels seems reasonable, as usually young tissue features high levels of CKs, which are often associated with high levels of nutrients (Rubio-Wilhelmi *et al.*, 2014). This is partially due to the fact that a CK gradient also mediates source-sink regulations, and higher levels of CKs increase the sink-strength of a given tissue (Richmond and Lang, 1957; Leopold and Kawase, 1964; Roitsch and Ehness, 2000; Body *et al.*, 2013). As young leaves have a higher potential fitness value for the plant due to their longer remaining time of carbon fixation (Harper, 1989), CKs could be correlated with the value of certain leaves. Based on our data, we suggest that CK levels reflect tissue age and hence the fitness value of the tissue, and infer that CKs influence defense allocation according to OD theory predictions (Figure 6).

Testing the OD theory has been thwarted by the challenge of manipulating developmentally regulated defense distributions (Baldwin, 1994). Elevations of CKs in older leaves of flowering plants by a senescence-activated promoter could restore their inducibility by herbivore feeding. Using a second approach with a DEX-inducible construct (i-ov*IPT*), we created short-term perturbations of the within-plant ontogenic gradients of CKs and observed the consequences for defense allocations to different tissues that followed the disturbances. CKs were shown to regulate the accumulation of specific defense metabolites in many different plant species (Smigocki *et al.*, 1993; Dervinis *et al.*, 2010; Schäfer *et al.*, 2015a) and we see similar distribution of CKs (Hewett and Wareing, 1973; Ori *et al.*, 1999) and defense metabolites (James, 1950; Kariñho-Betancourt

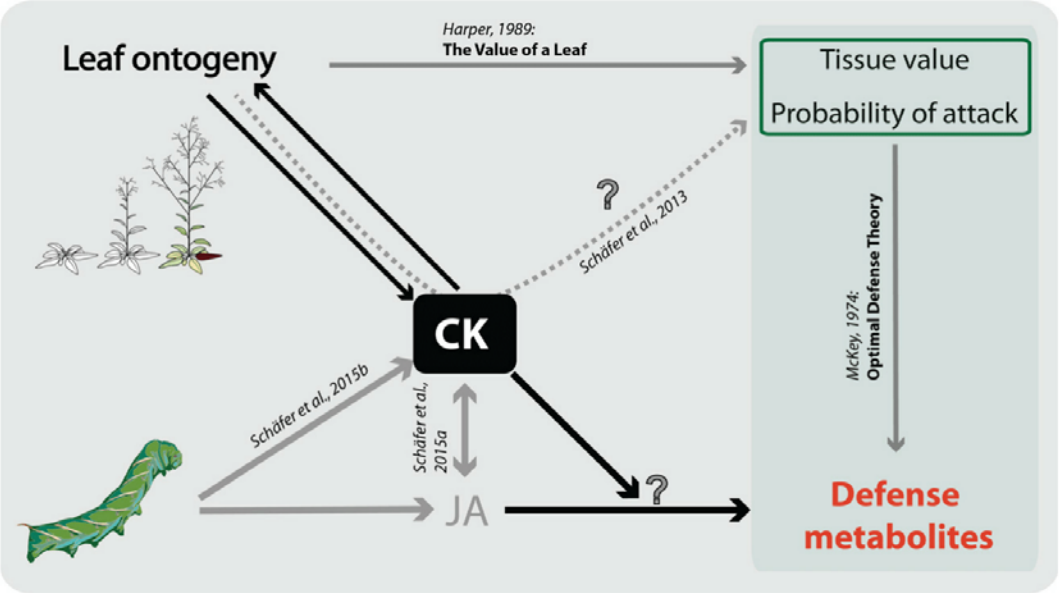

Figure 6. Cytokinins (CKs) influence the developmentally dependent distribution of defense metabolites in *Nicotiana attenuata*.
Black arrows: findings of this paper; gray arrows: previous publications as indicated next to the arrow. Leaf ontogeny/its developmental state change levels of CKs and vice versa. CK levels change levels of herbivory-inducible defenses. How exactly CKs influence herbivory-induced defenses remains to be discovered. We found evidence for transcriptional and post-transcriptional regulation. The main conclusion of McKey's Optimal Defense Theory is highlighted by the light green box: investment in defense metabolism in a tissue depends on its value and probability of attack. We hypothesize that leaf value and probability of attack are also influenced by growth hormones, such as CKs.

et al., 2015). Therefore we assume that the correlation between CK levels and defense metabolite accumulations is a general phenomenon. We think that our strategy to manipulate defense distribution would be appropriate in other species as well. Further studies with mono- and dicot species need to be carried out to examine how general this phenomenon is. We propose that CKs metabolically link nutrient content and defense allocation, and determine which defense strategy a plant uses: induced defenses or resource mobilization away from attacked tissues to reproductive or storage organs (i.e. tissues with higher sink strength). As such, growth-regulating hormones like CKs may link tissue value and the distribution of anti-herbivore defenses. CKs also regulate leaf ageing, and thus increasing CK levels in older leaves might have caused a general increase in the metabolic activities of these leaves. Because we mainly focused on the levels of defense metabolites, we cannot rule out that other metabolic pathways may have been altered as well by increasing CK levels. Future analyses are needed to more clearly separate CK-associated effects on the general metabolic activity of leaf tissues from their effect on defense metabolism. A possible strategy might be to target particular downstream components of the CK pathway. Possible targets could be extracellular invertases, as they have been manipulated to explore the CK-senescence connection (Balibrea Lara *et al.*, 2004), or different response regulators for CK-mediated effects as has been done to test effects on the plant immunity (Argueso *et al.*, 2012). In addition, CKs are known to be able to cause other physiological changes (for review, see Werner and Schmülling, 2009) that might influence the within-plant distributions of defense metabolites. CK pathway manipulations are often associated with strong alterations in plant architecture. Examples are dwarf phenotypes (Riefler *et al.*, 2006) or lateral shoot formation (Smigocki *et al.*, 1993). However, we did not observe such developmental changes in the short-term perturbations using the DEX-constructs. With the SAG promoter-driven constructs, we observed visible but not drastic morphological changes in flowering plants (slightly stunted growth, thicker stem and more side branches; Figure S10c). Analyzing early molecular markers of developmental changes might also help to further analyze the connection between the various CK-related processes. The exact mechanisms of the linkage between developmental patterns of CKs and defense metabolite accumulations remain unclear (Figure 6).

In addition to resource availability for defense biosynthesis, our data suggest the involvement of transcriptional and post-transcriptional mechanisms. Previous studies showed an enhanced induction of jasmonic acid (JA) upon

herbivore attack in plants with increased CK levels (Dervinis *et al.*, 2010; Schäfer *et al.*, 2015a). The JA pathway regulates most defense responses against herbivores (De Geyter *et al.*, 2012). JA signaling leads to the degradation of JAZ (JASMONATE ZIM DOMAIN) proteins (Chini *et al.*, 2007; Oh *et al.*, 2012). JA-Ile-induced JAZ degradation releases transcription factors, such as MYC2, which control the expression of JA-inducible genes (for review, see De Geyter *et al.*, 2012). In *N. attenuata* MYC2 regulates the PA pathway, including the expression of *Myb8*, *AT1*, *DH29* and *CV86* (Woldemariam *et al.*, 2013). The expression of these genes also correlates with the prediction of the OD theory (Figures 1, 2, S1 and S4). However, the experiments with MJ application and short-term manipulation of *tZ*-type CKs revealed that higher CK levels increased *AT1* expression but not the expression of *Myb8*, *DH29* and *CV86*, although at the metabolite level, CP and DCS levels were increased (Figures 4 and S7). In contrast, herbivore feeding and long-term changes in the levels of IP-type CKs also increased expression of *Myb8*, *DH29* and *CV 86*. Different treatments (MJ versus herbivory), other CKs (*tZ* versus IP) or the timing of the expression analysis may have caused the differential response in transcript accumulation in both experiments. Similar effects have been reported in previous work (Schäfer *et al.*, 2015a), where *Myb8*, *DH29* and *CV86* also did not respond to short-term increases in *tZ*-type CKs in i-ov*IPT* plants even though the associated PAs were increased. It is likely that post-transcriptional or other downstream mechanisms, such as changes in substrate availability, may govern the accumulation of PAs. While CK levels and perception regulate JA concentrations after wounding and simulated herbivory, levels of JA-Ile are not promoted (Schäfer *et al.*, 2015a). Furthermore, MeJA spraying of whole flowering plants was not sufficient to induce defense levels in older leaves without simultaneously increasing CK levels. Therefore, it seems likely that CKs regulate JA signaling downstream of JA-Ile perception. A possible mechanism might be that CKs mediate developmental control of herbivory and JA-regulated defenses upstream of *Myb8*, possibly at the level of JAZ-MYC2 interaction. Analyzing JAZ stability in developmental gradients and in response to CK manipulation would help to test this hypothesis. The identification of CK signaling elements associated with changes in defense responses provides another route towards a mechanistic understanding of OD patterns. CKs are perceived by cyclases/histidine kinases-associated sensing extracellular (CHASE)-domain-containing His kinases (CHKs; Stolz *et al.*, 2011; Gruhn and Heyl, 2013). CHK2 and CHK3 modulate jasmonate-dependent defense responses in *N. attenuata*, including PA accumulations (Schäfer *et al.*, 2015a). CK signaling downstream of the receptors is regulated by specific response regulators (RRs; Hwang *et al.*, 2012). While the type-B RRs (RRB) are transcription factors, the type-A RRs (RRA) are known as negative feedback regulators of the CK pathway. Although RRs have been shown to regulate pathogen defense in *Arabidopsis* (Choi *et al.*, 2010; Argueso *et al.*, 2012), their role in jasmonate-dependent defenses is currently unknown. We have previously identified RRs in *N. attenuata* that are regulated by wounding and herbivory (Schäfer *et al.*, 2015b). Expression profiling, phosphoproteomics and genetic manipulation of herbivory- and developmentally regulated RRs will be required to analyze their roles in establishing OD patterns. In addition to JA-mediated regulation of defense metabolites, CKs might also regulate defenses via sugar metabolism. CKs have been shown to regulate the levels of free sugars by altering invertase activities (Balibrea Lara *et al.*, 2004), thus increasing glucose and fructose levels. Sugar signaling has been linked to defense against herbivores (Schwachtje and Baldwin, 2008; Machado *et al.*, 2013). Whether CKs influence developmental patterns of defenses via sugar signaling requires further work.

Although CK overproduction recovered the induction of defense responses, the levels did not reach those observed in the youngest tissues (Figure 4e). Clearly factors other than CKs also play a role in the developmental regulation of defense metabolites. These may include the presence of precursors, nutrient availability, overall physiological activity of a leaf, and interaction with other phytohormones. Other growth hormones have been shown to be involved in JA-mediated defense regulation. Auxin, for example, regulates JA signaling at the level of JAZ/Myc2 via the regulatory protein TOPLESS (TPL) and Novel Interactor of JAZ (NINJA; Pauwels *et al.*, 2010). Gibberellin (GA) signaling reduces JA responses by changing the interaction between JAZ and MYC2 through DELLA proteins (negative regulators of GA signaling; Hou *et al.*, 2010; Hong *et al.*, 2012; Wild *et al.*, 2012). GAs promote growth stage transitions, such as vegetative to flowering stage (Blazquez *et al.*, 1998), and reduced GA levels accelerate the accumulation of herbivory-induced defenses (Yang *et al.*, 2012). Other hormones, such as brassinosteroids, abscisic acid, salicylic acid and ethylene might also play a role in the developmental control of defense metabolites (for review, see Robert-Seilaniantz *et al.*, 2011; Erb *et al.*, 2012; Meldau *et al.*, 2012). The regulation of plant defense strategies as a whole is likely to be regulated by a combination of multiple hormone pathways (Heath *et al.*, 2014; Mason and Donovan, 2014; Ochoa-López *et al.*, 2015). The analysis of these hormones and the manipulation of their developmental regulation will help to further illuminate the molecular mechanisms responsible for the commonly observed OD patterns. Interestingly, the basal levels of CP and TPI activity partially behaved opposing to their induced levels. They were higher in rosette leaves of flowering than of rosette stage plants (Figures 2 and S5), and were

suppressed by CK overexpression (WT versus SAG:IPT; Figures 5 and S12). This raises the question if parts of the CK pathway might also act as negative regulators of the herbivore defense under certain conditions, for example, in the absence of a respective stimulus. Similar effects were also observed for CK function in pathogen defense (Argueso *et al.*, 2012).

Optimal defense theory not only predicts an unequal distribution of defenses, but that the distribution depends on the attack risk and fitness value of a tissue. It has been shown before that CK manipulation increases levels of primary metabolites (Rubio-Wilhelmi *et al.*, 2014). This could even lead to a higher attractiveness to herbivores and a greater rate of attack. Indeed, in a previous study we demonstrated that increasing CK levels in individual leaves increased their attractiveness and attack rates from natural herbivores (Schäfer *et al.*, 2013). From these results we infer that CKs can also influence this aspect of the OD theory (Figure 6). Whether CKs also regulate the relative contribution of a given tissue to plant reproduction and hence fitness remains to be determined.

At first glance it seems to be a contradiction that CKs increase levels of defenses without significantly reducing growth of the plants, as it would be expected according to the growth-differentiation hypothesis. One possibility could be that nutritional resources are not limited in our greenhouse setup, which makes it possible to invest in both: growth and defense. In addition, all defense levels we found to be influenced by CKs are inducible (by MJ treatments or herbivore feeding). Inducible defenses are often considered as resource demanding. However, in our experimental setup plants were raised without defense induction until 2–3 days before the end of the experiment. We would expect to observe negative effects on growth and fitness (i.e. seed-capsule production) only if plants are screened for a prolonged time after defense induction.

Many studies suggest that changing CK levels may help to improve crop plants, especially drought tolerance (Werner *et al.*, 2010). Our method of changing the distribution of secondary metabolites through CK manipulation could also be explored further for a use in engineering crops. Our method might apply for plants, which produce pharmaceutically active compounds or specific metabolites used in the food industry, whose concentrations in leaves show ontogenic patterns. This study demonstrates that manipulating CK pathways could also facilitate the engineering of crop varieties with an altered secondary metabolite distribution.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

We used the 31st inbred generation of *N. attenuata* (Torr. ex S. Wats.) originating from a population in the Great Basin desert (Washington County, Utah, USA) as WT plants. Transgenic plants were generated from WT *N. attenuata* as described by Krügel *et al.* (2002) by Agrobacterium-mediated transformation.

SAG-IPT4 plants were transformed with a construct consisting of the cDNA of the *IPT4* gene from *A. thaliana* (*AtIPT4, IPT4;* AT4G24650) driven by the promotor of the senescence-activated gene 12 (*AtSAG12;* AT5G45890) from *A. thaliana* (*AtSAG12*; construct map, Figure S10, cloning primers Table S17). Two independently transformed lines with single insertions of construct were selected for experiments: *SAG-IPT4-1* (line number A-10-566) and *SAG-IPT4-2* (line number A-10-558). Only *SAG-IPT4-1* is shown in the figures and designated as *SAG-IPT4;* results from *SAG-IPT4-2* are shown in Tables S12–S15. Senescence- and herbivory-induced transcript accumulation of *IPT4* is shown in Figure S10.

Generation of DEX-inducible i-ov*IPT* plants was described by Schäfer *et al.* (2013). We used the line number A-11-92 × A-11-61, which contains the pOp6/LhGR expression system resulting from the crossing of pSOL9LHGRC (GenBank JX185747) and pPOP6IPT (GenBank JX185749) containing plants.

Seed germination and growth under glasshouse conditions was performed as described in Krügel *et al.* (2002) with few modifications. Seeds were sterilized for 5 min in 5 mL 2% dichloroisocyanuric acid (w/v, DCCA: Sigma, St Louis, MO, USA), supplemented with 50 μL 0.5% (v/v) Tween-20 (Merck, Darmstadt, Germany). Afterwards, seeds were washed three times and incubated for 1 h in 5 mL 50 × diluted sterile liquid smoke (House of Herbs; Passaic, New Jersey; USA) with 1 mM GA_3, and were germinated on Gamborg's B5 medium (Sigma, http://www.sigmaaldrich.com) with plant agar (Sigma) at 26°C, transferred after 10 days to TEKU JP 3050 104 pots and finally to 1-L pots filled with soil 10 days later. Plants were kept under glasshouse conditions at 26–28°C and 16 h light supplemented by Master Sun-T PIA Agro 400 or Master Sun-T PIA Plus 600 W Na lights (Philips, Turnhout, Belgium), and fertilized by flood irrigation with additions of 240 g $Ca(NO_3)_2 \times 4H_2O$ (Merck, http://www.merck-chemicals.com/) and 120 g Ferty B1 (Planta Düngemittel, http://www.plantafert.com/) in a 400-L watering tank.

Manduca sexta colony

Tobacco horn worm (*M. sexta* L.) larvae were obtained from an in-house colony, which is derived from moths caught at the field station in Utah and refreshed each year with additional wild-caught moths from the same area.

Induction of herbivory-induced defenses by *Manduca sexta*

Herbivory-triggered defense responses were induced by placing five freshly hatched neonate caterpillars of *M. sexta* on the youngest mature rosette leaf. After 3 days of caterpillar feeding, the damaged leaves (and control leaves) were harvested without the midvein. Sample collection was done in the morning (09:00–10:00 hours).

Induction of JA-mediated anti-herbivore responses by spraying MJ

For spray applications of MJ, it was dissolved in EtOH (1 M stock solution) and diluted in an aqueous solution with 0.02% TWEEN-40 to a final concentration of 1 mM. The above-ground plant parts were sprayed for two consecutive days in the morning and evening, until all leaves were moistened on both abaxial and adaxial sides. Leaves without midveins were harvested on the third day in

the morning (after 48 h), 1 h after the last MJ spray application (09:00–10:00 hours).

DEX treatments of i-ov*IPT* plants

Dexamethasone application was performed as described by Schäfer *et al.* (2013); 5 μM DEX-containing lanolin with 1% DMSO (to dissolve the DEX) was applied to the petiole of leaves of flowering plants intended to be manipulated; 1% DMSO in lanolin without DEX was used as control (indicated as 0 μM DEX). DEX application was performed 24 h before MJ treatments started.

qPCR analysis

RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA was synthesized by reverse transcription using oligo(dT) primer and RevertAid reverse transcriptase (Invitrogen). qPCR was performed using actin as standard on a Stratagene Mx3005P qPCR machine using a SYBR Green reaction mix (Eurogentec; qPCR Core kit for SYBR Green I No ROX). The primer sequences are provided in Table S16.

Measurements of nicotine, CP and DCS

Caffeoylputrescine, nicotine and DCS in Figures 2, 4, 5, S4, S5, S7–S10, S12 and S13, as well as Table S12 were determined using the HPLC-ELSD method described by Onkokesung *et al.* (2012). Data presented in Figures 1, 3, S1–S3 and S6 were obtained by measurements on a UHPLC-ToF-MS by analyzing extracted ion chromatograms as described in Schäfer *et al.* (2015a).

MeOH, 80% (v/v) was used in all cases for extraction of approximately 100 mg of frozen and ground leaf material from each sample.

When external standard curves of nicotine and CP have been performed simultaneously with measurement of the samples, absolute values are presented in mg or μg * g FM^{-1}; otherwise, when internal standards for CP were not available, values are presented as peak area * g FM^{-1}. DCS is always presented as peak area * g FM^{-1}.

TPI activity radial diffusion assay

Trypsin protease inhibitor activity was determined using a radial diffusion assay described by Jongsma *et al.* (1994) with approximately 50 mg of frozen and ground leaf-material. TPI activity was normalized to leaf protein content. Protein content was determined with the Bradford assay (Bradford, 1976) in extracts used for the TPI assay.

CK analysis

Cytokinin extraction for experiments with *SAG-IPT4* plants was performed according to the method described by Dobrev and Kaminek (2002). CK extraction in all other experiments was performed according to Dobrev and Kaminek (2002) and Kojima *et al.* (2009) with the modifications by Schäfer *et al.* (2013). The measurements were done via liquid chromatography coupled to a triple quadrupole MS (LC-MS/MS). A detailed description of the extraction and measurement can be found in the method published by Schäfer *et al.* (2014). Data for Figures 1, 4, S6 and S9, as well as Tables S3, S7 and S8 were obtained with a Bruker EVOQ Elite (www.bruker.com) triple quadrupole mass spectrometer with a heated electrospray ionization source accordingly. This method is described in detail in Schäfer *et al.* (2016).

Herbivory-induced defense responses and CK levels in two different growth stages

Two batches of WT plants were germinated in intervals of 4 weeks. Experiments began when plants reached the age of 30 or 58 days after germination, respectively, for the two different developmental stages used in the experiments comparable to the first and fifth growth stage used by Kaur *et al.* (2010; Figure 2). The youngest plants were in a vegetative rosette stage and not yet elongating (rosette), and the oldest plants had reached a height of about 70 cm and produced first seed capsules (flowering) at the start of the experiment.

For the induction of herbivore responses, five neonate *M. sexta* larvae were placed on the youngest fully expanded rosette leaf (leaf -1) or the corresponding leaf position in flowering plants. After 72 h of caterpillar feeding, the attacked leaf was harvested without midvein and samples were immediately shock frozen in liquid nitrogen. The sample collection was performed in the morning (09:00–10:00 hours). The samples were used for the analysis of herbivory-induced defense metabolites, such as nicotine, CP, DCS and TPI activity, as well as for transcript analyses and CK level quantifications.

Within-plant distribution of induced anti-herbivory defenses and CKs

To analyze the distribution of herbivory-induced defenses in different leaf classes of flowering plants, we used 58-day-old flowering plants. Leaf positions were numbered counting from the former source-sink transition leaf at the end of rosette stage (0), which corresponds to the youngest rosette leaf. Leaves above leaf 0 were numbered as S+1 to S+6, and rosette leaves below leaf 0 were numbered by R−1 to R−6. In the first experiment, we analyzed leaves from eight consecutive nodes (R−4 to S+4) separately (Figure 1a). In a second experiment, we separated the leaves of these plants into four different leaf classes of each three leaves: (i) old rosette leaves (R−4–6; R−4 to R−6), which showed visible signs of senescence (chlorophyll degradation) but were still photosynthetically competent; (ii) young rosette leaves (R−1–3; R−1 to R−3), which were the youngest three rosette leaves; (iii) the oldest three stem-leaves (S+1–3; S+1 to S+3); and (iv) the next three younger stem-leaves (S+4–6; S+4 to S+6; Figure S3a).

To simulate herbivore attack and induce JA-inducible defenses, we sprayed the above-ground parts of plants with 1 mM MJ or with a control solution as described above.

Manipulation of temporal CK distribution using *SAG-IPT4* plants

We used 58-day-old flowering WT and *SAG-IPT4* plants, and induced the youngest fully expanded rosette leaf by exposing the leaf to the feeding damage of five neonate *M. sexta* for 3 days as described above. Leaves were harvested after 72 h without their midveins. Samples were used for analysis of active CKs, gene expression and defense metabolites.

Manipulation of spatial CK distributions using i-ov*IPT* plants

We used 58-day-old *N. attenuata* i-ov*IPT* plants that were treated with 1 mM MJ for 2 days, and analyzed four different leaf age classes in each plant to determine the natural distribution of defense metabolites in the different leaf classes described above (R−4–6, R−3, R−2, R−1, S+1–3, S+4–6). In the first experiment, we induced one rosette leaf (R−2) with 5 μM (DEX) or 0 μM (control)

DEX to increase CK production locally in the treated leaf. All other leaves were treated with lanolin paste as controls. The remaining young rosette leaves were collected as older (R−3) and younger (R−1). Samples were used for the quantification of CKs and induced defense metabolites levels (Figure 4a).

In another experiment, we treated every second leaf with 5 μM DEX and harvested every leaf separately (Figure S9).

Chemicals

All chemicals used were obtained from Sigma-Aldrich (http://www.sigmaaldrich.com/), Merck (http://www.merck.com/), Roth (http://www.carlroth.com/) or VWR (http://www.vwr.com), if not mentioned otherwise in the text. CK standards were obtained from Olchemim (http://www.olchemim.cz), DEX from Enzo Life Sciences (http://www.enzolifesciences.com/), HCOOH for ultra-performance LC from Fisher Scientific (http://www.fisher.co.uk/), otherwise from Riedel-de Haën (http://www.riedeldehaen.com/) and GB5 from Duchefa (http://www.duchefa-biochemie.nl/).

Statistical analysis

Statistical analysis was performed using R 3.1.0 (http://www.r-project.org) with TWAs and Tukey HSD *post hoc* test, as well as *t*-tests, Wilcoxon rank sum tests and Pearson product moment correlation. If necessary, data were transformed to fit requirements of the particular test (homoscedasticity, normality). If homoscedasticity could not be achieved by transformation, we used a generalized least squares model [gls within the nlme package Pinheiro *et al.* (2014)], with the varIdent variance structure, which allows for corrections of different variances in each group. Statistical values for the main explanatory variables and their interaction were calculated by backward selection and comparison of the simpler with the more complex model with a likelihood ratio test (Zuur *et al.*, 2009). R version 3.1.1 R (R Core Team 2014) were used for all analyses.

Statistical tests, data transformations and number of biological replicates (*N*) are given in the figure legends. Mean values ± standard errors are given in the text. Differences were considered significant if $P < 0.05$.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. The herbivory-induced PA pathway in eight developmentally consecutive leaves of flowering *Nicotiana attenuata* plants follows a developmentally determined pattern.

Figure S2. Non-phenolic defenses in eight developmentally consecutive leaves only partially follow a developmental gradient within flowering plants of *Nicotiana attenuata*.

Figure S3. Herbivory-induced defense metabolites (HIDs) and CKs follow the same within-plant distributions in *Nicotiana attenuata*.

Figure S4. The developmentally regulated pattern of the herbivory-induced PA pathway of DCS in *Nicotiana attenuata*.

Figure S5. Developmental regulation of protease inhibitor activity and nicotine levels in leaves of *Nicotiana attenuata*.

Figure S6. Correlations of CK levels with the accumulations of different anti-herbivore defenses in *Nicotiana attenuata*.

Figure S7. Manipulating the within-plant CK gradient alters the distribution of DCS in *Nicotiana attenuata*.

Figure S8. Manipulating the within-plant CK gradient does not alter the distribution of nicotine and TPI activity in *Nicotiana attenuata*.

Figure S9. Manipulating the within-plant CK gradient alters the distribution of two PAs, but not of nicotine and TPI in *Nicotiana attenuata*.

Figure S10. Characterization of *SAG-IPT4* transgenic *Nicotiana attenuata* plants.

Figure S11. Restoring CK levels to an earlier developmental stage recovers inducibility of a major phenolic defense pathway in *Nicotiana attenuata*.

Figure S12. Protease inhibitor activity and nicotine levels in leaves of CK-overproducing *SAG-IPT4 Nicotiana attenuata* plants.

Table S1. CK levels in eight different leaf types of a flowering *Nicotiana attenuata* plant.

Table S2. Statistical analysis of CK levels in eight different leaf types of a flowering *Nicotiana attenuata* plant by TWAs.

Table S3. CK levels in different leaf classes of a flowering *Nicotiana attenuata* plant.

Table S4. Statistical analysis of CK levels in different leaf classes of a flowering *Nicotiana attenuata* plant by TWAs.

Table S5. CK levels in plants at two different growth stages of *Nicotiana attenuata*.

Table S6. Statistical analysis of CK levels at two different growth stages of *Nicotiana attenuata* with TWAs.

Table S7. CK levels in different leaf classes of a flowering i-ov*IPT* *Nicotiana attenuata* plant with a single DEX-treated leaf.

Table S8. CK levels in different leaf classes of a flowering i-ov*IPT* *Nicotiana attenuata* plant with alternatingly DEX-treated and control leaves.

Table S9. Statistical analysis of CK levels in different leaf classes of a flowering i-ov*IPT* *Nicotiana attenuata* plant with alternatingly DEX-treated and control leaves by TWAs.

Table S10. CK levels in WT and two transgenic *SAG-IPT4 Nicotiana attenuata* plants.

Table S11. Statistical analysis of CK levels in WT and two transgenic *SAG-IPT4 Nicotiana attenuata* plants by TWAs.

Table S12. Defense metabolites in WT and two transgenic *SAG-IPT4 Nicotiana attenuata* plants.

Table S13. Statistical analysis of defense metabolites in WT and transgenic *SAG-IPT4-2 Nicotiana attenuata* plants by TWAs.

Table S14. Relative transcript levels in two *SAG-IPT4 Nicotiana attenuata* plants.

Table S15. Statistical analysis of relative transcript levels in *SAG-IPT4-2 Nicotiana attenuata* plants.

Table S16. Sequences of primers used for qPCR.

Table S17. Cloning primers of *SAG-IPT4* construct used for generating *SAG-IPT4* lines.

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Zuur, A.F., Ieno, E.N., Walker, N.J., Saveliev, A.A., Smith, G.M., Zuur, A.F., Ieno, E.N., Walker, N.J., Saveliev, A.A. and Smith, G.M. (2009) *In Mixed Effects Models and Extensions in Ecology with R*. New York: Springer.

Supplementary figures

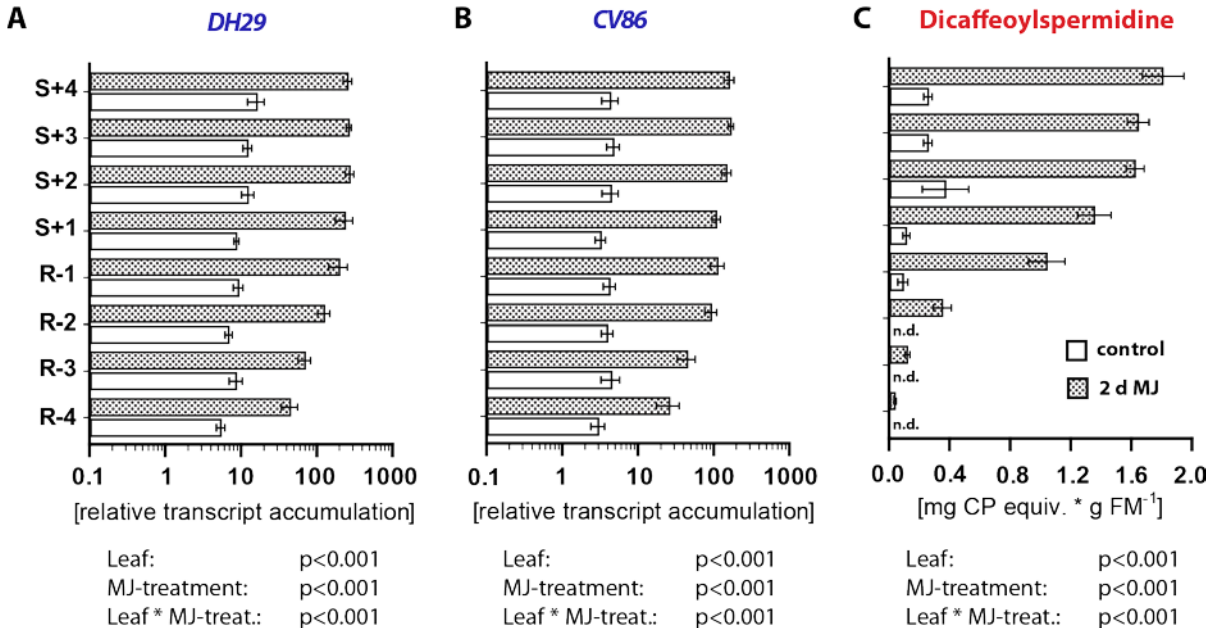

Fig. S1. The herbivory-induced phenolamide pathway in 8 developmentally consecutive leaves of flowering *Nicotiana attenuata* plants follows a developmentally determined pattern.

(A) Relative transcript accumulation of *NaDH29* and (D) *NaCV86* (biosynthesis of dicaffeoylspermidine). (C) Dicaffeoylspermidine. Levels were quantified in different leaf classes representing a developmental sequence from rosette leaves R-1 (youngest) to R-4 (oldest) and stem leaves S+1 (oldest) to S+4 (youngest). Plants were sprayed for two days with 1 mM methyl jasmonate (2 d MJ; dotted bars) or water as control (open bars). Data were analyzed by two-way ANOVAs (A) or generalized least squares models (B, C), *p*-values indicate influence of the single factors leaf and MJ-treatment or the interaction of both (Leaf * MJ-treat.). Error bars depict standard errors ($N \geq 5$). FM, fresh mass.

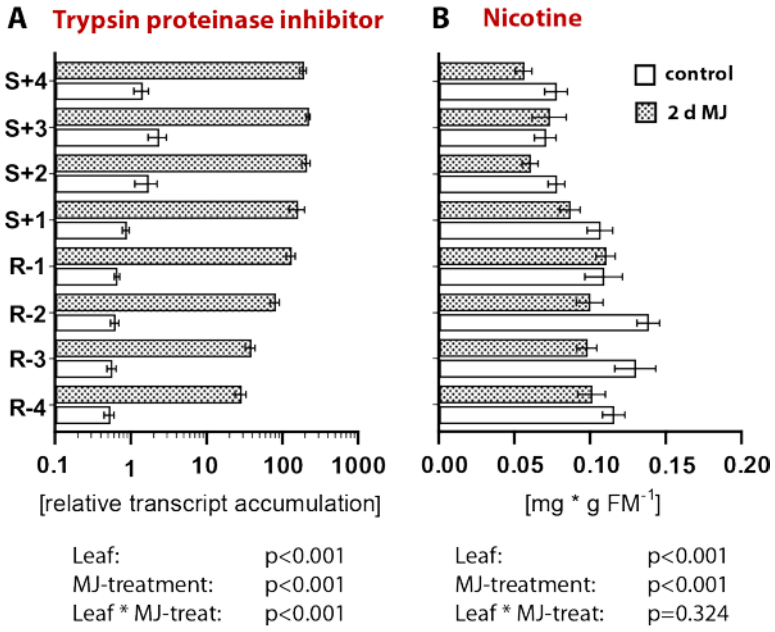

Fig. S2. Non-phenolic defenses in 8 developmentally consecutive leaves only partially follow a developmental gradient within flowering plants of *Nicotiana attenuata*.

(A) Relative transcript accumulations of *NaTPI* of trypsin proteinase inhibitor and (B) nicotine levels in different leaf classes (rosette leaves R-1 (youngest) to R-4 (oldest) and stem leaves S+1 (oldest) to S+4 (youngest) of flowering plants. Plants were sprayed for two days with 1 mM methyl jasmonate (2 d MJ; dotted bars) or water as control (open bars). Data were analyzed by two-way ANOVAs, *p*-values indicate influence of the single factors leaf and MJ-treatment or the interaction of both (Leaf * MJ-treat.). Error bars show standard errors ($N \geq 5$). FM, fresh mass.

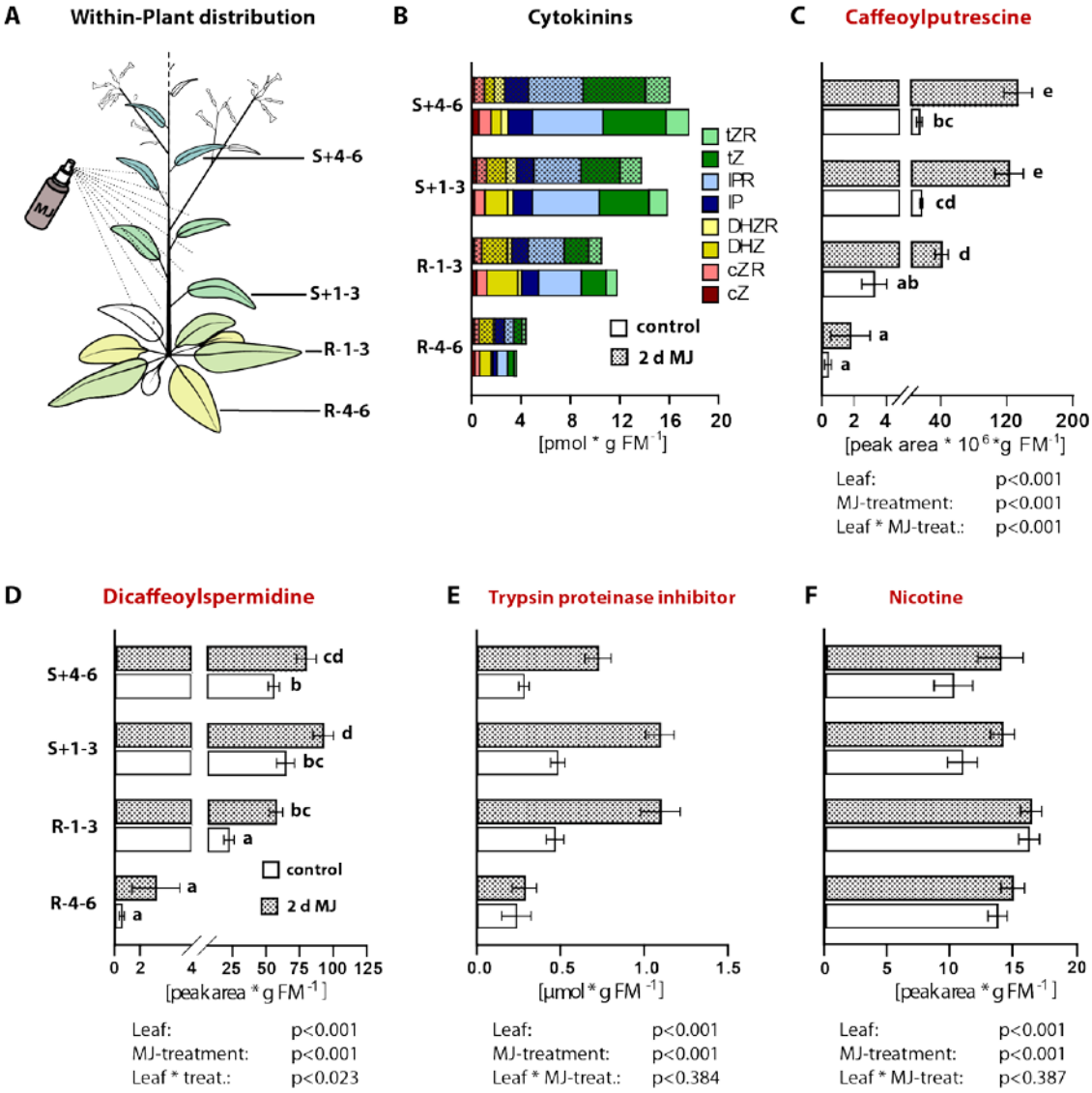

Fig. S3. Herbivory-induced defense metabolites (HIDs) and cytokinins follow the same within-plant distributions in *Nicotiana attenuata*.

(A) Experimental design. (B) CKs (*cis*-zeatin, *cZ*; *cis*-zeatin riboside, *cZR*; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, IP; isopentenyladenosine, IPR; *trans*-zeatin, *tZ; trans*-zeatin ribosides, *tZR*; other CKs in table S3), (C) caffeoylputrescine, (D) dicaffeoylspermidine, (E) trypsin proteinase inhibitor activity and (F) nicotine levels in different leaf-classes (youngest 3 rosette leaves R-1-3; next older rosette leaves 4-6, R-4-6; first 3 stem leaves, S+1-3; stem leaves 4-6, S+4-6) of a flowering plant. Plants were sprayed for two days with 1 mM methyl jasmonate (2d MJ) or water (control). Open bars: control values, dotted bars: MJ induced levels. Data were analyzed by two-way ANOVAs, *p*-values indicate influence of the single factors leaf and MJ-treatment or the interaction of both (Leaf * MJ-treat.).

Statistics for CKs can be found in table S4. Different letters indicate significant differences (if interaction was significant: Tukey HSD *post hoc* test: $p<0.05$). Error bars depict standard errors ($N \geq 5$). FM, fresh mass.

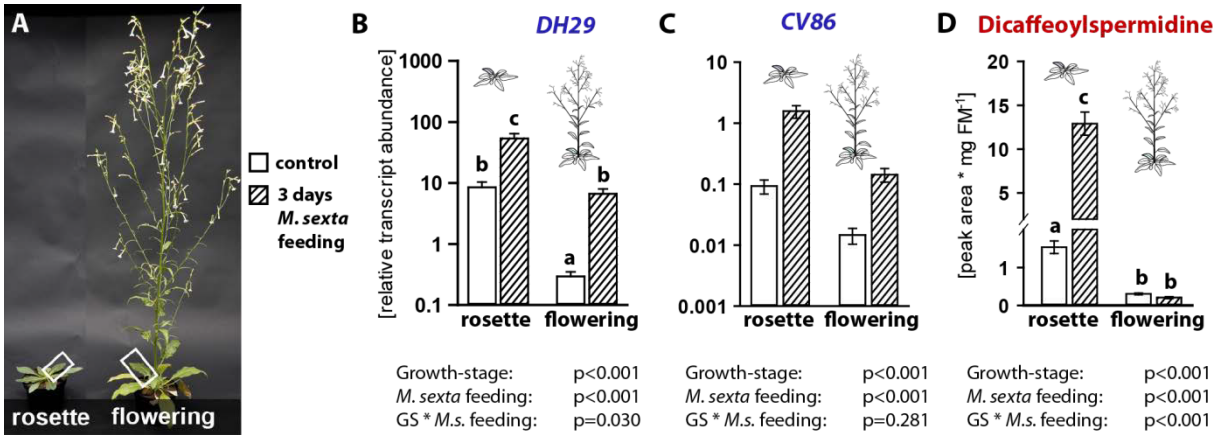

Fig. S4. The developmentally regulated pattern of the herbivory-induced phenolamide pathway of dicaffeoylspermidine in *Nicotiana attenuata*.

(A) Picture of the two growth stages of *N. attenuata* used for the experiments. Sampled leaves are highlighted by white boxes. (B) relative transcript abundance of *NaDH29* and (C) *NaCV86* (biosynthesis of dicaffeoylspermidine) and (D) dicaffeoylspermidine. Levels were determined in rosette leaves of vegetative rosette plants and reproductive flowering plants each in control plants (open bars) and after 3 d of *M. sexta* feeding (diagonal striped bars). Data were analyzed by two way ANOVAs, *p*-values indicate influence of the single factors growth-stage (GS) and *M. sexta* (*M.s.*) feeding or the interaction of both (GS * *M.s.*-feeding). Different letters indicate significant differences (if interaction was significant: Tukey HSD post hoc test: $p<0.05$). Error bars show standard errors ($N \geq 9$). FM, fresh mass.

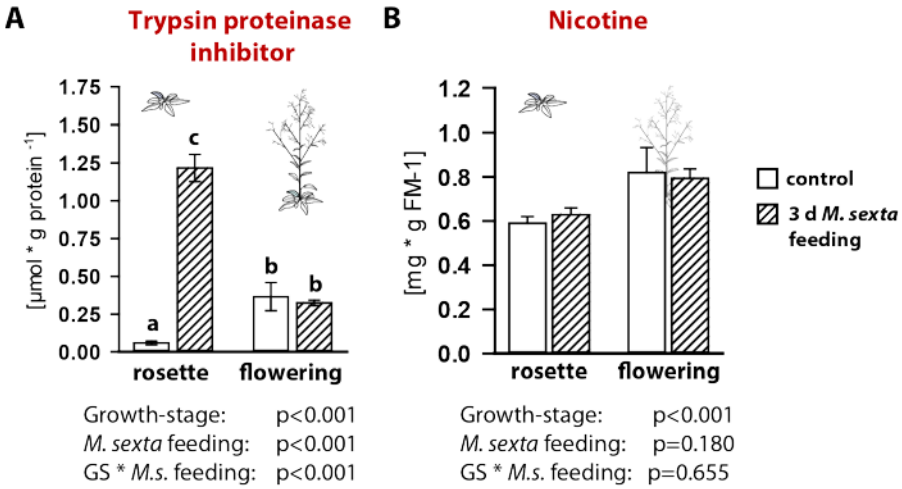

Fig. S5. Developmental regulation of protease inhibitor activity and nicotine levels in leaves of *Nicotiana attenuata*.

(A) Trypsin proteinase inhibitor activity and (B) levels of nicotine in leaves of plants in two different growth stages (rosette, flowering) after 3 d *Manduca sexta* feeding (diagonally striped bars) and in untreated control plants (open bars). Data were analyzed by two way ANOVAs, *p*-values indicate influence of the single factors growth-stage (GS) and *M. sexta* (*M.s.*) feeding or the interaction of both (GS * *M.s.*-feeding). Different letters indicate significant differences (if interaction was significant: Tukey HSD *post hoc* test: $p<0.05$). Error bars show standard errors ($N \geq 9$). FM, fresh mass.

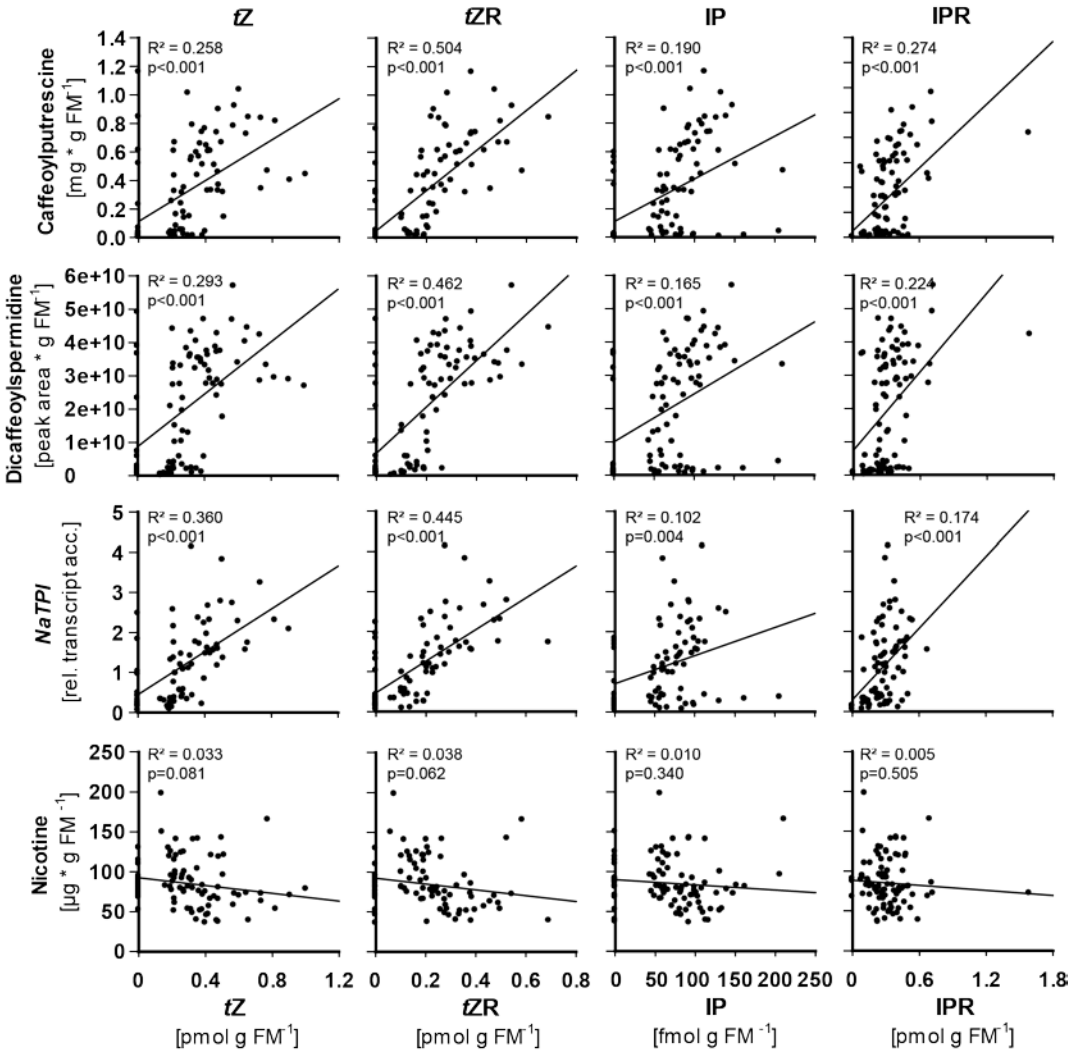

Fig. S6. Correlations of cytokinin levels with the accumulations of different anti-herbivore defenses in *Nicotiana attenuata*.

Caffeoylputrescine, dicaffeoylspermidine, *NaTPI* transcript levels and nicotine in different leaves of flowering plants were plotted against CKs (isopentenyladenine, IP; isopentenyladenosine, IPR; *trans*-zeatin, *tZ*; *trans*-zeatin riboside, *t*ZR) in the same leaves. Values are from leaves induced two days with 1 mM methyl jasmonate. *p*-values in the graphs represent results of a Pearson Product Moment Correlation. ($N \geq 78$). FM, fresh mass.

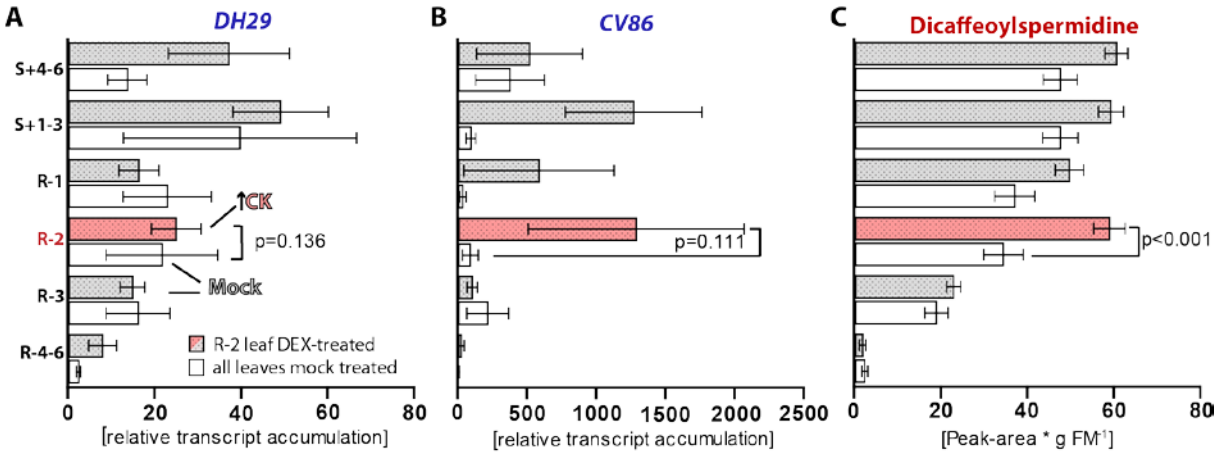

Fig. S7. Manipulating the within-plant cytokinin gradient alters the distribution of dicaffeoylspermidine in *Nicotiana attenuata*.

(A) Relative transcript accumulation of *NaDH29* and (B) *NaCV86* (biosynthesis of dicaffeoylspermidine) and (C) dicaffeoylspermidine in different leaf classes (rosette leaves 4-6, R-4-6,rosette leaf 3, 2 and 1 with R-1 being the youngest and R-6 being the oldest, first 3 stem leaves 1-3 (S+1-3) and stem leaves 4-6 (S+4-6)) of flowering plants transformed with a construct for dexamethasone-inducible expression of the CK biosynthesis enzyme isopentenyltransferase (*i-ovIPT*). One young rosette leaf (R-2) was treated with 5 µM dexamethasone and 1% DMSO in lanolin paste (DEX; red color; ↑CK) to increase levels of *tZ*-type CKs in the leaves or with 1% DMSO in lanolin as control (Mock, white color). All other leaves were mock-treated. Grey bars indicate levels from plants in which one leaf was DEX-treated. Plants were sprayed for two days with 1 mM methyl jasmonate (MJ). *p*-values from a *t*-test comparing the mock with the DEX treated R-2. Error bars depict standard errors ($N \geq 4$). FM, fresh mass.

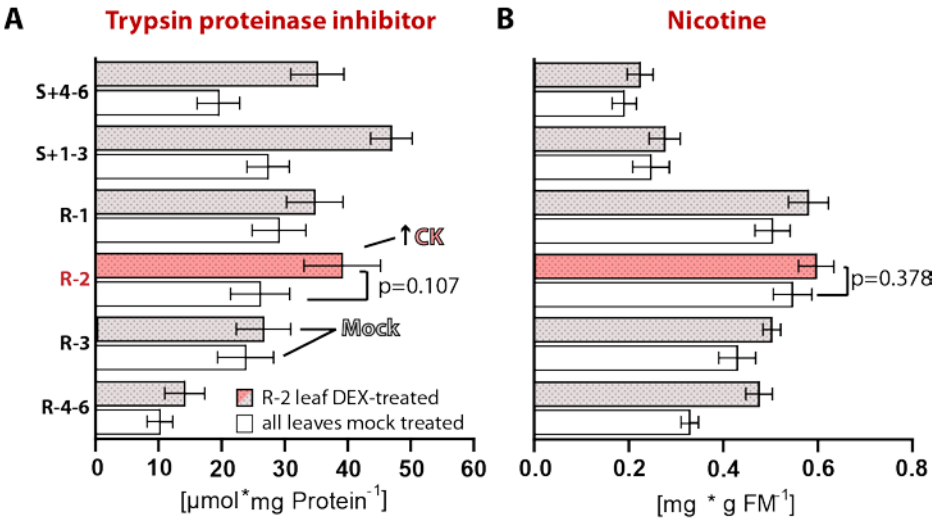

Fig. S8. Manipulating the within-plant cytokinin gradient does not alter the distribution of nicotine and trypsin proteinase inhibitor activity in *Nicotiana attenuata*.

(A) Trypsin proteinase inhibitor activity and (B) levels of nicotine in different leaf classes (Rosette leaves 4-6, R-4-6, rosette leaf 3, 2 and 1 with R-1 being the youngest and R-6 being the oldest, first 3 stem leaves 1-3 (S+1-3) and stem leaves 4-6 (S+4-6)) of flowering plants transformed with a construct for dexamethasone-inducible expression of the CK biosynthesis enzyme isopentenyltransferase (*i-ovIPT*). One young rosette leaf (R-2) was treated with 5 µM dexamethasone and 1% DMSO in lanolin paste (DEX; red color; ↑CK) to increase levels of *tZ*-type CKs in the leaves or with 1% DMSO in lanolin as control (Mock, white color). All other leaves were mock-treated. Grey bars indicate levels from plants in which one leaf was DEX-treated. Plants were sprayed for two days with 1 mM methyl jasmonate (MJ). *p*-values are derived from a *t*-test comparing the mock with the DEX treated R-2. Error bars depict standard errors ($N \geq 4$). FM, fresh mass.

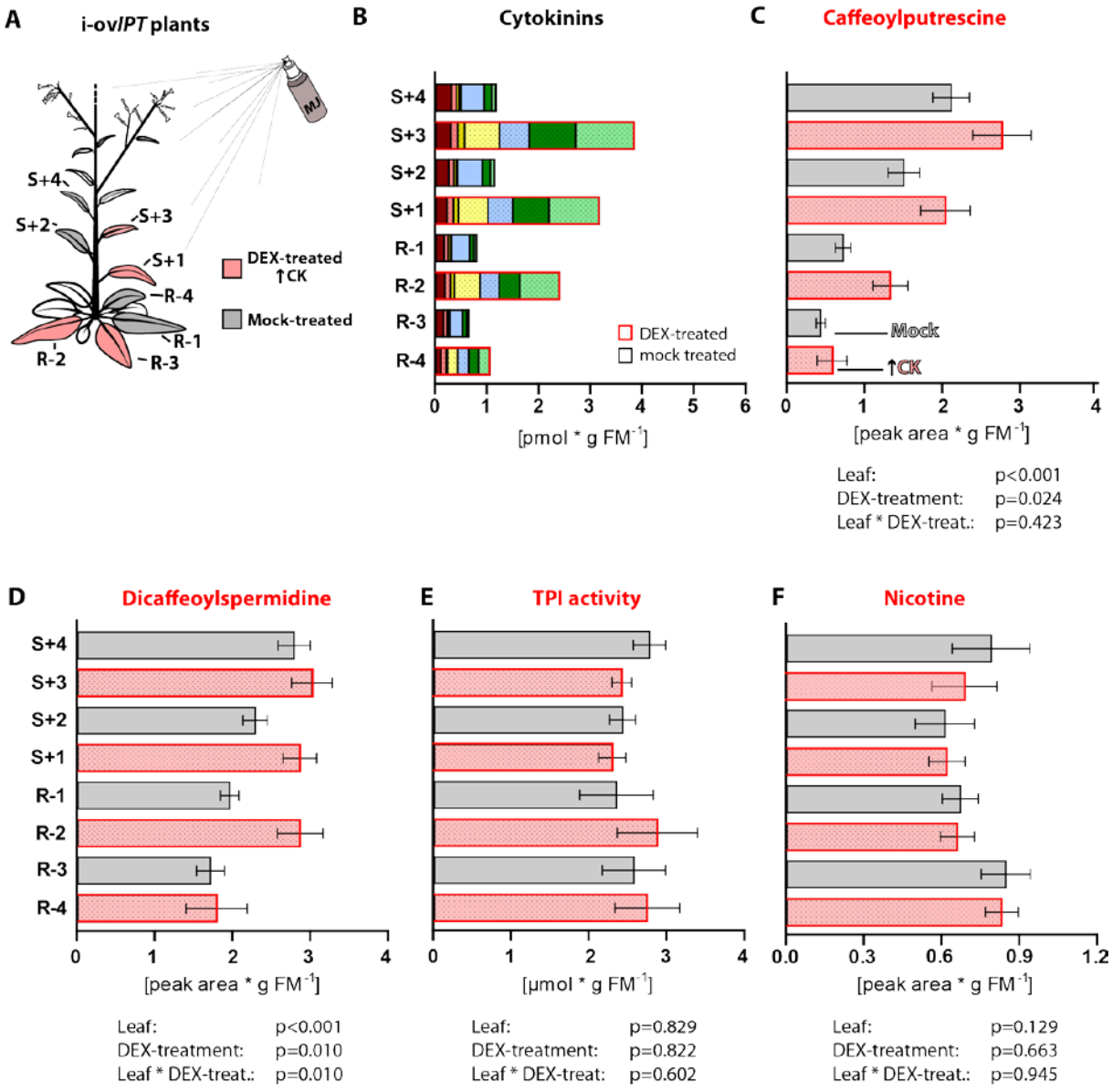

Fig. S9: Manipulating the within-plant cytokinin gradient alters the distribution of two phenolamides but not of nicotine and trypsin proteinase inhibitors (TPI) in *Nicotiana attenuata*. (A) Experimental setup (B) CKs (*cis*-zeatin, *cZ*; *cis*-zeatin riboside, *cZR*; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, IP; isopentenyladenosine, IPR; *trans*-zeatin, *tZ*; *trans*-zeatin riboside, *tZR*; ; other CKs in table S8). (C) Caffeoylputrescine, (D) dicaffeoylspermidine, (E) TPI-activity and (F) nicotine in different leaf classes (Rosette leaves R-2 and R-1 and stem leaves S+1 and S+2) of flowering plants transformed with a construct for dexamethasone-inducible expression of the CK-biosynthesis enzyme isopentenyltransferase (*i-ovIPT*). Leaves were either treated with 5 µM dexamethasone and 1% DMSO in lanolin paste (DEX; red color; ↑CK) to increase levels of *tZ*-type CKs in the leaves or with 1% DMSO

in lanolin as control (Mock, grey color). Plants were sprayed for two days with 1 mM methyl jasmonate (MJ). Data were analyzed by two-way ANOVAs (C-F), *p*-values indicate influence of the single factors leaf and DEX-treatment or the interaction of both (Leaf * DEX-treat.). Statistics for CKs can be found in table S9. Error bars depict standard errors ($N \geq 11$). FM, fresh mass.

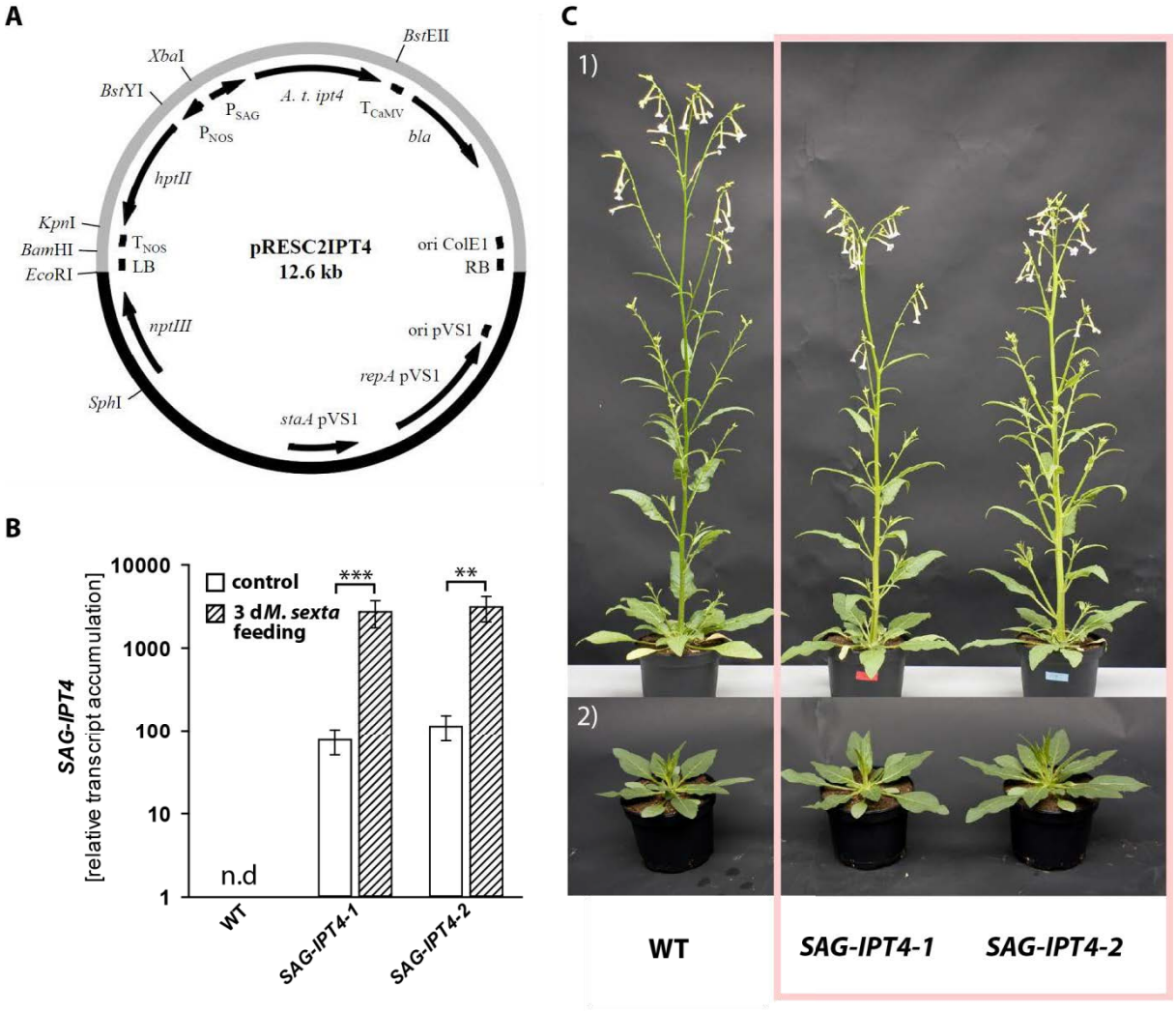

Fig. S10. Characterization of *SAG-IPT4* transgenic *Nicotiana attenuata* plants.

(A) Plasmid vector pRESC2IPT4 for the overproduction of the *Arabidopsis thaliana* isopentenyltransferase 4 (*AtIPT4*) driven by the promotor of *A. thaliana senescence-associated gene 12* (P_{SAG}). LB/RB: left- and right border of the T-DNA; PNOS/TNOS: promoter/terminator of the nopaline synthase gene from the Ti plasmid of Agrobacterium tumefaciens; hptII: hygromycin phosphotransferase gene from pCAMBIA-1301 (AF234297); TCaMV: terminator from cauliflower mosaic virus; bla: beta-lactamase gene (ampicillin resistance); nptII, aminoglycoside phosphotransferase class II. (B) P_{SAG} Promotor activity: relative transcript accumulation of *AtIPT4* in WT and *SAG-IPT4* lines (light red background). Levels were determined in youngest rosette leaf of a flowering plant after three days of *Manduca sexta* feeding (diagonal striped bars) and in leaves of unattacked control plants (open bars). Asterisks indicate significant differences between control and *M. sexta*-induced leaves ($p<0.05$; Wilcoxon rank sum

test). Error bars show standard errors (N ≥ 9). (C) Growth phenotype of *SAG-IPT4* plants in flowering (1) and rosette stage (2).

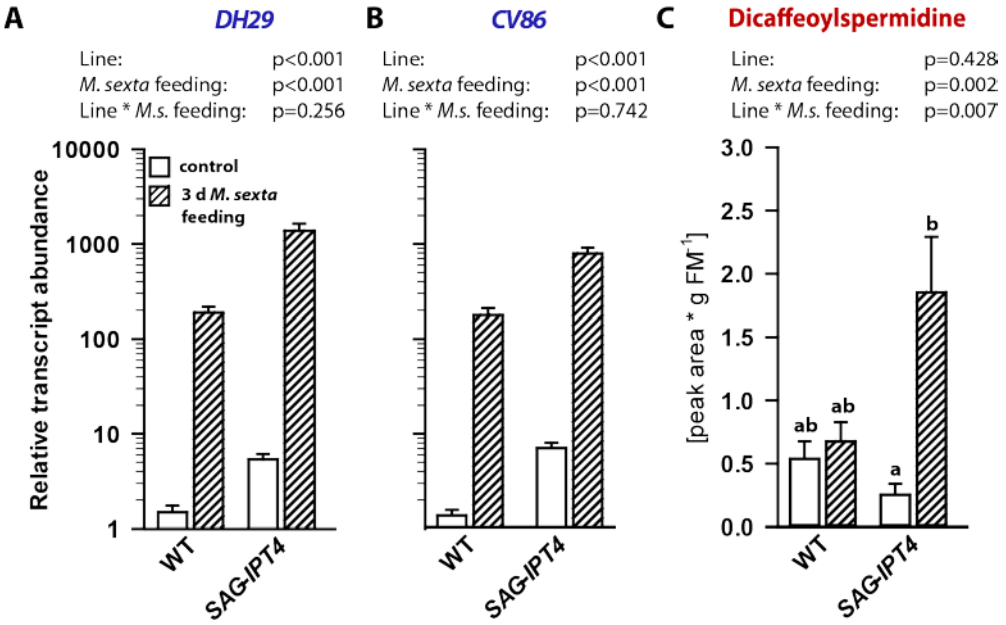

Fig. S11. Restoring cytokinin levels to an earlier developmental stage recovers inducibility of a major phenolic defense pathway in *Nicotiana attenuata*.

(A) Relative transcript accumulation of *NaDH29* and (B) *NaCV86* (biosynthesis enzymes of dicaffeoylspermidine) and (C) dicaffeoylspermidine in WT and senescence-activated CK overproducing *SAG-IPT4* lines. Levels were determined in the youngest rosette leaf of a flowering plant after three days of *Manduca sexta* feeding (diagonal striped bars) and in leaves from unattacked control plants (open bars). Data were analyzed by two-way ANOVAs (C) or generalized least squares-models (A, B), *p*-values indicate influence of the single factors genotype (line) and *M. sexta* (*M.s.*) feeding or the interaction of both (Line * *M.s.*-feeding). Results for line *SAG-IPT4-2* can be found in tables S12 – S15. Different letters indicate significant differences (if interaction was significant: Tukey HSD *post hoc* test, $p<0.05$). Error bars show standard errors ($N \geq 5$). FM, fresh mass.

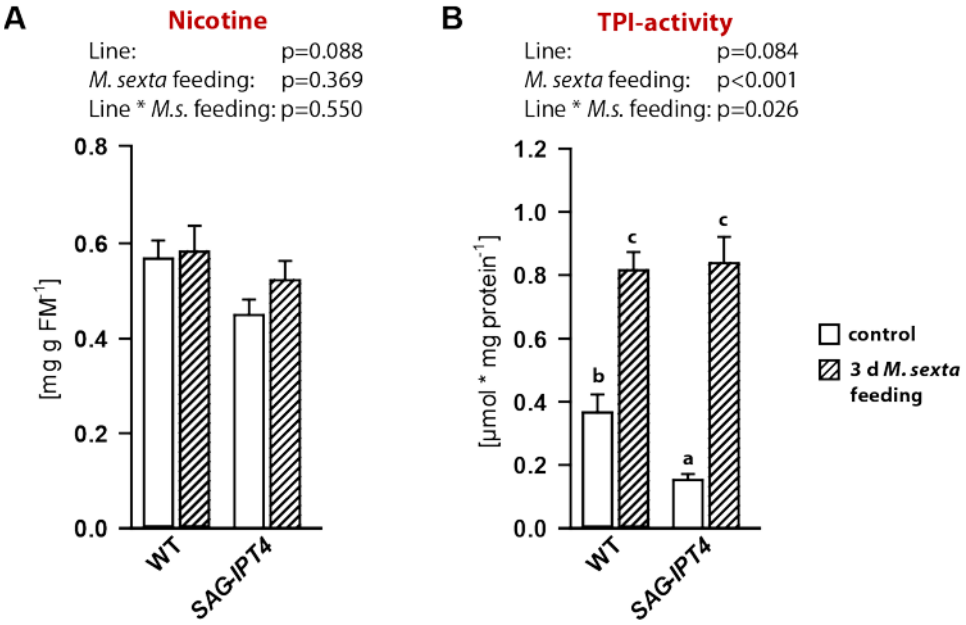

Fig S12. Protease inhibitor activity and nicotine levels in leaves of cytokinin-overproducing *SAG-IPT4 Nicotiana attenuata* plants.

(A) Nicotine and (B) trypsin proteinase inhibitor activity in WT and a senescence-activated CK overproducing *SAG-IPT4* line. Experiments were conducted with the youngest rosette leaf of a flowering plant after three days of *Manduca sexta* feeding (diagonal striped bars) and with the same leaf position on unattacked control plants (open bars). Data were analyzed by two-way ANOVAs, *p*-values indicate influence of the single factors genotype (line) and *M. sexta* (*M.s.*) feeding or the interaction of both (Line * *M.s.*-feeding). Results for line *SAG-IPT4-2* can be found in tables S12 and S13. Different letters indicate significant differences (if interaction was significant: Tukey HSD post hoc test: $p<0.05$). Error bars show standard errors ($N \geq 5$). FM, fresh mass.

Table S1. Cytokinin levels in 8 different leaftypes of a flowering *Nicotiana attenuata* plant. (part1)

CK-type	Cytokinins [pmol g FM^{-1}] in leaf																			
	R-4						R-3						R-2						R-1					
	Control			MJ-spray			Control			MJ-spray			Control			MJ-spray			Control			MJ-spray		
tZ	***0.12***	±	***0.05***	***0.06***	±	***0.04***	***0.08***	±	***0.04***	***0.20***	±	***0.03***	***0.12***	±	***0.04***	***0.22***	±	***0.05***	***0.27***	±	***0.03***	***0.32***	±	***0.05***
tZR	***0.03***	±	***0.02***	***0.07***	±	***0.03***	***0.08***	±	***0.03***	***0.10***	±	***0.03***	***0.04***	±	***0.02***	***0.13***	±	***0.03***	***0.10***	±	***0.03***	***0.18***	±	***0.02***
tZOG	n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.		
tZ7G	18.35	±	0.74	17.61	±	0.49	18.55	±	0.95	16.86	±	0.64	13.76	±	0.81	13.60	±	1.01	13.32	±	0.77	11.56	±	1.20
tZ9G	n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.		
tZROG	9.10	±	0.67	8.10	±	0.54	9.72	±	0.46	9.45	±	0.67	8.04	±	0.63	7.68	±	0.37	8.90	±	0.76	8.18	±	0.27
DHZ	***0.03***	±	***0.03***	***0.00***	±	***0.00***	***0.00***	±	***0.00***	***0.00***	±	***0.00***	***0.03***	±	***0.03***	***0.00***	±	***0.00***	***0.00***	±	***0.00***	***0.00***	±	***0.00***
DHZR	***0.00***	±	***0.00***	***0.00***	±	***0.00***	***0.00***	±	***0.00***	***0.01***	±	***0.01***	***0.00***	±	***0.00***	***0.00***	±	***0.00***	***0.00***	±	***0.00***	***0.00***	±	***0.00***
cZ	***0.12***	±	***0.05***	***0.27***	±	***0.02***	***0.19***	±	***0.05***	***0.15***	±	***0.05***	***0.12***	±	***0.03***	***0.27***	±	***0.06***	***0.18***	±	***0.06***	***0.39***	±	***0.06***
cZR	***0.21***	±	***0.02***	***0.36***	±	***0.04***	***0.21***	±	***0.02***	***0.36***	±	***0.07***	***0.22***	±	***0.02***	***0.39***	±	***0.06***	***0.23***	±	***0.01***	***0.41***	±	***0.02***
cZOG	3.14	±	0.96	5.81	±	0.91	5.63	±	0.92	8.23	±	0.83	5.05	±	0.38	7.27	±	0.34	6.11	±	0.80	7.92	±	0.87
cZ7G	32.24	±	1.29	35.31	±	1.34	32.70	±	1.59	37.07	±	1.45	29.69	±	1.66	33.88	±	1.70	32.60	±	1.40	36.12	±	1.37
cZ9G	0.81	±	0.03	0.89	±	0.11	0.90	±	0.05	1.06	±	0.09	0.75	±	0.05	1.12	±	0.06	0.97	±	0.04	1.25	±	0.12
cZROG	8.91	±	0.85	12.91	±	1.44	12.80	±	2.84	17.64	±	2.02	10.71	±	0.84	19.05	±	1.98	19.20	±	1.23	26.03	±	1.83
IP	***0.10***	±	***0.03***	***0.05***	±	***0.02***	***0.04***	±	***0.03***	***0.08***	±	***0.03***	***0.09***	±	***0.03***	***0.06***	±	***0.01***	***0.09***	±	***0.01***	***0.05***	±	***0.01***
IPR	***0.44***	±	***0.08***	***0.25***	±	***0.03***	***0.52***	±	***0.04***	***0.29***	±	***0.03***	***0.56***	±	***0.09***	***0.28***	±	***0.04***	***0.69***	±	***0.06***	***0.33***	±	***0.04***

Table S1. (part2)

CK-type	Cytokinins [pmol g FM^{-1}] in leaf																			
	S+1						S+2						S+3						S+4					
	Control			MJ-spray			Control			MJ-spray			Control			MJ-spray			Control			MJ-spray		
tZ	***0.20***	±	***0.04***	***0.31***	±	***0.06***	***0.28***	±	***0.03***	***0.39***	±	***0.07***	***0.36***	±	***0.04***	***0.47***	±	***0.10***	***0.33***	±	***0.04***	***0.53***	±	***0.08***
tZR	***0.14***	±	***0.01***	***0.18***	±	***0.04***	***0.15***	±	***0.03***	***0.28***	±	***0.05***	***0.17***	±	***0.02***	***0.30***	±	***0.07***	***0.23***	±	***0.02***	***0.29***	±	***0.05***
tZOG	n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.		
tZ7G	11.45	±	0.63	7.92	±	0.33	11.10	±	0.31	6.74	±	0.62	10.19	±	0.67	6.95	±	0.82	9.78	±	0.25	6.31	±	0.55
tZ9G	n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.		
tZROG	7.17	±	0.73	6.32	±	0.39	6.76	±	0.50	6.34	±	0.57	5.79	±	0.37	5.66	±	0.99	5.66	±	0.21	6.68	±	0.85
DHZ	***0.00***	±	***0.00***	***0.00***	±	***0.00***	***0.00***	±	***0.00***	***0.00***	±	***0.00***	***0.00***	±	***0.00***	***0.06***	±	***0.06***	***0.00***	±	***0.00***	***0.00***	±	***0.00***
DHZR	***0.00***	±	***0.00***	***0.00***	±	***0.00***	***0.00***	±	***0.00***	***0.00***	±	***0.00***	***0.00***	±	***0.00***	***0.00***	±	***0.00***	***0.00***	±	***0.00***	***0.00***	±	***0.00***
cZ	***0.19***	±	***0.02***	***0.43***	±	***0.08***	***0.17***	±	***0.04***	***0.51***	±	***0.11***	***0.23***	±	***0.04***	***0.47***	±	***0.08***	***0.22***	±	***0.03***	***0.62***	±	***0.09***
cZR	***0.28***	±	***0.02***	***0.39***	±	***0.03***	***0.28***	±	***0.03***	***0.38***	±	***0.07***	***0.30***	±	***0.03***	***0.34***	±	***0.04***	***0.32***	±	***0.03***	***0.58***	±	***0.05***
cZOG	6.34	±	0.85	8.42	±	1.10	7.03	±	0.74	9.42	±	1.86	8.96	±	1.63	11.16	±	1.87	10.98	±	1.02	13.80	±	1.67
cZ7G	30.84	±	0.99	29.34	±	1.15	29.93	±	1.30	25.07	±	2.71	29.89	±	1.23	26.81	±	2.95	28.49	±	1.19	26.10	±	1.74
cZ9G	1.02	±	0.05	1.18	±	0.10	1.26	±	0.12	1.00	±	0.09	1.19	±	0.04	0.94	±	0.09	1.27	±	0.06	1.03	±	0.11
cZROG	19.90	±	2.43	28.02	±	1.57	27.66	±	2.47	27.97	±	2.89	25.47	±	1.03	29.65	±	4.15	27.82	±	0.76	36.89	±	3.04
IP	***0.09***	±	***0.02***	***0.07***	±	***0.01***	***0.12***	±	***0.02***	***0.05***	±	***0.02***	***0.11***	±	***0.02***	***0.08***	±	***0.01***	***0.12***	±	***0.01***	***0.09***	±	***0.01***
IPR	***0.76***	±	***0.05***	***0.31***	±	***0.02***	***0.88***	±	***0.05***	***0.28***	±	***0.05***	***0.99***	±	***0.12***	***0.30***	±	***0.05***	***0.99***	±	***0.08***	***0.40***	±	***0.05***

n.d. = not determined

Table includes CKs shown in Fig. 1 (bold; *cis*-zeatin, *cZ*; *cis*-zeatin riboside, *cZR*; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, IP; isopentenyladenosine, IPR; *trans*-zeatin, *tZ*; *trans*-zeatin riboside, *tZR*) and glucosylated forms of those CKs. Levels were determined in different leaf classes (rosette leaves R-1 (youngest) to R-4 (oldest) and stem leaves S+1 (oldest) to S+4 (youngest) of flowering plants. Plants were sprayed for two days with 1 mM methyl jasmonate (MJ) or with water as control. Table shows mean values ± standard errors (N ≥ 5). FM, fresh mass.

Table S2. Statistical analysis of cytokinin levels in 8 different leaftypes of a flowering *Nicotiana attenuata* plant by two-way ANOVAs.

	P-values derived from two-way ANOVA							
	IP	**IPR***	***t*Z**	***t*ZR**	***c*Z**	***c*ZR***	**DHZ**	**DHZR**
Leaf	0.342	0.000	0.000	0.000	0.003	0.011	n.d.	n.d.
MJ-treatment	0.000	0.000	0.561	0.022	0.001	0.000	n.d.	n.d.
Interaction Leaf * MJ-treatment	0.326	0.207	0.605	0.693	0.825	0.332	n.d.	n.d.

*=log transformed

n.d.= not determined (too many 0 values)

Table includes the analysis for CKs shown in Fig. 1 and Tab. S1 (*cis*-zeatin, *c*Z; *cis*-zeatin riboside, *c*ZR; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, IP; isopentenyladenosine, IPR; *trans*-zeatin, *t*Z; *trans*-zeatin riboside, *t*ZR). The statistics analysed the effect of different leaf classes within a flowering plant (Leaf), the influence of being sprayed for two days with 1 mM methyl jasmonate (MJ), as well as their interaction.

Table S3. Cytokinin levels in different leaf classes of a flowering *Nicotiana attenuata* plant.

CK-type	Cytokinins [pmol g FM^{-1}] in																			
	Old rosette leaves R-4-6						Young rosette leaves R-1-3						Stem leaves S+1-3						Stem leaves S+4-6					
	Control			MJ-spray			Control			MJ-spray			Control			MJ-spray			Control			MJ-spray		
tZ	***0.55***	±	***0.16***	***0.67***	±	***0.29***	***1.99***	±	***0.15***	***1.99***	±	***0.94***	***4.03***	±	***0.35***	***3.13***	±	***0.83***	***5.10***	±	***0.76***	***5.04***	±	***1.37***
tZR	***0.19***	±	***0.05***	***0.33***	±	***0.19***	***0.88***	±	***0.17***	***1.02***	±	***0.58***	***1.46***	±	***0.21***	***1.74***	±	***0.58***	***1.84***	±	***0.26***	***1.98***	±	***0.55***
tZOG	n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.		
tZ7G	19.47	±	2.09	19.20	±	0.75	21.64	±	0.79	20.65	±	2.94	19.46	±	0.81	22.37	±	3.04	17.66	±	1.54	19.40	±	1.31
tZ9G	33.58	±	1.56	34.82	±	2.38	43.17	±	3.91	41.42	±	5.03	48.18	±	2.41	55.31	±	1.79	46.93	±	5.46	51.98	±	3.36
tZROG	11.91	±	0.96	12.70	±	1.84	16.55	±	1.58	18.17	±	1.50	15.59	±	1.39	22.30	±	4.01	12.19	±	1.16	21.08	±	2.59
DHZ	***0.95***	±	***0.22***	***1.14***	±	***0.24***	***2.51***	±	***0.41***	***2.09***	±	***0.75***	***1.81***	±	***0.22***	***1.58***	±	***0.29***	***0.82***	±	***0.13***	***0.79***	±	***0.18***
DHZR	***0.12***	±	***0.02***	***0.14***	±	***0.05***	***0.32***	±	***0.08***	***0.37***	±	***0.06***	***0.47***	±	***0.09***	***0.83***	±	***0.33***	***0.57***	±	***0.09***	***0.86***	±	***0.20***
cZ	***0.26***	±	***0.07***	***0.24***	±	***0.17***	***0.40***	±	***0.17***	***0.22***	±	***0.17***	***0.24***	±	***0.07***	***0.39***	±	***0.20***	***0.60***	±	***0.21***	***0.21***	±	***0.09***
cZR	***0.38***	±	***0.06***	***0.36***	±	***0.09***	***0.83***	±	***0.11***	***0.57***	±	***0.11***	***0.84***	±	***0.03***	***0.81***	±	***0.07***	***0.96***	±	***0.08***	***0.84***	±	***0.03***
cZOG	3.19	±	1.10	7.38	±	2.19	46.36	±	30.01	87.73	±	21.66	74.36	±	6.81	673.67	±	402.30	29.93	±	7.74	444.45	±	206.56
cZ7G	0.52	±	0.21	0.55	±	0.18	0.85	±	0.33	1.79	±	1.39	0.69	±	0.20	6.60	±	5.82	1.71	±	1.21	0.57	±	0.06
cZ9G	4.92	±	0.41	5.48	±	0.55	5.15	±	0.28	7.38	±	1.06	6.17	±	0.64	10.07	±	2.51	3.92	±	0.37	7.94	±	1.38
cZROG	26.24	±	2.32	30.07	±	7.06	79.09	±	7.21	79.32	±	3.80	103.16	±	5.29	82.12	±	1.75	104.78	±	6.08	83.17	±	4.87
IP	***0.34***	±	***0.08***	***0.78***	±	***0.47***	***1.37***	±	***0.07***	***1.32***	±	***0.32***	***1.56***	±	***0.34***	***1.41***	±	***0.34***	***1.97***	±	***0.22***	***1.92***	±	***0.05***
IPR	***0.84***	±	***0.13***	***0.74***	±	***0.38***	***3.47***	±	***0.17***	***2.92***	±	***1.15***	***5.41***	±	***0.20***	***3.84***	±	***0.95***	***5.70***	±	***0.66***	***4.43***	±	***0.88***

n.d. = not determined

Table includes CKs shown in main Fig. S3 (bold; *cis*-zeatin, *cZ*; *cis*-zeatin riboside, *cZR*; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, IP; isopentenyladenosine, IPR; *trans*-zeatin, *tZ*; *trans*-zeatin riboside, *tZR*) and their glucosylated forms. Plants were sprayed for two days with 1mM methyl jasmonate (MJ-spray) or water as control. Rosette leaves R-4-6; rosette leaves, R-1-3; first 3 stem leaves, S+1-3; stem leaves 4-6, S+4-6. Mean values ± standard error. FM, fresh mass. N≥5.

Table S4. Statistical analysis of cytokinin levels in different leaf classes of a flowering *Nicotiana attenuata* plant by two-way ANOVAS.

	P-values derived from two-way ANOVA							
	IP	**IPR***	***t*Z**	***t*ZR***	***c*Z***	***c*ZR***	**DHZ**	**DHZR***
Leaf	0.000	0.000	0.000	0.000	0.789	0.000	0.000	0.000
MJ-treatment	0.755	0.031	0.652	0.992	0.095	0.127	0.655	0.119
Interaction Leaf * MJ treatment	0.713	0.991	0.863	0.966	0.806	0.620	0.776	0.866

*=log transformed

Table includes the analysis for CKs shown in Fig. S3 and Tab. S3 (*cis*-zeatin, *c*Z; *cis*-zeatin riboside, *c*ZR; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, IP; isopentenyladenosine, IPR; *trans*-zeatin, *t*Z; *trans*-zeatin riboside, *t*ZR). The statistics analyzed the effect of different leaf classes within a flowering plant (Leaf), the influence of being sprayed for two days with 1 mM methyl jasmonate (MJ), as well as their interaction.

Table S5. Cytokinin levels in plants at two different growth stages of *Nicotiana attenuata*.

CK-type	Cytokinins [pmol g FM^{-1}] in rosette leaf of plants in			
	Rosette stage		Flowering stage	
	Control	*M. sexta*	Control	*M. sexta*
tZ	***0.312 ± 0.056***	***0.599 ± 0.060***	***0.138 ± 0.067***	***0.081 ± 0.012***
tZR	***0.230 ± 0.024***	***0.199 ± 0.022***	***0.024 ± 0.005***	***0.017 ± 0.002***
tZOG	1.552 ± 0.123	2.361 ± 0.191	1.572 ± 0.238	1.398 ± 0.173
tZ7G	4.871 ± 0.333	5.166 ± 0.183	7.479 ± 0.853	6.412 ± 0.467
tZ9G	1.971 ± 0.054	3.196 ± 0.215	20.124 ± 1.865	19.703 ± 1.023
tZROG	8.393 ± 0.483	10.033 ± 1.092	7.350 ± 0.724	6.811 ± 0.564
DHZ	***0.557 ± 0.105***	***0.486 ± 0.073***	***0.434 ± 0.070***	***0.320 ± 0.045***
DHZR	***0.126 ± 0.011***	***0.181 ± 0.023***	***0.045 ± 0.011***	***0.030 ± 0.004***
cZ	***0.202 ± 0.048***	***0.562 ± 0.049***	***0.246 ± 0.036***	***0.203 ± 0.025***
cZR	***0.178 ± 0.019***	***0.481 ± 0.055***	***0.160 ± 0.027***	***0.303 ± 0.036***
cZOG	4.569 ± 0.963	25.627 ± 5.583	1.191 ± 0.311	0.874 ± 0.163
cZ7G	0.098 ± 0.015	0.151 ± 0.019	0.285 ± 0.029	0.257 ± 0.014
cZ9G	1.767 ± 0.286	1.145 ± 0.119	4.236 ± 0.311	3.731 ± 0.396
cZROG	17.842 ± 1.517	29.917 ± 2.031	57.339 ± 5.004	59.356 ± 6.325
IP	***0.054 ± 0.003***	***0.057 ± 0.003***	***0.057 ± 0.009***	***0.029 ± 0.002***
IPR	***0.449 ± 0.033***	***0.322 ± 0.030***	***0.176 ± 0.028***	***0.134 ± 0.008***

Table includes CKs depicted in Fig. 2 (bold; *cis*-zeatin, *c*Z; *cis*-zeatin riboside, *c*ZR; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, IP; isopentenyladenosine, IPR; *trans*-zeatin, *t*Z; *trans*-zeatin riboside, *t*ZR) and their glucosylated forms. Plants were induced by 3 d *Manduca sexta* feeding or not induced (control). Mean values ± standard error. FM, fresh mass. N≥9.

Table S6. Statistical analysis of cytokinin levels at two different growth stages of *Nicotiana attenuata* with two-way ANOVAs.

	P-values derived from two-way ANOVA							
	IP*	**IPR***	***tZ***	***tZR****	***cZ****	***cZR****	**DHZ***	**DHZR**
Growth-stage	0.000	0.000	0.000	0.000	0.009	0.008	0.070	0.000
***M.s.* feeding**	0.004	0.007	0.032	0.299	0.003	0.000	0.227	0.815
Interaction Growth-stage * *M.s.* feeding	0.001	0.447	0.002	0.966	0.000	0.201	0.581	0.119

*=log transformed

Table includes the analysis for CKs shown in Fig. 2 and Tab. S5 (*cis*-zeatin, *cZ*; *cis*-zeatin riboside, *cZR*; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, IP; isopentenyladenosine, IPR; *trans*-zeatin, *tZ*; *trans*-zeatin riboside, *tZR*). The statistics analysed the effect of different growth stages (Growth-stage), the influence of being infested with *M. sexta* neonates for 3 d (*M.s.* feeding), as well as their interaction.

Table S7. Cytokinin levels in different leaf classes of a flowering i-ov*IPT Nicotiana attenuata* plant with a single dexamethasone treated leaf. (part 1)

	Cytokinins [pmol g FM^{-1}] in flowering i-ov*IPT* plants																				
	1 leaf DEX treated all others LAN treated																				
CK-type	LAN						DEX			LAN											
	R-4-6			R-3			R-2			R-3			S+1-3			S+4-6				
tZ	***0.59***	±	***0.24***	***1.29***	±	***0.32***	***2.84***	±	***0.73***	***1.25***	±	***0.20***	***1.25***	±	***0.25***	***1.45***	±	***0.64***			
tZR	***0.06***	±	***0.01***	***0.15***	±	***0.06***	***0.83***	±	***0.25***	***0.15***	±	***0.02***	***0.07***	±	***0.01***	***0.16***	±	***0.04***			
tZOG	2.56	±	0.86	1.16	±	0.16	3.90	±	1.07	2.86	±	1.95	2.92	±	1.61	32.03	±	27.71			
tZ7G	20.40	±	0.94	16.71	±	1.14	27.44	±	3.75	15.03	±	2.46	8.22	±	0.43	7.06	±	0.93			
tZ9G	1.53	±	0.59	15.52	±	3.07	76.82	±	6.05	67.54	±	13.82	84.66	±	4.29	76.45	±	6.01			
tZROG	16.98	±	0.88	19.23	±	1.21	33.58	±	6.29	41.87	±	22.66	18.12	±	2.13	19.76	±	1.55			
DHZ	***0.51***	±	***0.14***	***1.16***	±	***0.26***	***1.03***	±	***0.16***	***2.12***	±	***0.63***	***0.66***	±	***0.17***	***1.73***	±	***0.50***			
DHZR	***0.08***	±	***0.01***	***0.04***	±	***0.01***	***1.07***	±	***0.42***	***0.14***	±	***0.05***	***0.08***	±	***0.01***	***0.04***	±	***0.02***			
cZ	***1.24***	±	***0.35***	***0.92***	±	***0.14***	***1.06***	±	***0.30***	***1.19***	±	***0.20***	***1.47***	±	***0.52***	***1.94***	±	***0.50***			
cZR	***0.14***	±	***0.02***	***0.38***	±	***0.07***	***0.38***	±	***0.08***	***0.32***	±	***0.04***	***0.36***	±	***0.09***	***0.39***	±	***0.06***			
cZOG	249.7	±	73.9	243.6	±	8.3	273.1	±	27.0	264.7	±	12.7	260.9	±	9.2	7363.2	±	7118.4			
cZ7G	0.67	±	0.16	8.25	±	4.35	54.74	±	4.30	46.61	±	10.64	42.83	±	13.79	57.46	±	5.66			
cZ9G	21.48	±	3.02	22.00	±	2.65	13.32	±	1.32	15.30	±	1.60	10.03	±	1.21	5.30	±	0.37			
cZROG	23.82	±	2.69	80.83	±	3.79	99.52	±	8.22	97.96	±	5.57	108.69	±	6.38	107.17	±	4.97			
IP	***0.93***	±	***0.21***	***0.98***	±	***0.12***	***0.55***	±	***0.12***	***0.65***	±	***0.11***	***2.07***	±	***0.68***	***1.36***	±	***0.42***			
IPR	***0.10***	±	***0.00***	***0.19***	±	***0.05***	***0.17***	±	***0.03***	***0.16***	±	***0.05***	***0.28***	±	***0.07***	***0.43***	±	***0.01***			

Table S7. (part 2)

CK-type	Cytokinins [pmol g FM^{-1}] in flowering i-ov*IPT* plants																	
	All leaves LAN treated																	
	LAN																	
	R-4-6			R-3			R-2			R-1			S+1-3			S+4-6		
tZ	***0.89***	±	***0.18***	***1.41***	±	***0.44***	***1.09***	±	***0.18***	***0.81***	±	***0.30***	***1.51***	±	***0.26***	***1.38***	±	***0.31***
tZR	***0.04***	±	***0.01***	***0.12***	±	***0.01***	***0.07***	±	***0.04***	***0.10***	±	***0.03***	***0.14***	±	***0.06***	***0.14***	±	***0.03***
tZOG	2.13	±	0.70	1.27	±	0.45	0.98	±	0.24	2.80	±	0.99	1.89	±	0.82	128.67	±	127.87
tZ7G	20.19	±	1.00	14.79	±	0.57	14.51	±	1.67	13.86	±	0.61	9.00	±	0.39	6.87	±	0.44
tZ9G	1.23	±	0.40	10.19	±	5.79	37.91	±	14.60	32.55	±	14.11	84.09	±	15.47	85.88	±	15.61
tZROG	15.95	±	1.85	14.49	±	2.22	17.00	±	1.55	17.12	±	2.28	16.04	±	3.91	17.18	±	3.33
DHZ	***0.36***	±	***0.06***	***1.28***	±	***0.48***	***2.22***	±	***0.67***	***1.39***	±	***0.41***	***0.61***	±	***0.21***	***1.56***	±	***0.42***
DHZR	***0.04***	±	***0.01***	***0.07***	±	***0.01***	***0.04***	±	***0.01***	***0.09***	±	***0.03***	***0.07***	±	***0.01***	***0.08***	±	***0.02***
cZ	***0.40***	±	***0.03***	***1.05***	±	***0.29***	***0.70***	±	***0.33***	***1.08***	±	***0.29***	***1.57***	±	***0.30***	***1.40***	±	***0.11***
cZR	***0.14***	±	***0.02***	***0.20***	±	***0.03***	***0.30***	±	***0.04***	***0.29***	±	***0.05***	***0.32***	±	***0.12***	***0.44***	±	***0.07***
cZOG	217.3	±	7.2	269.1	±	8.2	253.4	±	5.4	314.1	±	30.2	262.0	±	21.0	6054.8	±	5896.7
cZ7G	1.71	±	0.37	7.61	±	4.96	20.33	±	11.09	4.73	±	2.45	30.95	±	16.07	61.42	±	10.68
cZ9G	21.88	±	2.42	21.69	±	1.09	18.11	±	4.07	23.57	±	4.27	10.94	±	0.64	5.48	±	1.22
cZROG	23.59	±	3.25	62.48	±	9.73	82.57	±	10.14	93.14	±	7.56	118.16	±	10.29	100.19	±	5.69
IP	***2.09***	±	***0.63***	***0.84***	±	***0.14***	***1.07***	±	***0.28***	***1.41***	±	***0.41***	***1.44***	±	***0.29***	***1.71***	±	***0.19***
IPR	***0.09***	±	***0.01***	***0.18***	±	***0.06***	***0.12***	±	***0.04***	***0.17***	±	***0.05***	***0.27***	±	***0.05***	***0.31***	±	***0.03***

Table includes CKs depicted in main Fig. 4 (bold; *cis*-zeatin, *c*Z; *cis*-zeatin riboside, *c*ZR; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, IP; isopentenyladenosine, IPR; *trans*-zeatin, *t*Z; *trans*-zeatin riboside, *t*ZR) and their glucosylated forms. Rosette leaves 4-6, R-4-6,rosette leaf 3, 2 and 1 with R-1 being the youngest and R-6 being the oldest, first 3 stem leaves 1-3 (S+1-3) and stem leaves 4-6 (S+4-6) of flowering plants. R-2 leaf was either treated with 5 µM dexamethasone and 1% DMSO in lanolin paste (DEX; red color) to increase levels of *t*Z-type CKs in the leaves or with 1% DMSO in lanolin as control (LAN). All other leaves were mock-treated. Plants were sprayed for two days with 1 mM methyl jasmonate. Mean values ± standard error are shown. N ≥ 4; FM, fresh mass.

Table S8. Cytokinin levels in different leaf classes of a flowering i-ov*IPT* *Nicotiana attenuata* plant with alternatingly dexamethasone treated and control leaves.

CK-type	Cytokinins [pmol g FM^{-1}] in flowering i-ov*IPT* plants in																					
	R-2						R-1						S+1						S+2			
	LAN			DEX			LAN			DEX			LAN			DEX			LAN			DEX		
tZ	***0.08***	±	***0.01***	***0.19***	±	***0.04***	***0.08***	±	***0.01***	***0.40***	±	***0.08***	***0.16***	±	***0.02***	***0.70***	±	***0.16***	***0.14***	±	***0.02***	***0.90***	±	***0.25***
tZR	***0.04***	±	***0.01***	***0.22***	±	***0.05***	***0.06***	±	***0.01***	***0.77***	±	***0.18***	***0.08***	±	***0.01***	***0.97***	±	***0.30***	***0.09***	±	***0.01***	***1.13***	±	***0.33***
tZOG	n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.		
tZ7G	5.81	±	0.57	7.12	±	0.74	3.77	±	0.25	6.19	±	0.61	3.04	±	0.16	5.26	±	0.43	2.82	±	0.16	5.27	±	0.64
tZ9G	n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.		
tZROG	6.11	±	0.68	7.56	±	0.75	5.16	±	0.40	12.50	±	1.72	4.52	±	0.37	11.97	±	1.48	4.72	±	0.41	14.19	±	2.75
DHZ	***0.03***	±	***0.00***	***0.03***	±	***0.00***	***n.d.***			***0.07***	±	***0.02***	***0.05***	±	***0.01***	***0.10***	±	***0.01***	***0.06***	±	***0.01***	***0.13***	±	***0.03***
DHZR	***0.02***	±	***0.00***	***0.19***	±	***0.07***	***0.02***	±	***0.00***	***0.50***	±	***0.12***	***0.03***	±	***0.00***	***0.57***	±	***0.16***	***0.03***	±	***0.01***	***0.68***	±	***0.18***
cZ	***0.16***	±	***0.03***	***0.12***	±	***0.02***	***0.18***	±	***0.02***	***0.19***	±	***0.03***	***0.27***	±	***0.04***	***0.23***	±	***0.04***	***0.31***	±	***0.04***	***0.31***	±	***0.05***
cZR	***0.07***	±	***0.00***	***0.09***	±	***0.01***	***0.09***	±	***0.01***	***0.11***	±	***0.01***	***0.10***	±	***0.01***	***0.13***	±	***0.01***	***0.10***	±	***0.01***	***0.13***	±	***0.01***
cZOG	n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.		
cZ7G	n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.		
cZ9G	4.10	±	0.45	3.51	±	0.63	2.72	±	0.26	2.81	±	0.47	1.30	±	0.21	1.60	±	0.33	1.75	±	0.29	1.51	±	0.16
cZROG	28.85	±	2.89	22.80	±	2.31	33.94	±	2.81	34.08	±	2.65	41.21	±	2.56	39.23	±	2.25	47.92	±	3.06	46.07	±	2.64
IP	***n.d.***			***n.d.***			***n.d.***			***n.d.***			***n.d.***			***n.d.***			***n.d.***			***n.d.***		
IPR	***0.25***	±	***0.03***	***0.22***	±	***0.02***	***0.35***	±	***0.04***	***0.37***	±	***0.04***	***0.48***	±	***0.04***	***0.48***	±	***0.05***	***0.45***	±	***0.04***	***0.57***	±	***0.05***

n.d. = not determined

Table includes CKs depicted in Fig. S9 (bold; *cis*-zeatin, *c*Z; *cis*-zeatin riboside, *c*ZR; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, IP; isopentenyladenosine, IPR; *trans*-zeatin, *t*Z; *trans*-zeatin riboside, *t*ZR) and their glucosylated forms. Rosette leaves R-2 and R-1 and stem leaves S+1 and S+2 of flowering plants. Leaves were either treated with 5 µM dexamethasone and 1% DMSO in lanolin paste (DEX; red color) to increase levels of *t*Z-type CKs in the leaves or with 1% DMSO in lanolin as control (LAN). Plants were sprayed for two days with 1 mM methyl jasmonate. Mean values ± standard error. N ≥ 14; FM, fresh mass.

Table S9. Statistical analysis of cytokinin levels in different leaf classes of a flowering i-ov*IPT Nicotiana attenuata* plant with alternatingly dexamethasone treated and control leaves by two-way ANOVAs.

	P-values derived from two-way ANOVA							
	IP	**IPR**	***t*Z***	***t*ZR***	***c*Z***	***c*ZR***	**DHZ***	**DHZR***
Leaf	n.d.	0.000	0.000	0.000	0.000	0.001	0.000	0.003
DEX-treatment	n.d.	0.325	0.000	0.000	0.198	0.000	0.000	0.000
Interaction Leaf * DEX- treatment	n.d.	0.292	0.372	0.175	0.904	0.981	0.084	0.575

*=log transformed
n.d.= not determined

Table includes the analysis for CKs shown in Fig. 1 and Tab. S8 (*cis*-zeatin, *c*Z; *cis*-zeatin riboside, *c*ZR; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, IP; isopentenyladenosine, IPR; *trans*-zeatin, *t*Z; *trans*-zeatin riboside, *t*ZR). The statistics analysed the effect of different leaf classes within a flowering plant (Leaf), the influence of the induction with dexamethasone (DEX-treatment), as well as their interaction.

Table S10. Cytokinin levels in wildtype and two transgenic *SAG-IPT4 Nicotiana attenuata* plants.

CK-type	Cytokinins [pmol g FM^{-1}] in youngest rosette leaf																	
	Wildtype						*SAG-IPT4*-1 (566)						*SAG-IPT4*-2 (558)					
	Control			*M. sexta*			Control			*M. sexta*			Control			*M. sexta*		
tZ	***0.676***	±	***0.073***	***0.539***	±	***0.128***	***1.833***	±	***0.644***	***1.080***	±	***0.222***	***0.572***	±	***0.116***	***0.687***	±	***0.097***
tZR	***0.237***	±	***0.044***	***0.107***	±	***0.027***	***1.454***	±	***0.601***	***0.625***	±	***0.178***	***0.269***	±	***0.065***	***0.208***	±	***0.044***
tZOG	0.246	±	0.055	0.390	±	0.180	0.767	±	0.402	0.801	±	0.104	0.312	±	0.091	0.922	±	0.174
tZ7G	6.970	±	0.575	5.572	±	0.567	9.662	±	0.641	10.761	±	0.252	6.400	±	0.415	6.957	±	0.692
tZ9G	0.533	±	0.088	0.380	±	0.078	0.849	±	0.239	1.060	±	0.090	0.913	±	0.099	1.131	±	0.163
tZROG	2.111	±	0.326	1.655	±	0.244	3.064	±	0.580	4.434	±	0.633	1.895	±	0.358	2.255	±	0.339
DHZ	***0.460***	±	***0.090***	***0.807***	±	***0.135***	***0.602***	±	***0.288***	***0.548***	±	***0.086***	***0.481***	±	***0.146***	***0.527***	±	***0.136***
DHZR	***0.077***	±	***0.017***	***0.063***	±	***0.019***	***0.246***	±	***0.133***	***0.127***	±	***0.037***	***0.040***	±	***0.007***	***0.071***	±	***0.013***
cZ	***1.226***	±	***0.422***	***1.964***	±	***0.264***	***1.828***	±	***0.477***	***1.975***	±	***0.356***	***2.310***	±	***0.444***	***2.227***	±	***0.596***
cZR	***0.572***	±	***0.040***	***1.018***	±	***0.201***	***0.525***	±	***0.110***	***0.847***	±	***0.104***	***0.418***	±	***0.057***	***1.086***	±	***0.218***
cZOG	1.527	±	0.165	1.726	±	0.192	1.520	±	0.268	1.385	±	0.342	1.331	±	0.134	1.374	±	0.120
cZ7G	34.586	±	2.169	31.522	±	0.863	29.777	±	1.788	34.390	±	2.369	27.155	±	0.793	28.632	±	1.768
cZ9G	0.260	±	0.122	0.484	±	0.064	0.783	±	0.118	0.651	±	0.211	1.052	±	0.039	1.000	±	0.103
cZROG	6.860	±	0.815	8.214	±	0.402	7.292	±	0.463	8.095	±	0.981	4.997	±	0.262	5.132	±	0.127
IP	***0.509***	±	***0.072***	***0.542***	±	***0.127***	***0.633***	±	***0.129***	***1.863***	±	***0.463***	***1.009***	±	***0.410***	***2.238***	±	***0.520***
IPR	***4.316***	±	***0.127***	***2.531***	±	***0.273***	***4.266***	±	***0.662***	***9.606***	±	***1.788***	***5.122***	±	***0.535***	***11.585***	±	***0.822***

Table includes CKs depicted in Fig. 5 (bold; *cis*-zeatin, *cZ*; *cis*-zeatin riboside, *cZR*; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, IP; isopentenyladenosine, IPR; *trans*-zeatin, *tZ*; *trans*-zeatin riboside, *tZR*) and their glucosylated forms. Plants were induced by 3 d *M. sexta* feeding or not induced (control). Mean values ± standard error. FM, fresh mass. N≥10.

Table S11. Statistical analysis of cytokinin levels in wildtype and two transgenic *SAG-IPT4 Nicotiana attenuata* plants by two-way ANOVAs.

	P-values derived from two-way ANOVA for *SAG-IPT4*-1 (566)								P-values derived from two-way ANOVA for *SAG-IPT4*-2 (558)							
	IP*	**IPR***	***t*Z***	***t*ZR***	***c*Z***	***c*ZR**	**DHZ**	**DHZR***	**IP**	**IPR**	***t*Z**	***t*ZR**	***c*Z**	***c*ZR**	**DHZ**	**DHZR**
Line	0.012	0.002	0.002	0.005	0.286	0.402	0.735	0.042	0.002	0.000	0.841	0.178	0.152	0.778	0.330	0.337
***M.s.* feeding**	0.058	0.500	0.103	0.077	0.128	0.008	0.403	0.331	0.079	0.000	0.917	0.058	0.475	0.002	0.145	0.566
Interaction Line * *M.s.*feeding	0.056	0.001	0.751	0.447	0.256	0.632	0.257	0.736	0.077	0.000	0.250	0.470	0.373	0.476	0.260	0.138

*=log transformed

Table includes the analysis for CKs shown in Fig. 5 and Tab. S10 (*cis*-zeatin, *c*Z; *cis*-zeatin riboside, *c*ZR; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, IP; isopentenyladenosine, IPR; *trans*-zeatin, *t*Z; *trans*-zeatin riboside, *t*ZR). The statistics analysed the effect of the two *SAG-IPT4* constructs compared to the wildtype (Line), the influence of being infested with *M. sexta* neonates for 3 d (*M.s.* feeding), as well as their interaction.

Table S12. Defense metabolites in WT and two transgenic *SAG-IPT4 Nicotiana attenuata* plants.

Defense metabolite	Wildtype				*SAG-IPT4*-1 (566)				*SAG-IPT4*-2 (558)			
	Control		*M. sexta* feeding		Control		*M. sexta* feeding		Control		*M. sexta* feeding	
CP [peak area * g FM^{-1}]	**799.64**	**± 90.73**	**963.73**	**± 73.80**	**481.54**	**± 38.69**	**2194.10**	**± 394.49**	582.85	± 53.18	918.77	± 74.38
DCS [peak area * g FM^{-1}]	**537.71**	**± 140.03**	**675.72**	**± 152.39**	**252.82**	**± 88.34**	**1852.56**	**± 438.58**	367.21	± 35.39	519.10	± 79.87
Nicotine [mg * g FM^{-1}]	**567.62**	**± 38.26**	**582.50**	**± 49.10**	**448.43**	**± 32.89**	**522.04**	**± 40.86**	473.35	± 23.81	476.17	± 33.77
TPI [nmol * mg Protein]	**1.95**	**± 0.32**	**3.51**	**± 0.26**	**1.26**	**± 0.16**	**5.97**	**± 0.58**	1.51	± 0.28	3.91	± 0.37

Table includes defense metabolites depicted in Fig. 5, S11 and S12 (bold) and results for line *SAG-IPT4-2*. CP: caffeoylputrescine, DCS: dicaffeoylspermidine, TPI: trypsin proteinase activity. Plants were induced by 3 d *Manduca sexta* feeding or not induced (control). Mean values ± standard error. FM, fresh mass. N≥10.

Table S13. Statistical analysis of defense metabolites in WT and transgenic *SAG-IPT4-2* *Nicotiana attenuata* plants by two-way ANOVAs.

	P-values derived from two-way ANOVA for *SAG-IPT4*-2 (558)			
	CP	DCS*	Nicotine*	TPI
Line	0.198	0.908	0.106	0.678
***M.s.* feeding**	0.004	0.646	0.655	0.000
Interaction Line **M.s.* feeding	0.306	0.941	0.968	0.256

*=log transformed

Table includes the analysis for plant defenses from WT and line *SAG-IPT4-2* shown in and Tab. S12 (caffeoylputrescine, CP; dicaffeoylspermidine, DCS; nicotine; trypsin proteinase activity, TPI). The statistics analysed the effect of the *SAG-IPT4* constructs compared to the wildtype (Line), the influence of being infested with *M. sexta* neonates for 3 d (*M.s.* feeding), as well as their interaction. Statistics for line *SAG-IPT4-1* in Fig. 5, S11, S12.

Table S14. Relative transcript levels in two *SAG-IPT4 Nicotiana attenuata* plants.

Gene	Relative transcript accumulation (actin as reference gene)															
	Wildtype						*SAG-IPT4*-1 (566)						*SAG-IPT4*-2 (558)			
	Control			*M. sexta*			Control			*M. sexta*			Control			*M. sexta*		
NaMyb8	**0.0013**	±	**0.0006**	**0.2840**	±	**0.1496**	**0.0016**	±	**0.0009**	**2.8614**	±	**0.8375**	0.0007	±	0.0002	0.9802	±	0.3901
NaAT1	**0.0067**	±	**0.0011**	**0.2336**	±	**0.0415**	**0.0263**	±	**0.0027**	**1.4953**	±	**0.2718**	0.0116	±	0.0014	0.4208	±	0.0801
NaDH29	**0.0015**	±	**0.0003**	**0.1885**	±	**0.0302**	**0.0054**	±	**0.0008**	**1.3763**	±	**0.2652**	0.0026	±	0.0003	0.4097	±	0.0797
NaCV86	**0.0013**	±	**0.0002**	**0.1713**	±	**0.0327**	**0.0068**	±	**0.0009**	**0.7661**	±	**0.1142**	0.0025	±	0.0004	0.3078	±	0.0477
AtIPT4	**n.d.**			**n.d.**			**0.0766**	±	**0.0262**	**2.7352**	±	**0.9834**	0.1133	±	0.0389	3.1253	±	1.0614

Table includes transcript levels shown in Fig. 5 and S11(bold). Transcript levels were normalized to *NaActin* as reference gene. Plants were induced by 3 d *Manduca sexta* feeding or not induced (control). Mean values ± standard error. FM, fresh mass. N≥10.

Table S15. Statistical analysis of relative transcript levels in *SAG-IPT4-2 Nicotiana attenuata* plants.

	P-values derived from two-way ANOVA for SAG-IPT2 (558)			
	NaMyb8*	***NaAT1****	***NaCV86****	***NaDH29***
Line	0.014	0.000	0.000	0.000
***M.s.* feeding**	0.000	0.000	0.000	0.000
Interaction Line **M.s.* feeding	0.919	0.889	0.874	0.958

*=log transformed

Table include the analysis for transcripts shown in Tab. S14. The statistics analysed the effect of the *SAG-IPT4* constructs compared to the wildtype (Line), the influence of being infested with *M. sexta* neonates for 3 d (*M.s.* feeding), as well as their interaction. Statistics for line *SAG-IPT4-1* in Fig. 5 and S11.

Table S16. Sequences of primers used for qPCR

Gene	Forward primer	Reverse primer
NaActin	5'ggtcgtaccaccggtattgtg3'	5'gtcaagacggagaatggcatg3'
NaMyb8	5'aacctcaagaaactcaggacatacaa3'	5'gatgaatgtgtgaccaaattttcc3'
NaAT1	5'tcacaaggttcacttgtggctctg 3'	5'gcatttgccttgagtttgcctagg3'
NaDH29	5'atcaactagccattagaatg3'	5'ccaaaaatgatttgcaaggtc3'
NaCV86	5'atcaaatagctgaagatgtc3'	5'ccaacaaagtagtgctgtact3'
NaTPI	5'ctcaggagatagtaaatatggctg3'	5'gcatctgcatgttccacattgct3'
AtIPT4	5'acgtggagtgccacatcacctt3'	5'gaatgtatgagttggatccaccg3'

Table S17. Cloning primers of *SAG-IPT4* construct used for generating *SAG-IPT4* lines

Gene	Forward primer	Reverse primer
SAG-IPT4	5'gcggcgtcatgaagtgtaatgacaaaatggttg3'	5'gcggcggagctctagttaagacttaaaaatcttttag3'

3.4 Manuscritp IV

***NaMYB8* regulates distinct, optimally distributed herbivore defense traits**

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Summary

When herbivores attack, plants specifically reconfigure their metabolism. Herbivory on the wild tobacco *Nicotiana attenuata* strongly induces the R2/R3 MYB transcriptional activator MYB8, which was reported to specifically regulate the accumulation of phenolamides (PAs). We discovered that transcriptional regulation of trypsin protease inhibitors (TPIs) and a threonine deaminase (TD) also depend on MYB8 expression. Induced distributions of PAs, TD and TPIs all meet predictions of optimal defense theory: their leaf concentrations increase with the fitness value and the probability of attack of the tissue. Therefore, we suggest that these defensive compounds have evolved to be co-regulated by MYB8.

Running Title: NaMYB8 as an "optimal defense" regulator

Keywords: MYB8, *Nicotiana attenuata*, *Manduca sexta*, trypsin protease inhibitor, threonine deaminase, phenolamides, optimal defense hypothesis, herbivory

As primary producers, plants have developed intricate strategies to defend themselves against herbivores, including chemical defenses that act as anti-digestives, toxins, or repellents, or attract predators and parasitoids of herbivores. These responses are often tailored to particular herbivores, who reveal themselves by the elicitors they secrete, and other feeding-associated traits. The tailoring of defense in response to herbivore elicitation helps plants to respond appropriately to different attackers, and to avoid fitness costs incurred by unnecessary production of defensive compounds (Baldwin 1998).

In the wild tobacco, *Nicotiana attenuata*, the recognition of fatty acid-amino acid conjugates, elicitors present in the oral secretions (OS) and regurgitant of its specialist herbivore *Manduca sexta* (Halitschke et al. 2001), induce the rapid accumulation of jasmonate hormones, which can activate the transcription of secondary regulators like MYB8 via transcription factors such as MYC2, to induce specific defense responses (Woldemariam et al. 2013). MYB8 was discovered as a homolog of the R2/R3-type MYB transcription factor NtMYBJS1 from cultivated tobacco (*Nicotiana tabacum*) and shown to regulate the accumulation of phenolamides (PAs) in BY-2 tobacco cell cultures in a JA-dependent manner (Galis et al. 2006). In *N. attenuata* MYB8 transcripts accumulate after herbivory, resulting in the transcription of genes related to phenolamide (PA) biosynthesis, in particular the three hydroxycinnamoyl-coenzyme A: polyamine transferases, AT1, DH29 and CV86 (Kaur et al. 2010; Onkokesung et al. 2012). Plants rendered deficient in MYB8 by RNA interference (RNAi, irMYB8) have drastically lower levels of PAs including caffeoylputrescine, but are similar to wild-type (WT) plants in their accumulation of other phenolic compounds, including rutin and chlorogenic acid. Kaur et al. (2010) showed that *M. sexta* and *Spodoptera littoralis* caterpillars grow faster on irMYB8 than on WT plants and that spraying caffeoylputrescine on irMYB8 plants reduces *M. sexta* growth, suggesting a defensive function of the PAs regulated by MYB8 (Kaur et al. 2010).

The microarray data presented by Kaur et al. (2010) also shows a reduction in trypsin proteinase inhibitor (TPI) transcripts, indicating that MYB8 might additionally regulate other plant defense responses. To follow up on that observation, we present experimental data that implicate MYB8 as a regulator of the within-plant distribution of multiple plant defense compounds. For detailed information about the experimental conditions see *Supplemental Material and Methods*.

To confirm the influence of MYB8 silencing on TPI transcript accumulation and activity, we conducted qPCR analysis (Fig. 1A) and a radial diffusion TPI activity assay (Fig. 1B, S1). Similar to the microarray results from Kaur et al. (2010), TPI transcript levels were attenuated to less than half of WT levels, as was basal and induced TPI activity. However,

hemizygous crosses with TPI-deficient plants indicate that the increased growth of *M. sexta* caterpillars on irMYB8 plants can be largely attributed to other MYB8-regulated factors, such as PAs, as proposed by Kaur et al. (2010) (Fig. S2). The dependence of TPI expression on MYB8 can elegantly explain the primed induction of PAs and TPIs but no other JA-mediated traits in *N. attenuata* plants that were oviposited by *Spodoptera exigua* (Bandoly et al. 2015).

To identify other potential MYB8-regulated herbivory responses we conducted a complete microarray analysis using a whole-transcriptome array. This comprehensive analysis confirmed the effect of MYB8 on genes related to PAs (Fig. 1 C, S3-S8). To identify further targets of MYB8 we conducted a promotor motif analysis for regions 2 kb upstream of genes. The identified motif (Fig. 1C) is similar to the motif for the tobacco homologue NtMYBJS1 identified by gel mobility shift assays (Galis et al. 2006). Forty out of sixty-one genes (65.6%) which were down-regulated in irMYB8 plants contained the promotor motif (Fig. 1 C, Table S2). We did not identify this motif in the 2kb region upstream of the TPI gene. The TPI gene contains repetitive regions (Wu et al. 2006) and thus the assembly of the gene sequence and upstream elements is less certain than for other target genes. Thus further investigation is required to determine whether the regulation of TPI by MYB8 is direct or indirect.

Instead we found that one of four threonine deaminase (TD) homologues had the motif located within 2kb upstream region and its transcripts were reduced in irMYB8 plants (Fig. 1D, S9). TDs catalyze the deamination of Thr to α-ketobutyrate, which is a precursor of Ile. TDs are thus required for accumulation of JA-Ile (Kang et al. 2006). TD activity is usually limited by substrate-level feedback, but Chen et al. (2005) described a JA-inducible TD isoform in *Solanum lycopersicum* that lacks its regulatory domain after proteolytic digestion and deaminates Thr in *M. sexta* guts, depriving larvae of this essential amino acid. Since irMYB8 plants were shown to be not impaired in JA-Ile formation (Kaur et al. 2010; Fig. S10) and the Thr and Ile levels were hardly affected in MYB8 silenced plants (Fig. S10), we propose an anti-nutritive function of the MYB8-regulated TD similar to that reported by Chen et al. (2005). This hypothesis is supported by an analysis of amino acids extracted from the guts content of *M. sexta* larvae, showing an increase in Thr as a molar percent of amino acids for larvae feeding on irMYB8 versus WT plants (Fig. 1E).

The data presented by Kaur et al. (2010) and Onkokesung et al. (2012) showed that the induced accumulation of PAs is specifically localized within the plant. The distribution follows patterns similar as described by the optimal defense hypothesis (ODH), which states that tissues which face a high attack risk and contribute the most to fitness should be most defended (McKey 1974). Recently, it was shown that cytokinins can regulate these PA distribution patterns,

likely via MYB8 (Schäfer et al. 2015; Brütting et al. 2017; Fig. 2B, C). TPI transcript levels were also associated with cytokinin content in leaves and had a distribution pattern similar to that of PAs (Schäfer et al. 2015; Brütting et al. 2017) Fig. 2D). Here, we show that TD2.1 transcripts also accumulate to higher levels in younger leaves than older leaves after jasmonate induction (Fig. 2E).

Steppuhn and Baldwin (2007) demonstrated that TPI activity induced compensatory feeding in *S. exigua*, increasing the susceptibility of larvae to nicotine-producing plants. MYB8 regulates not only the accumulation of compounds with a potential toxic effect (PAs, Kaur et al. 2010), but also one known, and one potential anti-nutritional defense (TPI, Zavala et al. 2004; TD, Chen et al. 2005 and Kang et al. 2006). This raises the question whether similar synergisms occur for MYB8-regulated defense responses. We hypothesize that TPI and TD activity increase the susceptibility of herbivores to PAs, or, alternatively, that the combination of TPI, TD and PAs acts to reduce protein availability for herbivores more than the summed effect of each individually.

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Author contribution:

Conceptualization: MS, CB, SX, AS, ITB and MCS; Methodology: MS, CB, SX, ZL, ITB and MCS; Software: SX and ZL; Validation: MS, CB and SX; Formal Analysis: MS, CB, SX and ZL; Investigation: MS, CB and MCS; Resources: SX, ZL and ITB; Data Curation: MS, CB, SX, ITB and MCS; Writing – Original Draft: MS, CB, SX, ITB and MCS; Writing – Review & Editing, MS, CB, SX, ZL, AS, ITB and MCS; Visualization: MS; Supervision: ITB and MCS; Project Administration: ITB and MCS; Funding Acquisition: ITB, SX and MCS.

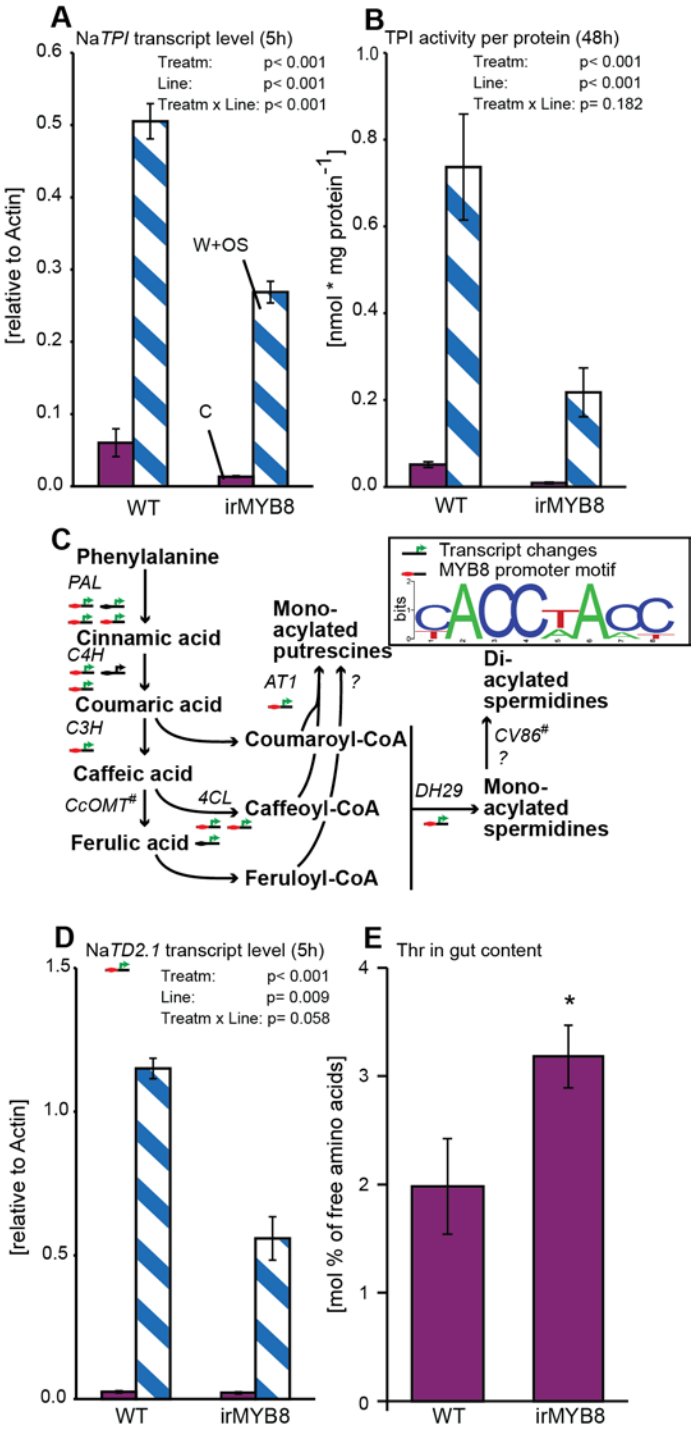

Figure 1: Trypsin protease inhibitor (Na*TPI*) transcripts and activity, and the herbivory-inducible threonine deaminase (TD) homolog Na*TD2.1* depend on *MYB8* expression

(**A**) Relative Na*TPI* transcript abundance in *Nicotiana attenuata* leaves 5 h after wounding and application of *Manduca sexta* oral secretions (W+OS) to the puncture wounding sites, as well as from untreated control plants (C). (**B**) TPI activity in *N. attenuata* leaves 48 h after W+OS treatment versus controls (C). (**C**) Model of the phenolamide pathway. Genes that are transcriptionally downregulated in MYB8 silenced plants (irMYB8) are marked with a green

arrow and those that contain the MYB8 specific promoter motive with a red dot. Different gene copies are indicated separately. Detailed transcript levels and promotor motif information are given in Fig. S3-S8 and Table S2. Genes that were not represented in the microarray are marked with a hash. (**D**) Transcript abundance of Na*TD2.1* in *N. attenuata* leaves 1 h after W+OS versus controls (C). (**E**) Thr level in the gut content of *M. sexta* caterpillars after 8 d feeding on WT or irMYB8 plants. The Thr level was expressed as proportion of the free amino acid content. All experiments were done with wild-type (WT) and MYB8 silenced plants (irMYB8).

(**A**, **B** and **C**) Line (WT, irMYB8) and treatment (C, W+OS) effects and their interactions were analyzed using univariate two-way ANOVAs. (**E**) Asterisks indicate significant differences among the WT and irMYB8 fed caterpillar (independent samples *t* test: * $p \leq 0.05$). Error bars are standard errors (A, D: N=3; B: N=5; E: N≥11).

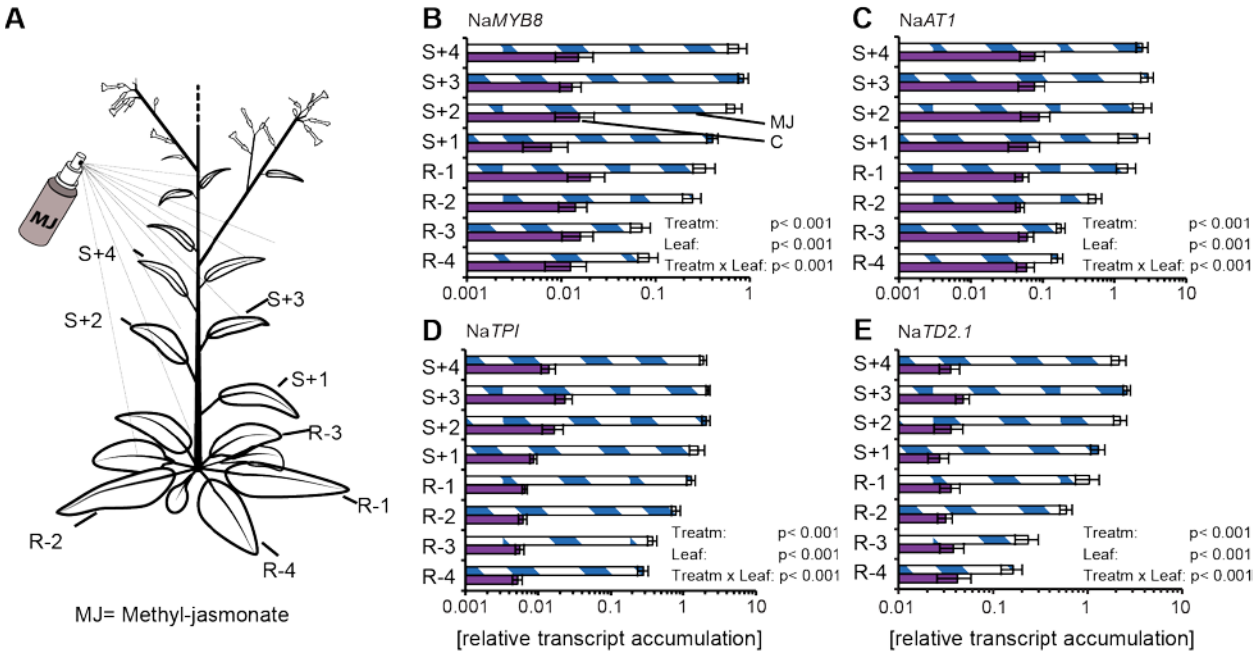

Figure 2: Transcripts of NaMYB8 and its target genes follow a within-plant distribution according to the optimal defense hypothesis

(**A**) Experimental design. Relative transcript abundance of Na*MYB8* (**B**), Na*AT1* (**C**), Na*TPI* (**D**) and Na*TD2.1* (**E**) in different leaf-classes of flowering *Nicotiana attenuata* plants: rosette leaves R-1 (youngest) to R-4 (oldest) and stem leaves S+1 (oldest) to S+4 (youngest). Plants were sprayed for two days with 1 mM methyl jasmonate (MJ) or water as control (C). **A-D** were modified from Brütting et al. (2017).

Leaf position and treatment (C, MJ) effects and their interactions were analyzed using univariate two-way ANOVAs. Error bars depict standard errors ($N \geq 5$). FM, fresh mass.

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TECHNICAL ADVANCE

'Real time' genetic manipulation: a new tool for ecological field studies

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SUMMARY

Field experiments with transgenic plants often reveal the functional significance of genetic traits that are important for the performance of the plants in their natural environments. Until now, only constitutive overexpression, ectopic expression and gene silencing methods have been used to analyze gene-related phenotypes in natural habitats. These methods do not allow sufficient control over gene expression for the study of ecological interactions in real time, of genetic traits that play essential roles in development, or of dose-dependent effects. We applied the sensitive dexamethasone (DEX)-inducible pOp6/LhGR expression system to the ecological model plant *Nicotiana attenuata* and established a lanolin-based DEX application method to facilitate ectopic gene expression and RNA interference-mediated gene silencing in the field and under challenging conditions (e.g. high temperature, wind and UV radiation). Fully established field-grown plants were used to silence phytoene desaturase and thereby cause photobleaching only in specific plant sectors, and to activate expression of the cytokinin (CK) biosynthesis gene isopentenyl transferase (*ipt*). We used *ipt* expression to analyze the role of CKs in both the glasshouse and the field to understand resistance to the native herbivore *Tupiocoris notatus*, which attacks plants at small spatial scales. By spatially restricting *ipt* expression and elevating CK levels in single leaves, damage by *T. notatus* increased, demonstrating the role of CKs in this plant–herbivore interaction at a small scale. As the arena of most ecological interactions is highly constrained in time and space, these tools will advance the genetic analysis of dynamic traits that matter for plant performance in nature.

Keywords: pOp6, LhGR, dexamethasone, fieldwork, *Nicotiana attenuata*, *Tupiocoris notatus*, cytokinin, *Manduca sexta*, herbivory, *pds*, technical advance.

INTRODUCTION

Experiments with transgenic plants in natural environments are often indispensable for ecological research, because the complex blend of abiotic and biotic factors can reveal plant phenotypes which might be absent under the coddled conditions of the laboratory and glasshouse (Izawa *et al.*, 2011; Baldwin, 2012; Kaur *et al.*, 2012; Dinh *et al.*, 2013). Until now the genetic tools for ecological field studies have been mainly restricted to the use of mutants and constitutive silencing or overexpression technologies. These techniques allow only functional analysis of genes that do not cause strong developmental defects, since these confound the analysis of traits that are important for ecological interactions with other organisms. Constitutive techniques also do not allow restricted fine-scale transcriptional regulation in specific plant tissues or developmental stages that is necessary to address basic questions about the spatial dynamics of herbivore feeding or to study season-specific interactions with herbivores. Additionally they complicate the work with plant traits whose ecological functions are dose dependent or are tightly regulated in time. Expression systems using tissue-, stress- or developmental-specific promoters (Potenza *et al.*, 2004; Moore *et al.*, 2006), like the stress- and ontogeny-regulated SARK (senescence-activated protein kinase) promoter and the

stress-inducible HVA22P promoter have been used in the field (Xiao *et al.*, 2009; Qin *et al.*, 2011), but are limited to specific tissues, stresses or developmental stages. Chemically inducible expression systems provide the flexibility required for studies of ecological interaction, since they allow immediate control over spatial, temporal and quantitative construct expression. To the best of our knowledge, such expression systems have only been used under controlled laboratory and glasshouse conditions in several plant species, and their use in ecological field research remains untested.

Various chemically inducible systems, which express the target construct only in the presence of specific compounds like estradiol, alcohol or dexamethasone (DEX), have been developed (Moore *et al.*, 2006; Corrado and Karali, 2009). There are several reports of conditional basal expression (the alc system; Salter *et al.*, 1998; Roslan *et al.*, 2001), chimeric patterns (the Cre/loxP recombination system; Guo *et al.*, 2003) and side-effects to the plant from the chemical elicitors (ethanol; Camargo *et al.*, 2007) or the activated expression system itself (the GVG system; Kang *et al.*, 1999). One of the most sensitive expression systems, which allows for the regulation of gene expression with minimal side-effects on the plant's physiology, is based on the DEX-inducible pOp6/LhGR system.

The pOp6/LhGR expression system was developed from the pOp/LhG4 system (Moore *et al.*, 1998) by Craft *et al.* (2005) and Samalova *et al.* (2005). The system comprises a constitutively expressed chimeric transcription factor (LhGR) containing a high-affinity DNA binding lac-repressor domain, a Gal4 transcription activator region and the ligand-binding domain of a glucocorticoid receptor. The target construct is under control of a minimal CaMV promoter downstream of an array of lac-operator repeats. In the presence of DEX, the transcription factor dissociates from heat shock proteins (Picard, 1993), binds to the lac-operator array and activates the otherwise inactive minimal CaMV promoter, leading to expression of the target construct.

The system was tested for heterologous gene expression as well as for RNA interference (RNAi)-mediated gene silencing (Craft *et al.*, 2005; Samalova *et al.*, 2005; Wielopolska *et al.*, 2005), for example to regulate the expression of the *Agrobacterium tumefaciens* isopentenyl transferases (*ipt*) coding gene *Tumor morphology root* (*Tmr*), which catalyzes the rate-limiting step in the biosynthesis of the cytokinin (CK) *trans*-zeatin (*tZ*; Heidekamp *et al.*, 1983; Craft *et al.*, 2005; Samalova *et al.*, 2005; Ueda *et al.*, 2012). Since minor CK changes already affect plant development (Medford *et al.*, 1989; Bohner and Gatz, 2001), *ipt* represents a sensitive visual marker for analyzing dose-dependent induction. Gene silencing was tested by silencing phytoene desaturase (*pds*), which is involved in carotenoid biosynthesis, resulting in visually observable photobleaching (Chamovitz *et al.*, 1993; Wielopolska *et al.*, 2005; Zhang *et al.*, 2010). In the traditional application methods, such as spraying or soil drenching (Aoyama and Chua, 1997; Samalova *et al.*, 2005), the use of this steroid-based system has been restricted to the controlled environment of a laboratory (Moore *et al.*, 2006; Corrado and Karali, 2009). The pOp6/LhGR system has found limited use for field studies, mostly due to the biological activity of DEX in humans (Walton, 1959) and other organisms (Miller *et al.*, 1994), as well as the high risk of contaminating the environment.

Nicotiana attenuata is intensively used for ecological field experiments (e.g. Kessler *et al.*, 2004, 2008; Allmann and Baldwin, 2010; Long *et al.*, 2010; Weinhold and Baldwin, 2011; Meldau *et al.*, 2012a; Schuman *et al.*, 2012). The extensive use of transgenic *N. attenuata* plants in their native habitat in the analysis of plant–animal and plant–microorganism interactions has made it one of the best-characterized model organisms for understanding the genetic traits responsible for ecological performance under natural conditions.

Many ecologically important plant traits, such as defense responses against specific herbivores, are regulated by temporal and tissue-specific changes in plant hormones (Erb *et al.*, 2012). Cytokinins, for example, mediate many developmental and stress-related processes (Werner and Schmülling, 2009). Yet the physiological responses to CKs are highly concentration-dependent and specific to different tissues and developmental stages. Unfettered manipulation of CKs induces severe developmental perturbations (Klee *et al.*, 1987), which may confound the analysis of their role in the interactions of plants with native herbivores. A field-applicable pOp6/LhGR system, with locally restricted dose-dependent CK manipulations, would allow for rigorous tests of many hypotheses about the roles of CKs in plant ecological interactions (Giron *et al.*, 2013).

Here we describe the application of the DEX-inducible pOp6/LhGR system for precisely controlled overexpression and gene silencing in *N. attenuata* and present an approach to DEX application that can be used in the field.

RESULTS

Establishment of the pOp6/LhGR system in *N. attenuata*

We tested the pOp6/LhGR system for field experiments in *N. attenuata* using two different pOp6 constructs (Figure 1). The i-ir*pds* expresses a construct for RNAi-mediated gene silencing (Wesley *et al.*, 2001) of the *N. attenuata pds*, leading to visible photobleaching, whereas the i-ov*ipt* line ectopically expresses an *ipt* from *A. tumefaciens* (Heidekamp *et al.*, 1983), leading to higher levels of CKs (Figure 1). Both lines contain a specific pOp6 construct (pOp6ir*pds* for i-ir*pds* and pOp6*ipt* for i-ov*ipt*), as well as the LhGR construct (Figure 1 and Figure S1 in Supporting Information), which were combined by crossing plants

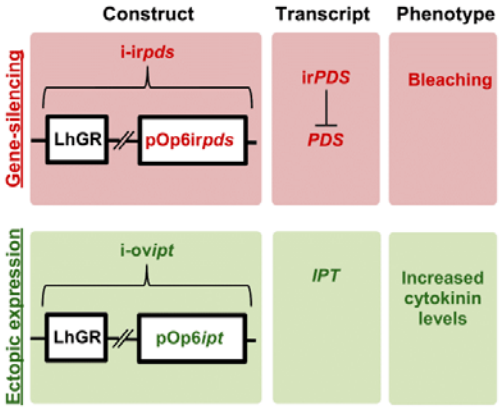

Figure 1. The pOp6/LhGR system for inducible construct expression. Constructs used for ectopic expression and gene silencing, their regulated transcripts and the expected phenotype. The color code (red, gene silencing; green, ectopic expression) is used consistently for all figures in the online version. 'ir' stands for inverted repeat, 'pds' for phytoene desaturase and 'ipt' for isopentenyl tranferase.

homozygous for each of the individual constructs. All pOp6 and LhGR lines were independently screened (Figure S2) before the crossings. Separate screening allowed us to combine the most promising lines for both constructs, reuse the screened LhGR line and to combine the pOp6 constructs with inducer lines with tissue- or developmental-specific expression in the future (Moore *et al.*, 2006). Hemizygosity also reduces possible insertion site effects by maintaining one wild-type (WT) allele. We modified the screening procedure optimized for *N. attenuata* (Figure S2; Gase *et al.*, 2011) because of the high levels of *hptII* (Figure S3) silencing, which thwarted efficient hygromycin-based screening. After crossing the pOp6 with homozygous LhGR plants, we visually screened the resulting seedlings on agar plates containing DEX for photobleaching and CK over-accumulation phenotypes (Figure S4). The pOp6 and LhGR lines were selected by choosing the transformed lines that showed the highest inducibility. An example of line optimization is shown for LhGR in Figure S5 (line 92 was chosen for final crosses). Photobleaching in *pds*-silenced plants is only achieved in lines with high silencing efficiency. This allows efficient screening of functional LhGR lines by analyzing crosses with the corresponding i-ir*pds* lines. Lines with insufficient phenotypes in the presence of DEX or with phenotypic changes in the absence of DEX were excluded. The final selected lines exhibit highly DEX-inducible seedling phenotypes, but are phenotypically normal in the absence of DEX (Figure S4).

The DEX application procedure

We next established a DEX treatment procedure for mature plants for use under field conditions. We first tested the stability of DEX under conditions relevant for studies in the plant's natural environment (Table S1; Dinh *et al.*, 2013). To test the effect of elevated temperature, a DEX-containing MeOH solution was incubated at 37°C for several days in the dark. Even after 6 days at 37°C only a small proportion of DEX was degraded (11% degradation; Figure S6a). However, when exposed for 6 days to the light environment of the glasshouse at 22°C, we found strong DEX degradation (63% degradation; Figure S6b), suggesting that DEX is sensitive to light. Since our field site in the Great Basin Desert (UT, USA) is characterized by light irradiation (Dinh *et al.*, 2013) that is much higher than in the glasshouse, the stability of DEX was considered as a limiting factor under these conditions. Therefore DEX should be applied to areas of the plant that are protected from direct sunlight and repeatedly applied for long-term treatments. Spray application is not a useful field technique because wind increases the risks of DEX exposure to both the environment and the researcher. Lanolin is commonly used as a matrix for applying lipophilic substances to *N. attenuata* (Baldwin, 1996; Kessler and Baldwin, 2001; Meldau *et al.*, 2011; Kallenbach *et al.*, 2012). When the test tubes containing the DEX–MeOH solutions were covered with a thin layer of lanolin (about 1 mm), the degradation of DEX in the light environment of the glasshouse was significantly reduced (45% degradation; Figure S6b). Thus lanolin can help to increase the stability of DEX in high-light environments. To dissolve substances in lanolin, it is usually liquefied at 60°C and then mixed with the compound of interest. We tested whether heating leads to the breakdown of DEX. Since we were not able to purify DEX from lanolin, we analyzed its stability when dissolved in MeOH. Our results show that short exposures of DEX to 60°C did not affect its stability (Figure S6c). Therefore it is unlikely that degradation of DEX occurs during dissolution in lanolin. However, we cannot rule out that DEX has different stabilities in lanolin compared with in MeOH. To dissolve the DEX in lanolin, we used DMSO as the primary solvent. This is expected to improve absorption by the plant tissue (Williams and Barry, 2012), and DMSO has minimal side-effects on plant physiology at low doses when compared with other solvents such as ethanol (Samalova *et al.*, 2005; Robison *et al.*, 2006). Since we mainly manipulated the gene expression in leaves, most experiments described here involved the application of a thin layer of DEX-containing lanolin paste to the lower side of the petiole of the targeted leaves (Figure S7).

The pOp6/LhGR system under glasshouse conditions

Before performing experiments in the field, we tested the system under glasshouse conditions. To quantify DEX-induced silencing, we measured ir*PDS* and *PDS* transcript accumulations, while for DEX-induced heterologous expression we analyzed *IPT* transcript accumulation and

CK levels. The i-ir*pds* plants showed 10-fold increases in ir*PDS* transcripts 12 h after treatment with 100 μM DEX (Figure 2a) and this increased to more than 100-fold 1 day after DEX application (Figure S8a). Accumulation of the *PDS* transcript was only reduced by approximately 10% at both time points (Figures 2a and S8a). The i-ov*ipt* plants increased *IPT* transcript abundance 250-fold after 12 h, leading to increases of more than 15-fold in *tZ* levels (Figure 2d). After 1 day, *IPT* transcripts increased more than 1000-fold and *tZ* by nearly 35-fold. Three days after treatment of a single petiole with 100 μM DEX, the bleaching in i-ir*pds* plants and growth effects due to CK overproduction in i-ov*ipt* plants were clearly visible (Figure S8b,d). The corresponding changes in transcript and metabolite level were found to be similar in the basal and apical part and the midvein of the leaves (Figure 2b,e). Three days after DEX treatment, the ir*PDS* transcripts accumulated 10–40-fold in i-ir*pds* plants compared with control levels, leading to reductions of 60–80% in *PDS* transcript levels (Figure 2b). Even if the photobleaching was only visible in newly established leaf tissue, completely green leaf parts showed strong *pds* silencing (apical parts in Figure 2b; compare Ruiz *et al.*, 1998). The *IPT* transcript abundance increased 4000-fold 3 days after DEX application, leading to increases in *tZ* levels of 60–200 fold (Figure 2e). The application of different DEX concentrations showed a strong dose-dependent response with a high dynamic range (1–100 μM DEX) for gene silencing and for ectopic expression (Figure 2c,f,g). In addition to the clearly observable growth phenotypes (Figure 2c,f), *tZ*-type CKs increased 5–15-, 15–30-, 45–100- or 250–2500-fold 12 days after the application of 1, 5, 20 or 100 μM DEX, respectively (Figure 2g). We also observed that treatment of single leaves did not affect adjacent leaves, indicating that the pOp6/LhGR system can be used to manipulate spatially restricted responses in plants (Figure S9). To test if the DEX system can also be used to manipulate responses in roots, plants were grown in hydroponic cultures and DEX was applied directly to the hydroponic solutions. The induction of i-ir*pds* plants by treating roots with DEX induced bleaching in the leaves (Figure S10a). Treatment of i-ov*ipt* plants with DEX through the roots reduced root growth and changed the growth of the shoots (Figure S10b,c).

The pOp6/LhGR system under field conditions

After establishing the pOp6/LhGR system under glasshouse conditions, we tested its utility for fieldwork. When i-ir*pds* plants were treated with 100 μM DEX in the field, the first signs of photobleaching were visible after 3 days (Figure 3a), leading to strongly bleached plants after 2 weeks (Figure S11). We tested if gene expression in single branches of pOp6/LhGR lines could be silenced without affecting the other branches. After decapitation, plants developed several equally sized side branches, which were treated individually with different DEX concentrations (Figure S12a). The application of DEX to side branches of LhGR plants did not influence plant growth (Figure S12b, c), indicating minimal side-effects of the DEX treatment under field conditions. Notably, treatments with 0.5 μM DEX were sufficient to induce visible photobleaching in the field (Figure 3b). The application of 0, 5, 20 or 100 μM DEX to different side branches of i-ir*pds* plants induced strong concentration-dependent photobleaching (Figure 3c). The ir*PDS* and *PDS* transcript abundances mirrored the visual photobleaching patterns (Figure 3d). Twelve days of treatment with 100 μM DEX induced 2000-fold induction of ir*PDS* transcript abundance and more than 80% *pds* silencing (Figure 3d). No photobleaching was observed on the control branch which was directly adjacent to the branch treated with 100 μM DEX (Figure 3c). Single treatments of complete plants also showed reliable, dose-dependent photobleaching phenotypes (Figure 3e). The observed visible bleaching was also correlated with a strong decrease in chlorophyll content, as expected for *pds* silencing (Figure S13; Qin *et al.*, 2007). To evaluate the risks of contamination for surrounding plants, adjacent untreated plants and control plants were also monitored, but no signs of cross-contaminations were found.

We also analyzed if DEX treatments of different branches of i-ov*ipt* plants led to branch-specific changes in the levels of CKs or CK-related phenotypes. Different side branches were treated with 0, 0.05 and 0.1 μM DEX-containing lanolin paste, which was refreshed every 3 days. The treatment increased *tZ* levels only in DEX-treated branches but not after lanolin treatments (Figure 4a). Previous work has shown that external application of CKs to poplar leaves increases wound-induced levels of oxylipins (Dervinis *et al.*, 2010). We found wound-induced levels of the oxylipin 12-oxo-phytodienoic acid (OPDA) to be significantly higher in leaves of branches treated with DEX, compared with the controls (Figure 4b).

Using the pOp6/LhGR system to study plant–herbivore interactions

Since we were able to subtly increase CK levels in specific plant tissues, we analyzed if this influences the resistance of the plant to native herbivores, as has been suggested (Smigocki *et al.*, 1993, 2000; Mujer and Smigocki, 2001; Dervinis *et al.*, 2010; Erb *et al.*, 2012; Meldau *et al.*, 2012b; Giron *et al.*, 2013). We quantified herbivore damage to lanolin- and DEX-treated branches of i-ov*ipt* plants. We found that one of the most abundant herbivores in the field, the mirid bug *Tupiocoris notatus,* caused significantly more damage to the DEX-treated side branches of i-ov*ipt* plants than branches treated with lanolin (Figure 4c). The growth of the analyzed i-ov*ipt* plants was not altered by these gentle increases in CK levels (Figure S14), excluding possible

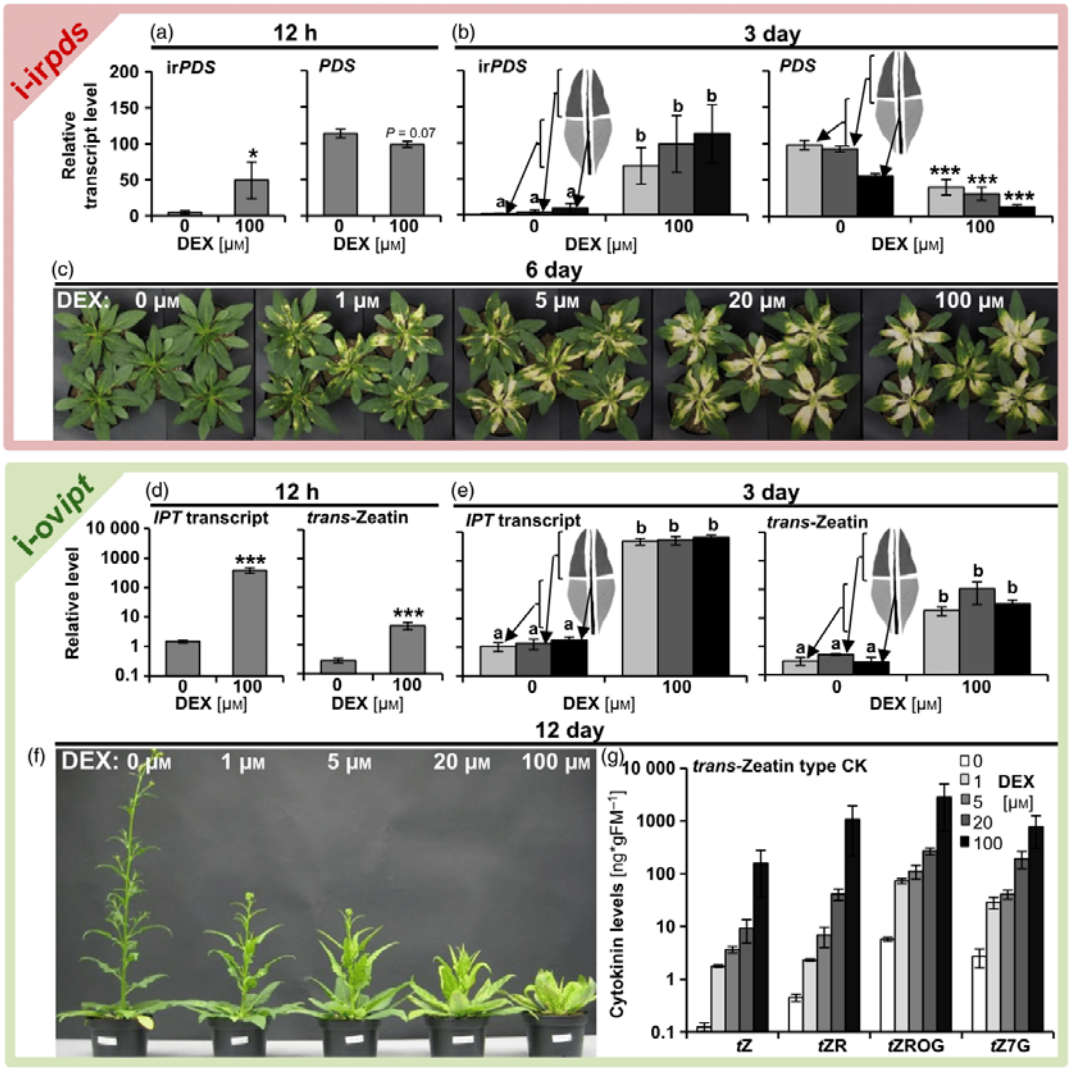

Figure 2. pOp6/LhGR system in *Nicotiana attenuata* under glasshouse conditions.
(a) Inverted repeat *PDS* (ir*PDS*) and *PDS* transcript level in i-ir*pds* plants 12 h after application of lanolin paste containing dexamethasone (DEX) to the midvein.
(b) The ir*PDS* and *PDS* transcript levels in the basal and apical parts of the leaf lamina, as well as in the midvein of i-ir*pds* plants 3 days after application of lanolin paste containing DEX.
(c) The i-ir*pds* plants 6 days after application of lanolin paste containing DEX.
(d) *IPT* transcript and *trans*-zeatin [ng per g fresh mass (g FM^{-1})] levels in i-ov*ipt* plants 12 h after application of lanolin paste containing DEX to the midvein.
(e) *IPT* transcript and *trans*-zeatin (ng gFM^{-1}) levels in the basal and apical parts of the leaf lamina, as well as in the midvein of i-ov*ipt* plants 3 days after application of lanolin paste containing DEX.
(f) i-ov*ipt* plants 12 days after application of lanolin paste containing DEX.
(g) *trans*-Zeatin (*tZ*), *trans*-zeatin riboside (*tZR*), *trans*-zeatin riboside *O*-glycoside (*tZROG*) and *trans*-zeatin 7-glycoside (*tZ7G*) levels in i-ov*ipt* plants after 12 days of treatment with lanolin paste containing different concentrations of DEX.
Lanolin paste was applied every 3 days. Asterisks indicate significant differences between DEX-treated samples and the corresponding control (0 µM) (independent samples *t*-test: $*P < 0.05$; $***P < 0.001$). ir*PDS*, *IPT* transcript and *trans*-zeatin data, 12 h after DEX treatment were $\log_{10}$-transformed before *t*-test analysis. Small letters indicate significant differences between samples (one-way ANOVA, Turkey honestly significant difference, $P < 0.05$). Error bars show standard errors (a, b, $n = 6$; d, $n = 5$; e, g, $n \geq 3$). FM, fresh mass.

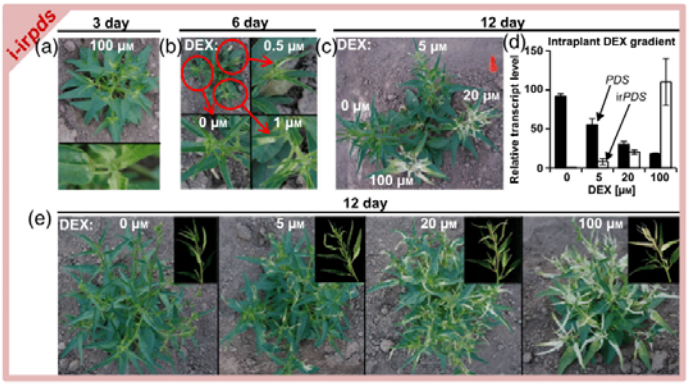

Figure 3. Inducible gene-silencing in *Nicotiana attenuata* in its native habitat.
(a) i-ir*pds* plants 3 days after application of lanolin paste containing dexamethasone (DEX).
(b) i-ir*pds* plants 6 days after application of lanolin paste containing DEX on different side branches of the same plant.
(c) i-ir*pds* plant 12 days after application of lanolin paste containing DEX on different side branches of the same plant.
(d) *PDS* (solid bars) and inverted repeat *PDS* (ir*PDS*, open bars) transcript levels in leaves of side branches of i-ir*pds* plants treated with lanolin paste containing DEX.
(e) i-ir*pds* plants 12 days after a single application of lanolin paste containing DEX.
Lanolin paste was applied every 3 days. Experiments were performed in the Great Basin Desert, UT, USA. Error bars show standard errors ($n = 6$).

developmental perturbations as an explanation for the observed increase in susceptibility to mirids. To examine potential direct effects of DEX on plant susceptibility, we measured levels of leaf damage on LhGR plants treated with high concentrations (up to 20 µM) of DEX and found no difference in mirid damage (Figure S15).

We repeated the experiment under glasshouse conditions, using a highly accurate method for quantifying mirid damage (Figure S16). We compared the *T. notatus*-inflicted leaf damage between DEX-treated and control i-ov*ipt* plants in a paired design (Figure 4d). Additionally, a within-plant leaf choice assay was conducted by exposing i-ov*ipt* plants with alternating DEX-treated and control leaves to *T. notatus* (Figure 4e). In both experiments, the DEX-treated leaves with elevated CK levels showed greater damaged by *T. notatus* (Figure 4d,e). Interestingly, the interaction between treatment and leaf position significantly affected the mirid damage in the between-plant choice assays, indicating that the effects of DEX and/or cytokinins on mirid damage are dependent on leaf position (Figure 4d).

We also tested whether DEX itself influences the performance of the specialist lepidopteran herbivore *Manduca sexta* and did not find significant effects (Figure S17).

DISCUSSION

Genetic tools for ecological research

In addition to constitutive and stress-, tissue- or developmentally regulated manipulations, chemically inducible expression technologies are increasingly being used in basic research as well as agriculture (Corrado and Karali, 2009). As pointed out by Corrado and Karali (2009), for studying molecular processes in plants, an inducible expression system should show fast, strong and concentration-dependent activity after induction but have insignificant activity in the absence of the inducer. The inducer should also have no pleiotropic effects and be applied in a flexible manner. In contrast, most techniques used in commercial and field-based applications have been selected by considerations of cost efficiency and lack of impact on the ecosystem, but lack the necessary precision required for molecular biology research. Here, we have developed a system that provides both the precision needed for surgical manipulation of gene expression and the robustness required for fieldwork.

Adaptation of the pOp6/LhGR system to ecological field research

The pOp6/LhGR system is one of the most popular inducible expression systems used in plant molecular biology, but its use has been restricted to the laboratory, since treatments with steroids require controlled environments (Moore *et al.*, 2006; Corrado and Karali, 2009). The usual application methods for DEX include incorporation into the growth medium and spraying, as well as watering with aqueous DEX solutions (Aoyama and Chua, 1997; Samalova *et al.*, 2005). Since DEX is a glucocorticoid known for its anti-inflammatory activities in humans (Walton, 1959), strategies for the application of DEX should avoid the

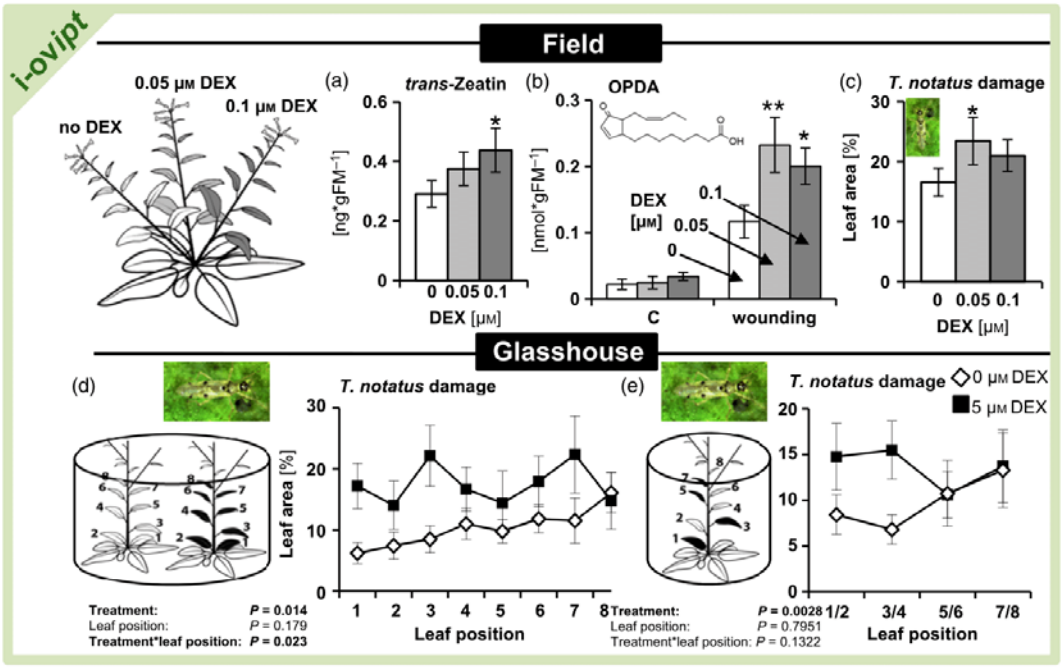

Figure 4. Tissue-specific elevated cytokinin levels increase *Tupiocoris notatus* damage in *Nicotiana attenuata* in its native habitat and in the glasshouse.
(a) *trans*-zeatin levels in leaves of different side branches of i-ov*ipt* plants 15 days after application of lanolin paste containing different concentrations of dexamethasone (DEX).
(b) The 12-oxo-phytodienoic acid (OPDA) levels in untreated control leaves and 60 min after wounding. Samples were taken from different side branches of i-ov*ipt* plants 15 days after application of lanolin paste containing different concentrations of DEX.
(c) Leaf damage caused by *T. notatus* on different side branches of i-ov*ipt* plants treated with lanolin paste containing different concentrations of DEX for 12 days.
(d) Between-plants choice assays. Leaf damage caused by *T. notatus* on i-ov*ipt* plants after a single application of lanolin paste containing either 0 (numbered white leaves) or 5 µM (numbered black leaves) DEX. Paired plants were exposed to *T. notatus* for 14 days.
(e) Within-plant choice assays. Leaf damage caused by *T. notatus* on i-ov*ipt* plants after a single alternating application of lanolin paste containing either 0 (numbered white leaves) or 5 µM (numbered black leaves) DEX. Plants were exposed to *T. notatus* for 14 days.
Lanolin paste was applied every 3 days. Experiments were performed under field conditions in the Great Basin Desert, UT, USA (a–c) or in transparent boxes under glasshouse conditions (d, e). Asterisks indicate significant differences between DEX-treated samples and the corresponding control (0 µM) (paired samples *t*-test: $*P < 0.05$; $**P < 0.01$). Damage by *T. notatus* under glasshouse conditions was analyzed by a mixed-effects model. Error bars show standard errors (a, $n = 8$; b, $n \geq 5$; c, $n \geq 15$; d, $n = 7$; e, $n = 10$). FM, fresh mass.

uncontrolled formation of aerosols as well as direct skin contact or ingestion. The influence of DEX on other study organisms inhabiting natural environments is also a consideration, as DEX can suppress the immune responses of insects like *M. sexta*, by inhibiting phospholipase A_2 and thereby eicosanoid biosynthesis (Miller *et al.*, 1994). Since field experiments are often done under unpredictable conditions with limited technical support, the method used to apply DEX should be simple and work under a variety of environmental conditions without posing risks to personnel and the environment.

The field site used in this study is located in the Great Basin Desert, UT, USA, which is a natural habitat for *N. attenuata* and is characterized by high light irradiation, high temperatures, drought and intense wind (Table S1; Dinh *et al.*, 2013), conditions which make the application of DEX by spraying or watering untenable. Many studies with *N. attenuata* have shown that lanolin can be used to apply substances to the plant (Baldwin, 1996; Kessler and Baldwin, 2001; Steppuhn *et al.*, 2004) in its natural environment. A lanolin-based application method for DEX was also mentioned by Borghi (2010), but no data were reported. We therefore applied DEX-containing lanolin paste to the plants, which prevents aerosol formation, remains locally restricted and is expected to protect DEX from light-mediated degradation (Figure S6b). Lanolin-based applications are also expected to continuously supply DEX to the plant, which ensures stable long-term effects. To reduce the exposure of herbivorous insects to DEX, we only applied a thin layer of DEX-containing lanolin paste to the lower side

of the petioles (Figure S7), which represents a shaded position and is less frequently visited by herbivores. This application also allows the DEX to access the leaf vasculature, leading to optimal distribution of DEX in the attached leaf. The application of DEX also did not affect the performance of *T. notatus* and *M. sexta* (Figures S15 and S17); however, control experiments should be performed when working with other herbivores. Since the DEX-treated plants were harvested at the end of the field season, DEX contamination of the environment is unlikely. Personal contact with DEX can be avoided by basic protection measures (protective clothes and gloves).

The efficiency of the pOp6/LhGR system in *N. attenuata*

We used the silencing of *pds* (i-ir*pds*) and the heterologous *ipt* expression (i-ov*ipt*) to evaluate the suitability of the pOp6/LhGR system for field studies. *pds* is highly expressed in leaf tissues and *pds* silencing results in the bleaching of newly developed above-ground tissues (Figures S8b and S11). Since the consequences of silencing are readily observable *in vivo,* it represents an ideal marker with which to track the effectiveness of silencing under field conditions. We used *ipt*-mediated changes in CK levels to test the sensitivity of the system, because even relatively small increases in *ipt* expression can lead to changes in plant development (Medford *et al.*, 1989; Bohner and Gatz, 2001). As such, *ipt* expression is a very sensitive marker for 'leaky' pOp6 activity. In addition to visual observations and transcript analysis, the CK levels of i-ov*ipt* plants were quantified by mass spectrometry.

In the absence of DEX, no physiological changes were observed in any of our selected lines, indicating negligible background activity, while the application of DEX induced transcript expression within hours (Figure 2a,d). Detailed analysis of different leaf parts revealed that petiole treatments are sufficient to regulate gene expression in the entire leaf (Figure 2b,e). The induced changes could be regulated in a temporal, spatial and quantitative manner and the dynamic range of the system was excellent (Figures 2, S8 and S9).

Phenotypes that are obtained in the field should be verified under controlled conditions in the glasshouse and the lab, and hence an inducible expression system should work with similar efficiencies under both conditions. Figure 3 shows that these requirements can be fulfilled. One major challenge for experiments with transgenic plants in natural habitats is that only a limited number of plants can be grown. When appropriate, eliciting the growth of equal-sized lateral branches by decapitation allows for branch-specific transcriptional manipulation (Figure S12a,b) and comparisons between DEX-induced and control side branches from the same plant (Figures 3b–d and 4a). In addition to reducing the number of replicate plants required, the use of the same individual plant for treatments and control also helps to compensate for environmental variation between the plants, as well as for insertion side-effects of the construct. Clearly, this approach should be used with caution when manipulating systemically transmitted traits.

The pOp6/LhGR system for studies of plant–herbivore interaction

Cytokinins are important targets for crop improvement as they influence plant traits such as leaf senescence (Richmond and Lang, 1957; Gan and Amasino, 1995), drought resistance (Werner *et al.*, 2010; Qin *et al.*, 2011) and resistance against pathogens (Choi *et al.*, 2010; Großkinsky *et al.*, 2011). The influence of CKs on traits important for resistance against insect herbivores in the natural environment remains unstudied. Constitutive *ipt* expression leads to abnormal development, which confounds the analysis of natural plant–herbivore interactions. Here we used the i-ov*ipt* plants to subtly elevate CK levels without affecting plant development and analyzed their role in resistance to natural herbivores in the field. We analyzed leaf damage after elevating CK levels in particular side branches of decapitated plants. As intended, only mild changes in CK levels were measured (Figure 4a). These subtly higher CK levels significantly increased the accumulation of jasmonate (JA) precursors after wounding (Figure 4b). Since the JA pathway is known to regulate the levels of defense metabolites involved in the resistance of *N. attenuata* to herbivores (Halitschke and Baldwin, 2003), increased CK levels in leaves were expected to correlate with an enhanced resistance to herbivores. However, the DEX treatment of the i-ov*ipt* plants increased the leaf damage by *T. notatus* in both the field and the glasshouse (Figure 4c,d,e). Since mirids are only slightly affected by most JA-mediated defense responses, except diterpene glycosides (Dinh *et al.*, 2013), increased OPDA levels might even induce metabolic changes that are favorable for mirids. Changes in OPDA were observed after mechanically wounding leaves, and it is not clear how far OPDA levels are affected by mirid feeding itself. The within-plant choice assays (Figure 4e) indicate that regulating CK levels are sufficient to shape the patterns of herbivore damage in a plant, which is an important aspect of ecological theories such as the optimal defense theory (Meldau *et al.*, 2012b). Since CKs regulate source–sink relationships (Kuiper, 1993; Ehness and Roitsch, 1997; Lara *et al.*, 2004), they likely enhance the nutritional quality of a tissue, which in turn attracts *T. notatus* feeding, resulting in higher levels of damage. Interestingly, Figure 4(d) indicates that the influence of changes in the CK levels on the mirid damage were dependent on leaf position, which might correlate with the inhomogeneous distribution of CKs between different plant parts (Hewett and Wareing, 1973; Ori *et al.*, 1999). Future experiments will reveal the mechanism behind

leaf-specific, CK-mediated plant susceptibility to insects and their contributions to herbivore resistance.

Potential future applications of the DEX-inducible pOp6/LhGR system

Since plants are the foundation for most food chains on the planet, the timing of their activities profoundly orchestrates most ecological interactions. Not surprisingly, many plant traits are not constitutively expressed throughout a plant's body, but are restricted to specific tissues or ontogenic stages and particular times. The expression of these traits is likely to reflect an evolutionary adaptation to the spatial and temporal activity patterns of interacting organisms (Figure 5). However, the tools available to ecologists for manipulating these interactions have been too hamfisted to disentangle these sophisticated environmental interactions. 'Real-time' genetic tools such as the pOp6/LhGR system allow for the manipulation of such conditionally expressed traits in ecologically relevant situations (Figure 5). Many traits important for interactions with pollinators, such as nectar production and scent emission, are only transiently expressed at particular stages of flower development. Using the DEX system to surgically manipulate these traits can prevent off-target effects in other tissues, which may confound interactions with non-target organisms.

The DEX system can also be applied to manipulate the spread of an induced defense, as its elicitation by herbivory. Analyzing the herbivore's feeding behavior on such plants will shed light on the role of complex spatio-temporal changes that are induced throughout a plant's body. This is especially valuable for the analysis of defense and signaling components whose constitutive manipulations do not yield viable plants (Meldau *et al.*, 2011).

The pOp6 lines also offer the possibility for crosses with other activator lines with stress-, tissue- or developmentally regulated promoters, generating various functional combinations (Moore *et al.*, 2006). This provides the possibility to broaden application to specific cell types, such as trichomes. Additionally the use of constitutive activators

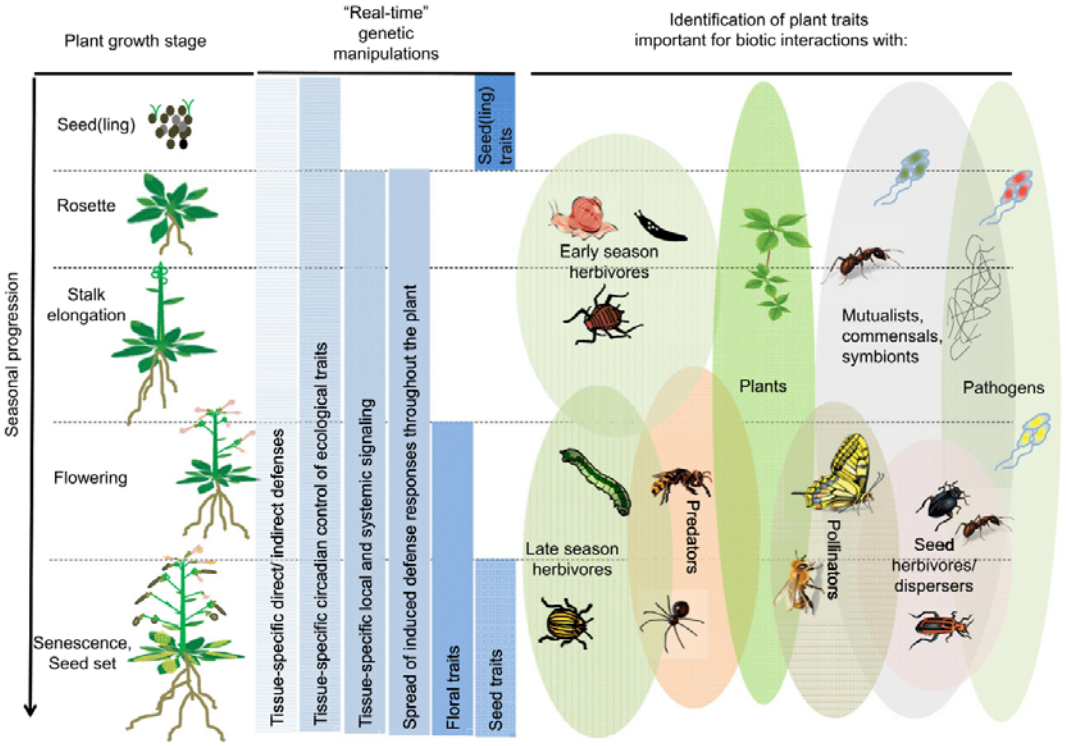

Figure 5. 'Real-time' genetic manipulations for studying plant ecology.
'Real-time' genetic manipulations can be used to study traits involved in biotic interactions of plants in different growth stages during seasonal progression. Black broken lines separate different plant growth stages. The shaded (blue) boxes illustrate the manipulation of tissue-specific target traits in different growth stages. Differentially colored ovals are examples of season-specific biotic interactions of plants.

(Moore *et al.*, 1998) might be beneficial for the analysis of tissues, such as roots, which are difficult to treat with DEX under field conditions.

The presented method could also be applied for field experiments with other transformable plant species like petunia (Kessler *et al.*, 2013), peanut (Qin *et al.*, 2011) or rice (Xiao *et al.*, 2009). Next-generation sequencing technologies allow us to gain the genetic information required for the preparation of species-specific RNAi constructs from many plant species. This genetic information is not required for DEX-mediated ectopic expressions. We hope that the possibilities offered by the pOp6/LhGR system will encourage researchers to develop transformation systems for non-model plant species.

Conclusion and outlook

Flexible control over gene expression is highly desirable for ecological experiments in natural environments. Due to its ease of use and the experimental flexibility, affording precise manipulation of gene expression, we predict that the DEX-pOp6/LhGR system described here will allow scientists to revisit hypotheses about the function of traits whose analyses were previously thwarted by the inflexibility of the available technologies.

EXPERIMENTAL PROCEDURES

Vector construction

Genomic DNA from transgenic tobacco plants harboring the *A. tumefaciens tmr* gene for isopentenyltransferase (IPT) under the control of the DEX-inducible LhGR/pOp6 promoter system was used as template to amplify the LhGR cassette and the pOp6-*ipt* cassette by PCR with primer pairs described in Table S3. After digestion with *Sal*I the LhGR fragment was cloned in vector pSOL8DC2 cut with *Sal*I and *Eco*RV, yielding pSOL9LHGRC (GenBank JX185747; Figure S1a). The pOp6-*tmr* cassette was cloned after digestion with *Sac*I and *Hin*dIII in the vector pVKH18 digested with the same enzymes, yielding pPOP6IPT (GenBank JX185749, Figure S1b). The pPOP6IRPDS vector (GeneBank JX185750; Figure S1c) was constructed by replacing the 0.7-kb *Sal*I-partial-*Bam*HI fragment of pPOP6IPT with a 0.3 kb inverted repeat of a part of the *N. attenuata pds* gene (GeneBank JX185751), separated by an intron.

The plants were kindly provided by Bretislav Brzobohaty (Masaryk University, Brno, Czech Republic) and the pVKH18 vector by Ian Moore (Oxford University, UK).

Plant transformation and growth

Plants from the 30th inbred generation of the inbred 'UT' line of *N. attenuata* (Torr. ex S. Wats.) were transformed with the vectors mentioned above. The transformation with *A. tumefaciens* (strain LBA 4404), seed germination and growth under glasshouse conditions were done as described by Krügel *et al.* (2002). Field experiments were conducted under the US Department of Agriculture Animal and Plant Health Inspection Service permission number 11-350-101r for the LhGR, i-ir*pds* and i-ov*ipt* plants. For field experiments, plants were grown as described by Schuman *et al.* (2012). The LhGR and i-ov*ipt* plants for herbivore damage analysis were grown in pairs on a field plot located at latitude 37.141 and longitude 114.027 (Figure S11).

Application of DEX

The DEX was dissolved in DMSO and diluted to 100 times the final concentration of the lanolin paste or 2000 times that of the GB5 medium and hydroponic medium, respectively. Aliquots were stored at −20°C. The final DEX concentration for GB5 medium was 20 μM and for hydroponic solution 1 μM. Lanolin was liquefied at 60°C, DEX was added, and after thorough mixing the lanolin paste was taken up by a syringe (1 ml, Omnifix), in which the lanolin solidifies after cooling. These syringes can be directly used for plant treatments. As control treatments, the plants were treated with the corresponding amount of DMSO in lanolin without DEX. For phenotypic analyses of seedlings, the seeds were germinated on GB5 medium containing DEX. Applications of DEX to the hydroponic medium were performed 1 week after plants were transferred to the pots. Lanolin treatments were performed at the earliest 3 weeks after germination, when plants were already transferred to pots. The time of treatments varied according to the experiment. If not stated otherwise, for glasshouse experiments plants were treated in the early rosette stage of growth. For *T. notatus* experiments and under field conditions, plants were treated during the early flowering stages. Side branches of decapitated plants were treated after exceeding lengths of 3–5 cm. If not stated otherwise, lanolin paste was applied to the lower side of a petiole (Figure S7). Depending on leaf size, between 10 and 30 μl of lanolin paste was applied per petiole. In contrast, for the short-time experiments (12 and 24 h) the entire midveins of the leaves were treated.

The DEX analysis

For DEX degradation experiments a 50 mM DEX stock solution in DMSO was dissolved in pure MeOH to a concentration of 20 μM DEX. Aliquots were placed in clear 1.5-ml glass vials (Machery Nagel, http://www.mn-net.com/) and were incubated at 37°C in the dark, in a 60°C water bath or under glasshouse conditions with or without a lanolin layer of about 1 mm covering the vial. Samples were taken at indicated time points. The samples were diluted 1:20 with MeOH and stored at −80°C. Before measurement, 200 ng of [9,10-^{2}H]dihydro-JA was added per sample as an internal standard for relative quantification. Samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a Varian 1200 Triple-Quadrupole-LC-MS system (Varian, http://www.varian.com/). Separation was done on a Kinetex C18 column (50 × 2.10 mm, 2.6 μm, 100 A; Phenomenex, http://www.phenomenex.com/). The mobile phase comprised solvent A (water, 0.05% HCOOH, 0.1% acetonitrile) and solvent B (MeOH) used in a gradient mode: 0–1 min, 95% A; 1–8 min 5–98% B in A; 8–15.5 min 98% B; 15.5–17 min, 2–95% A; 17–20 min, 95% A; with a flow of time/flow (ml min^{-1}): 0–0.5 min, 0.4 → 0.2 ml min^{-1}, 0.5–15.5 min, 0.2 ml min^{-1}, 15.5–16 min, 0.2 → 0.4 ml min^{-1}, 17–20 min, 0.4 ml min^{-1}. For detection, the mass spectrometer was operated in negative mode and multireaction monitoring (MRM) was performed to monitor analyte parent ion → product ion (Table S4). Varian MS Workstation Version 6.6 software was used for data acquisition and processing.

Cytokinin analysis

Cytokinins were analyzed by extraction of plant tissue in acidified aqueous MeOH followed by two solid-phase extraction (SPE) steps and subsequent measurement with LC-MS/MS. The

methodology was adapted according to Dobrev and Kaminek (2002) with the modifications by Kojima *et al.* (2009).

In brief, 100 mg of ground frozen plant tissue was extracted twice with 800 µl MeOH:H_2O:HCOOH (15:4:1) at −20°C. Labelled internal standards were supplemented in the first extraction step. Extraction and SPE were performed in 96-well BioTubes (1.1 ml individual tubes; Arctic White LLC, http://www.arcticwhiteusa.com/) and Nunc 96-well Deep Well Plates (Thermo Scientific, http://www.thermoscientific.com/). The first SPE step was performed on a Multi 96 HR-X column (96 × 25 mg) (Macherey-Nagel) conditioned with extraction buffer. The flow-through was collected and the MeOH was evaporated under constant nitrogen flow in an Evaporator system (Glas-Col, http://www.glascol.com/) at 42°C. After replenishment with 850 µl 1 M HCOOH, the samples were loaded on a Multi 96 HR-XC column (96 × 25 mg) (Macherey Nagel), conditioned with 1 M HCOOH. After washing with (i) 1 ml 1 M HCOOH, (ii) 1 ml MeOH and (iii) 1 ml 0.35 M NH_4OH, (iv) the CK-ribosides, free bases and glucosides were eluted with 1 ml 0.35 M NH_4OH in 60% MeOH. The SPE were performed using a Chromabond Multi 96 vacuum chamber (Macherey-Nagel). After evaporation, samples were reconstituted in 50 µl 0.1% acetic acid.

Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies, 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% formic acid) and solvent B (acetonitrile) with the following 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 min 95% A, with a flow rate of 1.1 ml min^{-1}. The column temperature was maintained at 25°C. The liquid chromatograph was coupled to an API 5000 tandem mass spectrometer (Applied Biosystems, http://www.appliedbiosystems.com/absite/us/en/home.html) equipped with a Turbospray ion source. For detection the mass spectrometer was operated in positive ionization mode (MRM modus) to monitor analyte parent ion → product ion (Table S5). Settings were as follows: ion spray voltage, 5500 eV; turbo gas temperature, 700°C; nebulizing gas, 70 p.s.i.; curtain gas, 25 p.s.i.; heating gas, 60 p.s.i.; collision gas, 6 p.s.i. 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*, *tZ*ROG and *tZ*7G were quantified by using deuterated internal standards (Table S5; Olchemim, http://www.olchemim.cz/).

The OPDA analysis

The OPDA was extracted and analyzed by LC-MS/MS as described by Kallenbach *et al.* (2010).

Chemicals

Methanol was purchased from Merck (http://www.merck.com/), HCOOH for ultra-performance LC by Fisher Scientific (http://www.fisher.co.uk/), otherwise by Riedel-de Haën (http://www.riedeldehaen.com/), DEX by Enzo Life Sciences (http://www.enzolifesciences.com/), lanolin, as well as methyl JA and DMSO, by Sigma-Aldrich (http://www.sigmaaldrich.com/), GB5 by Duchefa (http://www.duchefa-biochemie.nl/), acetic acid by Roth (http://www.carlroth.com/) and CK standards by Olchemim. The [9,10-^{2}H] dihydro-JA was synthesized by saponification and deuteration of methyl JA.

Transcript analysis

The RNA extraction was done with TRIZOL (Invitrogen, http://www.invitrogen.com/), according to the manufacturer's instructions. Complementary DNA was synthesized by reverse transcription using oligo(dT) primer and RevertAid reverse transcriptase (Invitrogen). Quantitative (q)PCR was performed using actin as standard on a Stratagene Mx3005P qPCR machine using a SYBR Green containing reaction mix (Eurogentec, http://www.eurogentec.com/; qPCR Core kit for SYBR Green I No ROX). The primer sequences are summarized in Table S2.

Statistics

Data were analyzed with SPSS STATISTICS 17.0. Either an independent sample *t*-test, paired samples *t*-test or one-way ANOVA followed by Tukey's honestly significant difference test were used as indicated. Use of data transformation is indicated. R 3.0.1 was used for statistical analysis of mirid-damage data obtained in the glasshouse experiments (Figure 4d,e). A mixed-effects model was applied with cage and plant as random factors and treatment (DEX application), leaf position and their interaction as fixed factors. The model was simplified by stepwise elimination of fixed factors. To achieve the influence of the different fixed factors, a maximum likelihood ratio test of the different models was done. Mirid damage data were arc sin square-root transformed.

Tupiocoris notatus damage

Tupiocoris notatus damage in the field was estimated as a percentage of the total leaf area. Glasshouse choice assays were carried out on matured *N. attenuata* plants with flowers removed. Plants were kept in 25 × 25 × 50 cm (length × width × height) sealed glass cages, with 50–100 *T. notatus* adults and older nymphs at the start of experiment. The *T. notatus* were obtained from an in-house colony. Damage was quantified after 14 days. The inter-plant choice assays were carried out with two plants per cage; one treated with 0 and the other with 5 µM DEX (Figure 4d). Damage to the first eight stem leaves was determined. The intra-plant choice assays were carried out with leaves alternately treated with 0 or 5 µM DEX. Odd-numbered leaves, beginning with the oldest stem leaf, were DEX induced, while even-numbered leaves served as controls (Figure 4e). To determine the rate of damage we took pictures of the leaves and used Adobe Photoshop CS5 (http://www.adobe.com/) for picture analysis. We quantified the area of each leaf and manually marked damaged parts and calculated the percentage of the damaged area. Leaf area was marked as damaged if the leaf showed clear signs of *T. notatus* feeding, such as locally bleached chlorotic spots or mirid frass (Figure S16).

Manduca sexta

For *M. sexta* performance, freshly hatched neonates were placed on early rosette-stage LhGR plants pre-treated for 1 day with 0 or 100 µM DEX. Lanolin paste was applied every 3 days. Caterpillar mass was measured at the indicated time points. The *M. sexta* larvae were obtained from in-house colonies.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

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Table S5. Multireaction-monitoring settings for cytokinin quantification in positive ionization mode.

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Supporting information

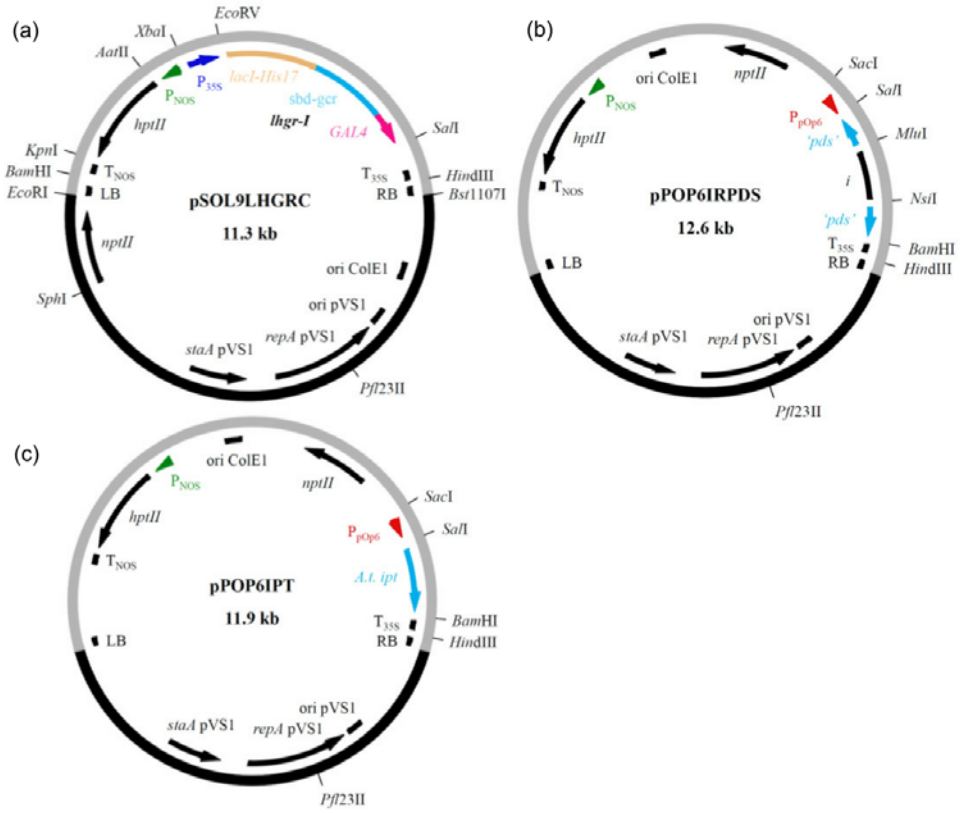


Figure S1. Plasmid vector overview.

Plasmid design of the LhGR (a, pSOL9LHGRC, GeneBank JX185747), pOp6ir*pds* (b, pPOP6IRPDS, GeneBank JX185750) and pOp6*ipt* (c, pPOP6IPT, GeneBank JX185749) constructs.

LB/RB, left/right border of T-DNA; P_{NOS}/T_{NOS}, promoter/terminator of the nopaline synthase gene from the Ti plasmid of *A. tumefaciens*; P_{35S}/T_{35S}, 35S promoter/

terminator from cauliflower mosaic virus; *hptII*, hygromycin phosphotransferase gene from pCAMBIA-1301 (AF234297); P_{pOp6}, six lac operators upstream of a minimal CaMV 35S promotor (-50 to +8); *lacI-His17*, high-affinity DNA binding lac repressor mutant (residues 1-330); sbd-ger, steroid-binding-domain (residues 508-795) of a glucocorticoid receptor from rat; *Gal4*, *Gal4* transcription-activator-domain-II from yeast (residues 768-881); i, intron 3 of Flaveria trinervia pdk gene for pyruvate, orthophosphate dikinase; 'pds', *N. attenuata* phytoene desaturase (positions 847-1195 in GeneBank JX185751); *A. t. ipt,* isopentenyl transferase of *A. tumifaciens;* nptII, aminoglycoside phosphotransferase class II; ori, origin of replication.

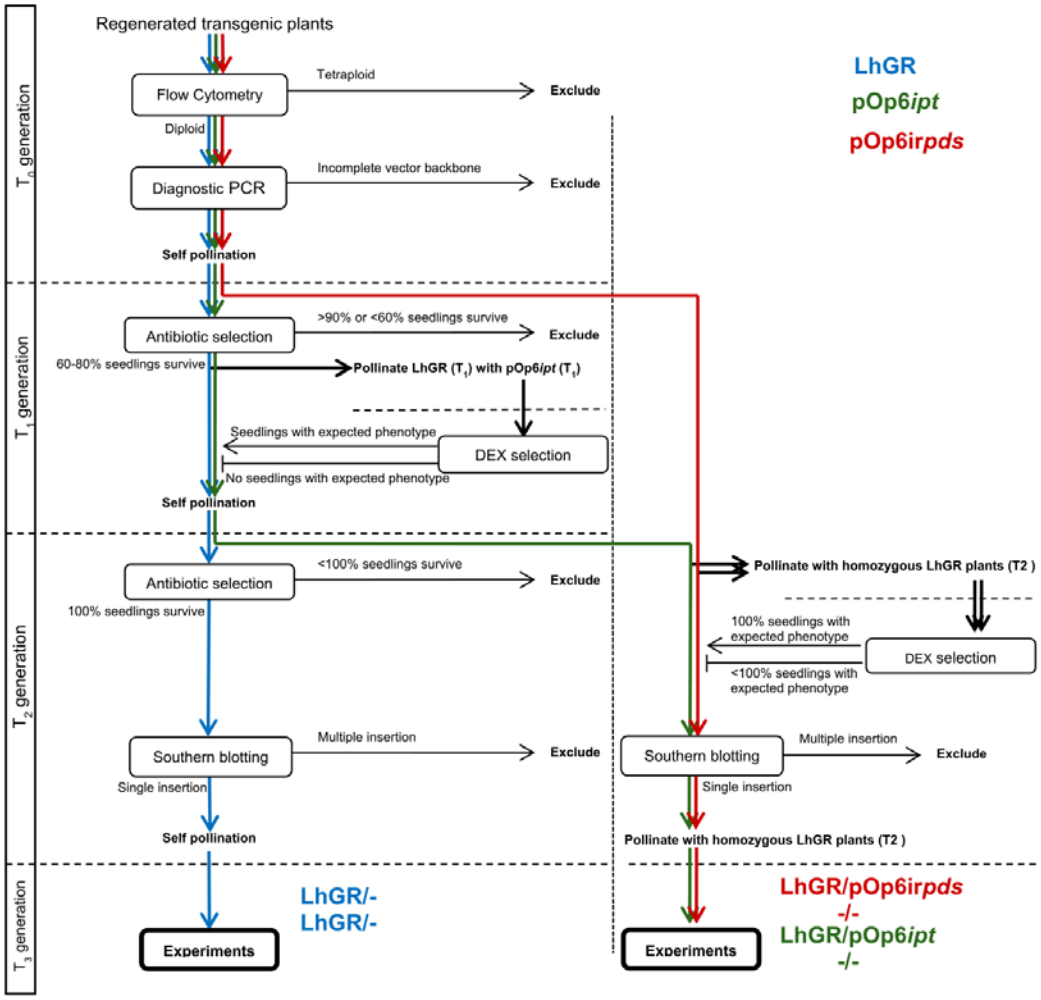


Figure S2. Screening overview.

Workflow for screening of the transgenic lines.

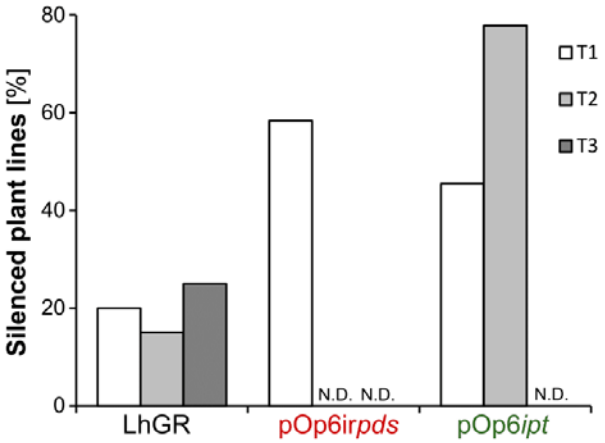


Figure S3. Hygromycin resistance silencing.

Ratio of lines with silenced *hptII* gene observed within the screening process. *HptII* silencing was detected by hygromycin sensitivity. T1 and T2 plants are considered as silenced if less than 60% of the seedlings were hygromycin resistant. Homozygous T3 were considered as silenced if hygromycin sensitive seedlings were observed. N.D., not determined.

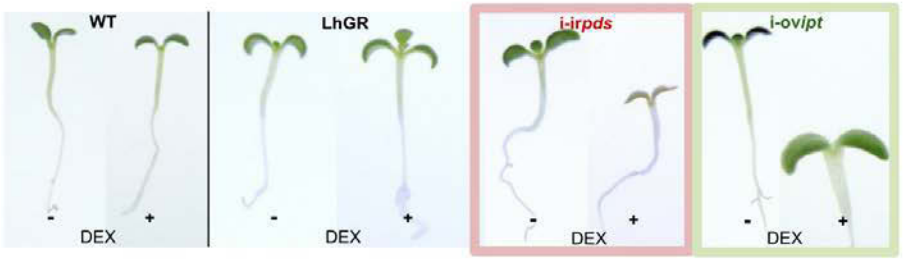
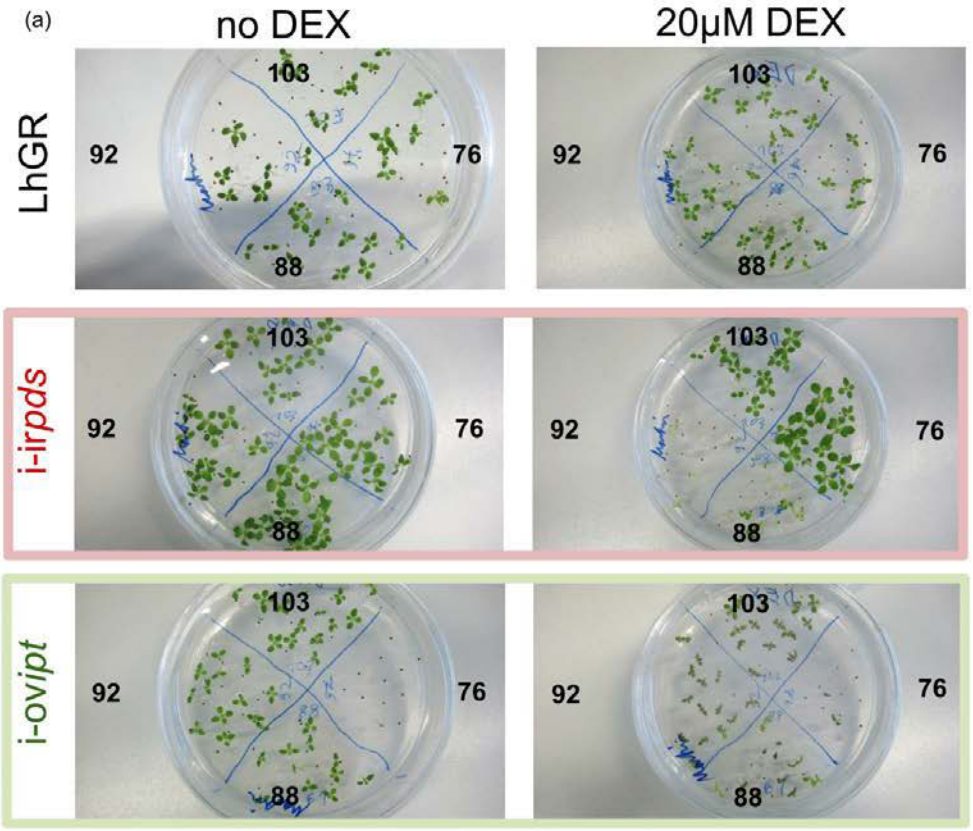


Figure S4. Seedling phenotype of i-ir*pds* and i-ov*ipt*.

Wildtype (WT), LhGR, i-ir*pds* and i-ov*ipt* seedlings two weeks after germination on 0 (-) or 20µM (+) DEX-containing GB5 plates.



(b)

	76	88	92	103
Effectivity (i-ir*pds*)	-	++	+++	+
Effectivity (i-ov*ipt*)	*germination problem*	+++	+++	+++

Figure S5. LhGR-efficiency screening.

(a) LhGR, i-ir*pds* and i-ov*ipt* seedlings two weeks after germination on 0 or 20µM DEX-containing GB5 plates. Different LhGR lines (76, 88, 92 and 103) were

compared before and after crossing with pOp6ir*pds* (-> i-ir*pds*) and pOp6*ipt* (-> i-ov*ipt*).

(b) Overview of the observed phenotypes. Expected phenotype: – absent, + weak, ++ medium, +++ strong.

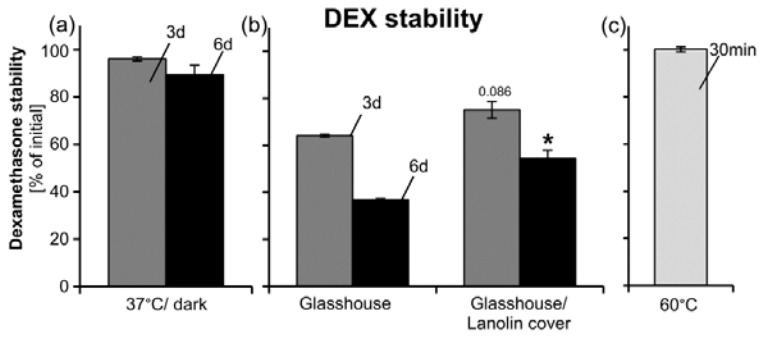


Figure S6. DEX stability under different temperature and light conditions.

Remaining DEX in a 100% methanol solution after indicated time

(a) in a dark 37°C temperature-chamber,

(b) under glasshouse conditions covered with or without a ~1mm lanolin layer or

(c) at 60°C.

Asterisks indicate significant differences between the same incubation times at different conditions. (independent samples t-test; *=p< 0.05). Error bars show standard errors (N=3). For additional statistical analysis, see text.

Figure S7. DEX application method.

Application of DEX-containing lanolin paste with a syringe to the petiole of a leaf.

Arrows indicate the lanolin paste after application.

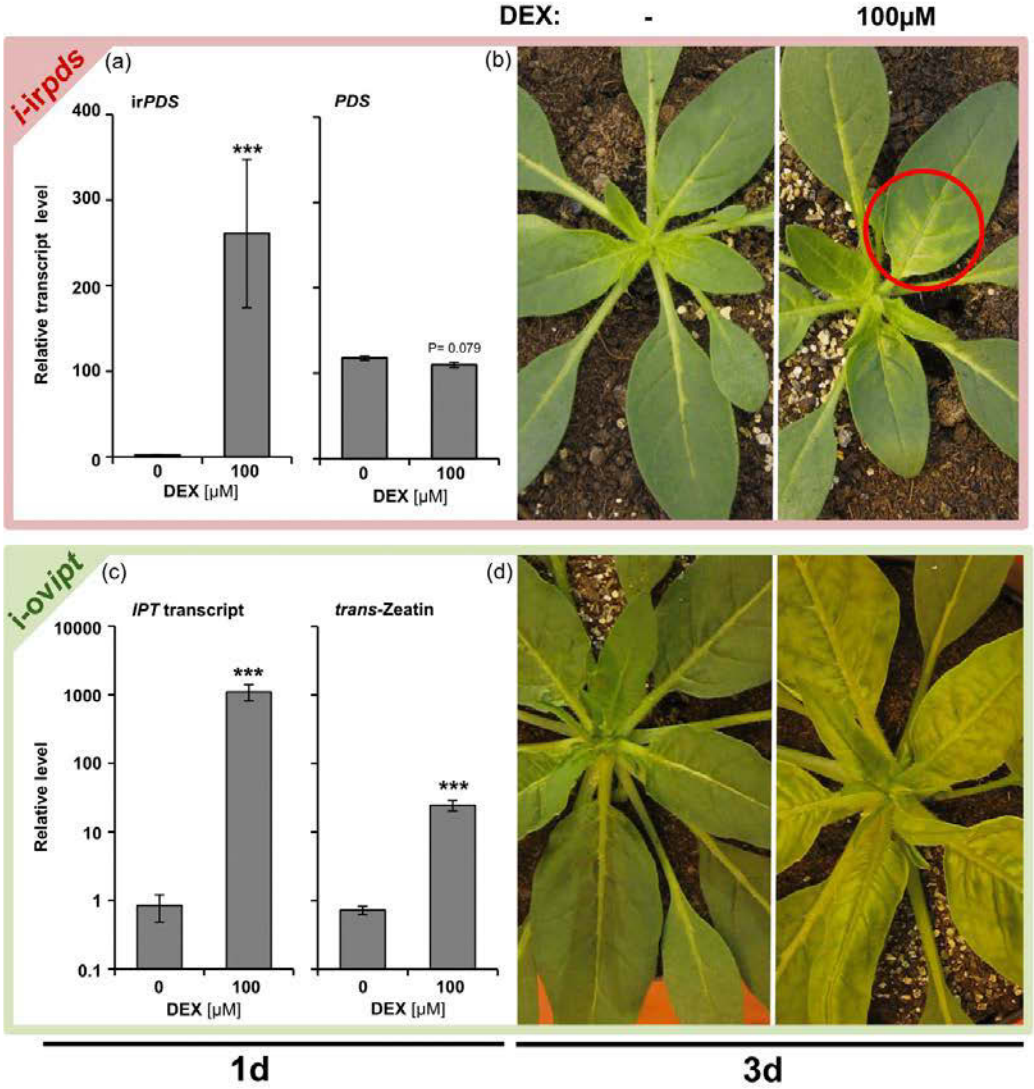


Figure S8. pOp6/LhGR system in *Nicotiana attenuata* under glasshouse conditions.

(a) Inverted repeat PDS (irPDS) and PDS transcript level in i-ir*pds* plants one day after application of 0 or 100µM DEX-containing lanolin paste to the midvein.

(b) i-ir*pds* plants three days after application of 0 or 100µM DEX-containing lanolin paste. Red circle indicates the first signs of bleaching.

(c) IPT transcript level and *trans*-zeatin level ($ng*gFM^{-1}$) in i-ov*ipt* plants one day after application of 0 or 100µM DEX-containing lanolin paste to the midvein.

(d) i-ov*ipt* plants three days after application of 0 or 100µM DEX-containing lanolin paste.

Asterisks indicate significant differences between DEX treated samples and the corresponding control (0µM) (independent samples *t* test: *** P<0.001). ir*PDS*, *IPT* transcript and *trans*-zeatin data, 24h after DEX treatment were log_{10} transformed before *t* test analysis. Error bars show standard errors (N≥4). FM, Fresh mass.

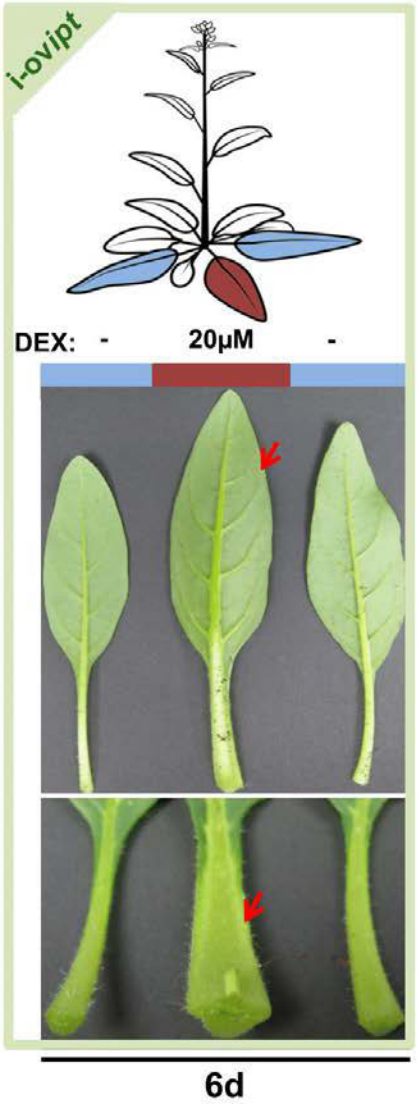


Figure S9. Spatial characteristics of the pOp6/LhGR system after induction with DEX-containing lanolin paste.

Comparison between a single DEX treated leaf with the next oldest untreated leaves. 20µM DEX-containing lanolin paste was applied to a single leaf for six

days. Arrows indicate the treated leaf. Experiments were performed under glasshouse conditions.

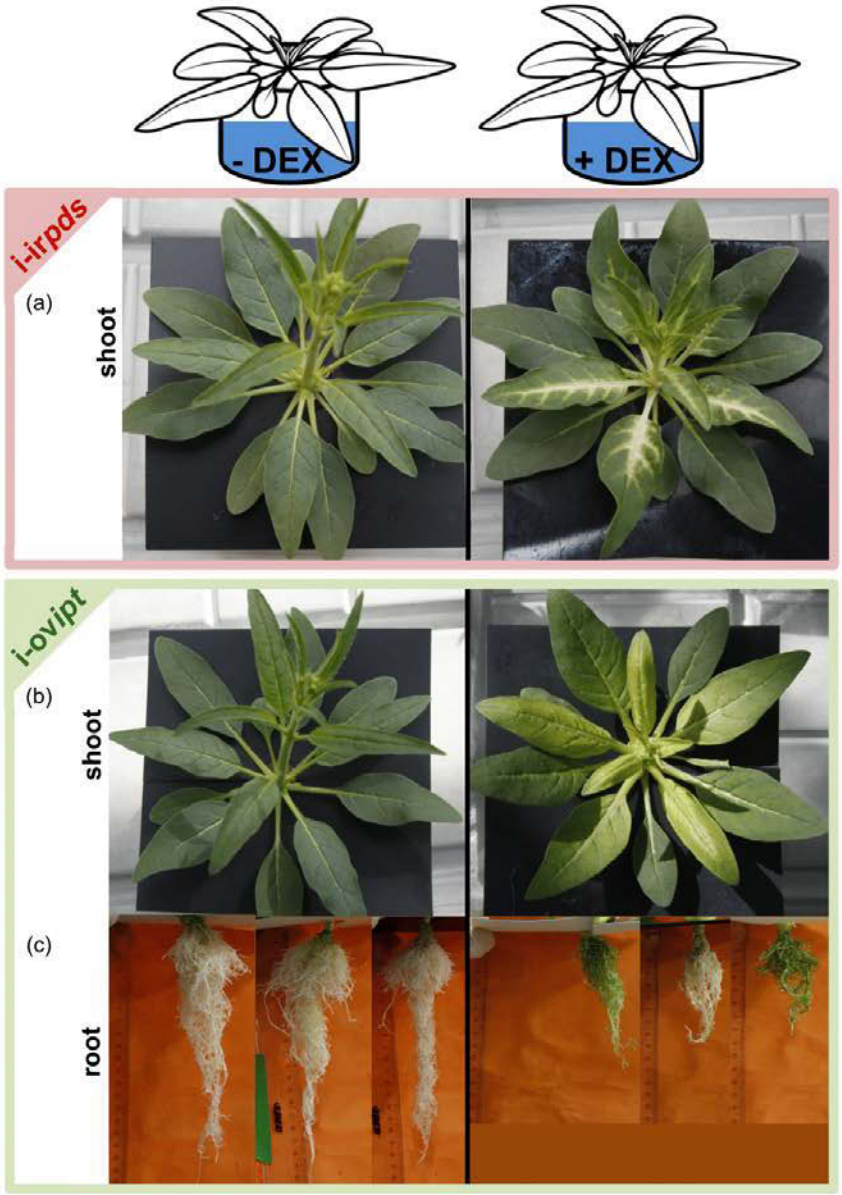


Figure S10. pOp6/LhGR system in *Nicotiana attenuata* - Hydroponic culture.

i-ir*pds* (a) and i-ov*ipt* (b, c) plants treated for six days (a, b) respectively 12 days (c) with 0 (-) or 1μM (+) DEX-containing hydroponic solution. Experiment was performed under glasshouse conditions.

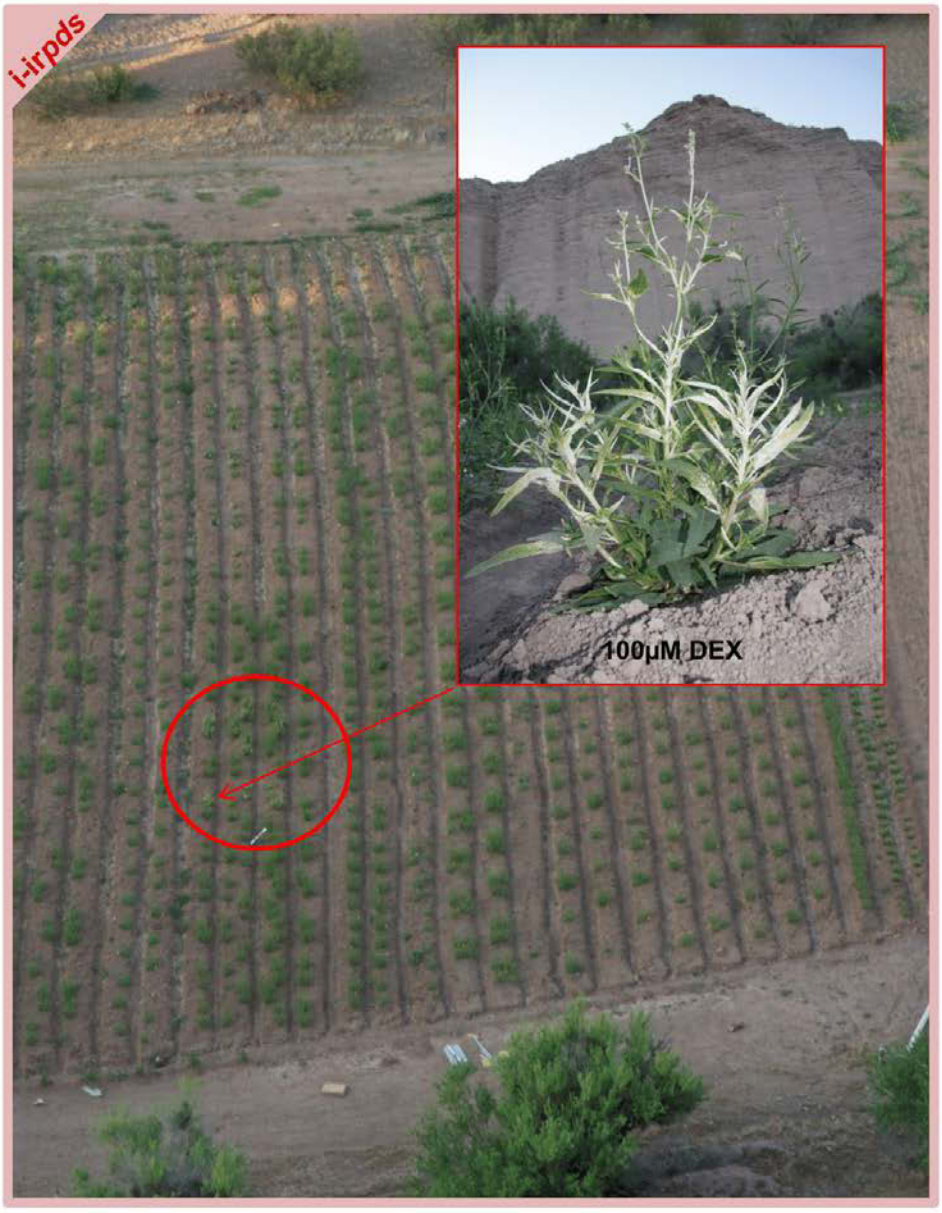


Figure S11. Inducible gene-silencing in the field.

Field plot in the Great Basin Desert, Utah. Red circle indicates i-ir*pds* plants two weeks after a single treatment with different concentrations of DEX-containing lanolin paste (0, 5, 20 and 100μM).

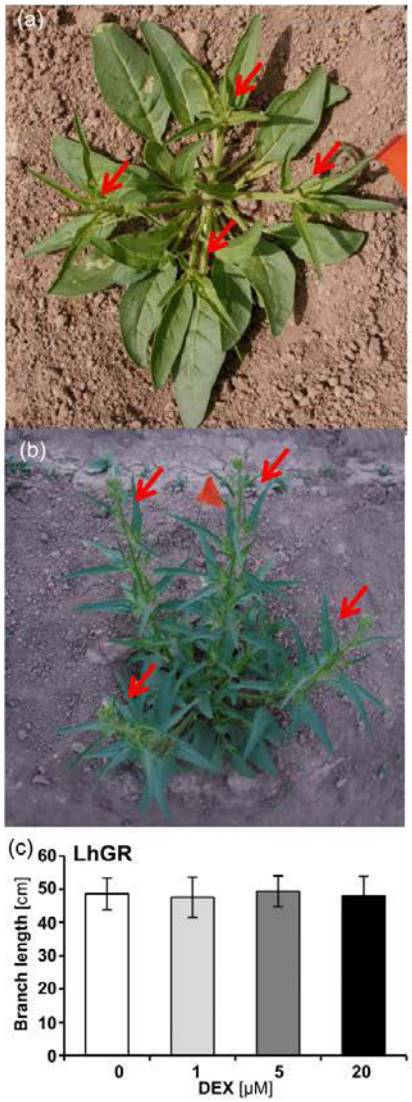


Figure S12. DEX treatments do not influence plant growth.

Representative decapitated plants at the stage of treatment begin (a) and 12d later (b). Side branch length of LhGR plants 12 days after application of 0, 1, 5 or 20µM

DEX-containing lanolin paste to different side branches. Lanolin paste was applied every three days. Error bars show standard errors (N≥15).

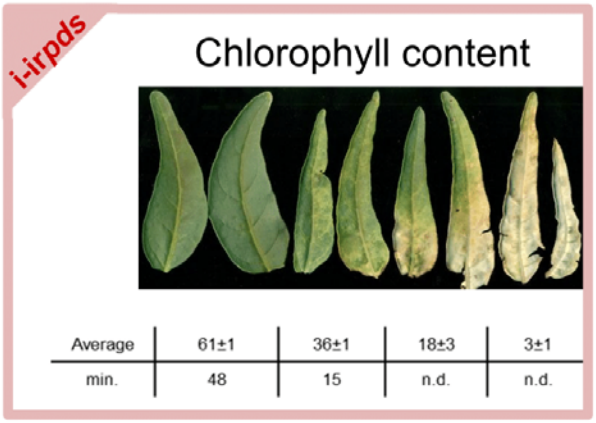


Figure S13. Chlorophyll content of leaves with different bleaching grades. Average amount ± SE (N=20) and minimal values (min.) are shown in relative units. Measurements were done with the SPAD502 Chlorophyll Meter from Konica Minolta Sensing, Inc. Bleaching was achieved by DEX-induced *pds* silencing (-> i-ir*pds* plants). Experiment was performed under field conditions in the Great Basin Desert, Utah. n.d., not detected.

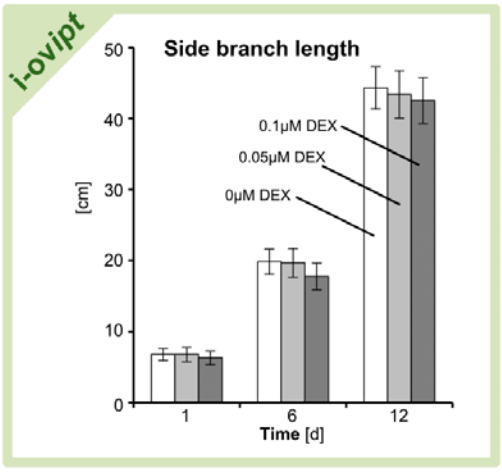


Figure S14. Subtle cytokinin induction in the field does not change plant growth. Side branch lengths of i-ov*ipt* plants after application of 0, 0.05 or 0.1µM DEX-containing lanolin paste to different side branches. Lanolin paste was refreshed every three days. Experiment was performed under field conditions in the Great Basin Desert, Utah. Error bars show standard errors (N≥15).

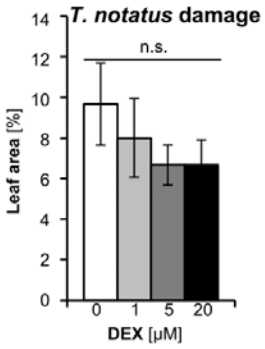


Figure S15. DEX treatment does not affect *Tupiocoris notatus* performance.

T. notatus leaf damage on different side branches of LhGR plants treated either with 0, 1, 5 or 20µM DEX-containing lanolin paste for 12d. Lanolin paste was refreshed every three days.

Experiments were performed under field conditions in the Great Basin Desert, Utah. Error bars show standard errors (N≥15). n.s. = no significant difference (paired samples *t* test: P>0.05).

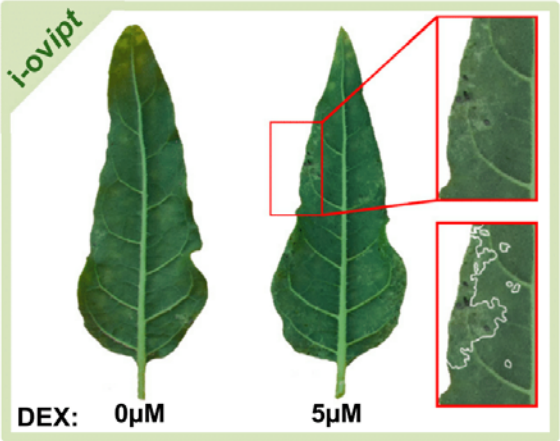


Figure S16. Quantification method for *Tupiocoris notatus* damage.

Leaves of i-ov*ipt* plants were treated with 0 or 5µM DEX-containing lanolin paste and exposed to *T. notatus* for 14 days. The red frame indicates the quantified damaged leaf area. Experiment was performed under glasshouse conditions.

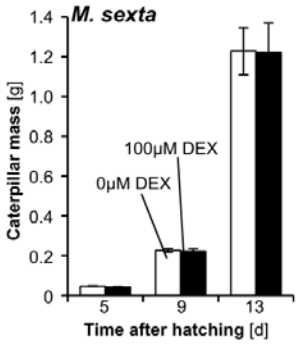


Figure S17. DEX treatment does not affect *Manduca sexta* performance. *M. sexta* caterpillar performance on LhGR plants treated with 0 or 100µM DEX-containing lanolin paste. Lanolin paste was applied every three days. Experiments were performed under glasshouse conditions. Error bars show standard errors (N≥8).

Table S1. Temperature conditions at the Utah field site at one day within the field season (4pm, 02.06.2012).

	Air[1]	Soil[2]	Petiole[3]
min. Temp [°C]	39	61	36
max. Temp [°C]	40	66	43

[1]Air temperature measured in the shade; [2]Soil temperature direct next to the test plants; [3]Surface temperature of the lower side of the petiole. Soil and petiole temperature was measured with the MiniTemp MT from Raytek.

Table S2. Sequences of primers used for qPCR.

Gene/construct	Forward primer	Reverse primer
NaActin	5‘ggtcgtaccaccggtattgtg3’	5‘gtcaagacggagaatggcatg3’
NaPDS	5‘gcattgattatccaagaccagagc3’	5‘cagacctgcaccagcaataaca3’
pOp6 driven construct[1]	5‘ccgcaaaaatcaccagtctctc3’	5‘catgagcgaaaccctataagaacc3’

[1]ir*pds*/*ipt* by detection of the corresponding terminator region

Table S3. Sequences of primers used for PCR.

Target cassette	Forward primer	Reverse prime
LhGR	5‘atctccactgacgtaagggatgacgc 3’	5‘gcggcggtcgaccagcttctgaataagccctcg3’
pOp6-*ipt*	5‘cgccagggttttcccagtcacgac3’	5‘cactgatagtttaaaccgaaggcggg3’

Table S4. Multi-reaction-monitoring settings for DEX quantification in negative ionization mode.

Analyte	Q1 [m/z] →	Q3 [m/z]	Capillary CID [V]	Collision energy [V]
DEX	437	361	-35	14
[9,10-^{2}H]dihydro-JA	213	59	-35	12

Table S5. Multi-reaction-monitoring settings for cytokinin quantification in positive ionization mode.

Analyte	Q1 [m/z] →	Q3 [m/z]	DP	CE	CXP
*t*Z	220.200	136.300	26	25	16
*t*ZR	352.200	220.300	76	25	30
*t*ZROG	514.100	382.100	96	25	16
*t*Z7G	382.100	220.000	71	31	16
D5-*t*Z	225.200	136.300	26	25	16
D5-tZR	357.200	225.300	76	25	30
D5-*t*ZROG	519.100	387.100	96	25	16
D5-*t*Z7G	387.100	225.000	71	31	16

3.6 Manuscript VI

Cytokinin transfer by the free living insect *Tupiocoris notatus* to its host-plant *Nicotiana attenuata* recapitulates a strategy of endophytic insects

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In preparation for eLIFE

ABSTRACT:

As plants have evolved strategies to respond to insect feeding, insects have countered to manipulate plants in their favor. It has been suggested that endophytic insects manipulate the host plants' source/sink relationships and increase the nutritional value of the infested tissue by transferring cytokinins (CK). Nevertheless, unambiguous tests of transfer and studies with free living insects have been elusive. Our study with the free living herbivore *Tupiocoris notatus* on *Nicotiana atteunata* revealed stable nutrient levels, increased CK levels and influences on CK-related transcripts in attacked leaves. With ^{15}N-isotope labeled plants, we found the CK 6-isopentenyladenine (IP) being transferred in significant amounts from the insects to the plant via its oral secretions. As plants with altered CK metabolism changed the feeding-preferences of *T. notatus*, we suggest insect triggered CK dependent manipulations of source/sink regulations an important part of the interaction of plants also with free living insects.

KEYWORDS:

Cytokinins, N6-isopentenyladenine, phytohormones, herbivores, plant defense, source/sink, plant manipulation, tolerance, effectors, *Tupiocoris notatus*, *Nicotiana attenuata*

ELIFE DIGEST:

As plants have evolved strategies to respond to insect feeding, insects have countered to manipulate plants in their favor. Endophytic insects, such as leaf miners and gall-forming species, may even create local resource sinks in the tissues they infest. One way they are thought to do this is by transferring cytokinin (CK) plant hormones which manipulate the host plants' source/sink relationships and increase the nutritional value of the infested tissue. However, unambiguous tests of CK transfer have been elusive. Furthermore, the majority of insect herbivores are free-living, yet the ability of mobile herbivores to manipulate host plants via CKs remains unstudied. We analyzed the CK-dependent interaction of the free-living cell-content feeding herbivore Tupiocoris notatus with its native host plant, Nicotiana attenuata. T. notatus attack elicits increases in transcripts related to CK degradation and decreases in biosynthetic genes, suggesting active CK manipulation. Interestingly, the levels of CKs increased or did not change in attacked leaves, and high levels of two of them, 6-isopentenyladenine and 6- isopentenyladenosine, were found in T. notatus bodies, which were likely produced either by the insects themselves, or their associated microbial community. To test whether these insect-derived CKs were transferred to the plant, we used 15N-isotope labeling experiments. After several days of heavy T. notatus feeding, 48% of the 6-isopentenyladenine content in leaves were identified as originating from the insects, likely transferred through oral secretions, which were highly enriched in CKs. Heavily attacked leaves showed only minor changes in its photosynthetic rate, protein, amino acid, sugar and starch content, even though jasmonic acid signaling was strongly induced, which usually results in decreased photosynthesis and triggers the onset of senescence. Plants with suppressed CK-dependent signaling were less attractive to T. notatus than wild-type plants and seemed to suffer more from an attack by the insects. We infer that free-living T. notatus use CKs to counteract herbivory-induced senescence processes and increase sink strength, thereby borrowing a page from the playbook of endophytic herbivores.

INTRODUCTION

Insect herbivores are under constant pressure from their host plants: they must adapt to toxic or anti-digestive defense compounds; low nitrogen to carbon ratios; and a food source whose nutritional value changes dramatically as leaves mature and senesce. Insects have developed strategies to overcome the low nutritional value of their diet and evolved specialized mechanisms to tolerate, or even employ toxic plant defense metabolites for their own uses, in an apparent evolutionary arms race (Despres *et al.* 2007, Winde and Wittstock 2011, Schuman and Baldwin 2016). As plant defense mechanisms against herbivores differ, so do the strategies that insects use to counter these defenses.

Generally, phytophagous insects are categorized as either endophytic or free-living. This distinction is not binary and many transitional forms exist even within the same taxa. However, this broad difference in lifestyle has resulted in various feeding strategies and different plant defense responses. Free-living insects are mobile on their hostplant, between plants, and frequently among different plant species. They can freely choose plant tissues that are most nutritious or least defended —the most nutritious tissues are often highly defended, resulting in a potential tradeoff for herbivores (Herms and Mattson 1992, Ohnmeiss and Baldwin 2000, Brütting *et al.* 2017). To avoid herbivore-induced defenses, mobile free-living insects often move to other plant parts or even other host plants in response to defense activation, a phenomenon readily seen when induced defenses are abrogated (Paschold *et al.* 2007) or manipulated independently of insect attack (Van Dam *et al.* 2001)

In contrast, endophytic insects have a more intimate relationship with their host plants since they spend a large portion of their life cycle within plant tissues. As a consequence, the effects of their feeding for the host plant is frequently less detrimental compared to the effects of free-living herbivores, although their high level of specialization to their host plant often makes them difficult to eliminate. There are many strategies to resist or tolerate free-living insects including production of toxins, attraction of predators or parasites of herbivores via indirect defense mechanisms like plant volatiles or extrafloral nectar, and re-allocation of nutrients away from attacked tissue into storage or reproductive organs (Forkner 2014, Schuman and Baldwin 2016). Endophytic herbivores, in contrast, must be contained or tolerated and if neither is successful, the infested tissues can be abscised to minimize negative impacts (Fernandes *et al.* 2008).

Intimacy is always challenging, but has benefits for herbivores: some endophytic species hijack their host's physiology, inhibit the production of defense compounds and manipulate their host to create nutritional resources, or even structures, which benefit the insects at the expense of plant growth and reproduction (Giron *et al.* 2016). To date, the best studied plant-manipulating species are those that spend a large portion of their life-cycle within the plant tissues: gall-forming

insects and leaf-miners, whose achievements are often featured in textbooks (Giron, et al. 2016). Gall-forming organisms, which include not only several orders of insects but also mites, nematodes and microbes, promote abnormal plant growth by reprogramming the expression of plant genes, to create novel organs that provide favorable environments for the exploiter (Stone and Schönrogge 2003, Shorthouse *et al.* 2005, Giron, et al. 2016). Advantages for the gall-formers range from an improved nutritional value, with reduced defense levels, to protection from disease, competitors, predators, parasitoids and unfavorable abiotic conditions (Hartley 1998, Stone and Schönrogge 2003, Allison and Schultz 2005, Harris *et al.* 2006, Saltzmann *et al.* 2008, Nabity *et al.* 2013, Giron, et al. 2016).

The manipulations of leaf-mining larvae are often less spectacular and rarely result in the formation of new macroscopic structures, but are often revealed in senescence of host tissues, where green islands appear around the active feeding sites (Engelbrecht 1968, Engelbrecht *et al.* 1969, Kaiser *et al.* 2010, Giron, et al. 2016). Such green islands maintain a high level of photosynthetic activity typical of non-senescent leaves, thus providing an adequate concentration of nutritional substances to the larvae, which feed on them (Behr *et al.* 2010, Body *et al.* 2013, Zhang *et al.* 2016). In this way green islands reflect the battle during the nutrient recovery phase that precedes abscission. The host plant tries to recover nutrients from the senescent leaf, whereas the insect tries to maintain a nutritious environment.

In addition to the observable effects on plant phenotype, manipulation strategies of gallers and miners share several attributes: 1) an improved nutritional value at the feeding sites, with increases in minerals, lipids, proteins, amino acids, sugar and starch contents; 2) a local decrease in allelochemicals; 3) a change in phytohormone balance compared to the rest of the plant (reviewed in Giron, et al. 2016). Although these effects have been observed and studied in a number of plant-insect interactions, little is known about the effectors used by insects to manipulate a plant's normal physiological response to wounding. Among the candidates, the prime suspects are phytohormones, since significant levels of some well-known wound-responsive phytohormones, including cytokinins (CKs), abscisic acid (ABA) and auxins, have been found in the body and salivary secretions of a number of gall-forming insects (Matsui *et al.* 1975, Mapes and Davies 2001, Dorchin *et al.* 2009, Straka *et al.* 2010, Tooker and De Moraes 2011b, Tooker and De Moraes 2011a, Yamaguchi *et al.* 2012, Tanaka *et al.* 2013), as well as in the labial glands of several leaf-mining larvae (Engelbrecht, et al. 1969, Body, et al. 2013). Amongst those phytohormones, CKs deserve additional discussion due to their long-presumed role in the formation of green islands.

CKs are a group of growth hormones, which are adenine derivatives and play a key role in the regulation of plant growth and development (Sakakibara 2006). They are known for their capacity to increase photosynthetic activity (Jordi *et al.* 2000), determine sink strength (Mok and Mok 2001) and inhibit senescence (Richmond and Lang 1957, Gan and Amasino 1995, Ori *et al.*

1999), as well as regulating herbivory-induced defense signaling (Schäfer *et al.* 2015c, Brütting, et al. 2017). The long history of investigating CKs in the formation of green islands date back to the late sixties, to reports of increased levels of CKs in affected tissues (Engelbrecht 1968, Engelbrecht, et al. 1969). In the last decade, studies on the leaf-mining larvae of *Phyllonorycter blancardella* species identified CKs as the causative factors for the "green island" phenomenon (Giron *et al.* 2007, Kaiser, et al. 2010, Body, et al. 2013). These studies suggested that insects could be the source of phytohormones used to manipulate plant physiological responses (Giron and Glevarec 2014). However clear demonstrations of the ability of insects to transfer CKs to a hostplant remains elusive (Giron, et al. 2016).

To date, reprogramming and manipulating of plant physiology via growth hormones is usually associated only with gall-forming and leaf-mining insects in literature. These species– at least during some developmental stages – are immobile and rely on their intimate relationships with specific hosts. In contrast, free-living herbivores that can select the best tissues for feeding are not thought to be in need to manipulate their hosts' physiology. As such, plant manipulation is thought to be evolved together with endophytophagy, which has been seen as an adaptive response to natural threats (Price *et al.* 1987, Giron, et al. 2016). In principle, all herbivores could benefit from manipulating their hosts; there is always some cost to movement and any strategy to subvert host plant defense may be adaptive. Thus plant manipulation by insects may be far more widespread amongst herbivores than we realize.

Here, we will address two major questions: 1) can a free-living insect actively manipulate a plant physiology, similarly to endophytic insects? 2) Does a free-living insect actively transfer CKs to the plant? To tackle these questions we worked with the well-established ecological model-plant *Nicotiana attenuata* and one of its most abundant herbivore, *Tupiocoris notatus* (Glawe *et al.* 2003). *N. attenuata* is a natural tobacco species native to the southwest of North America and well-studied for its interactions with herbivores like the lepidopteran *Manduca sexta* and the heteropteran *T. notatus* (Baldwin 1998, Kessler and Baldwin 2001, Kessler and Baldwin 2002, Voelckel and Baldwin 2004, Paschold, et al. 2007, Wu and Baldwin 2010). *T. notatus* is a free-living, small, 3-4 mm mirid bug (Miridae, Heteroptera). It is a piercing sucking cell-content feeder and is specialized to tobacco species and few other solanaceous plants like *Datura wrightii* (Gassmann and Hare 2005, Adam *et al.* 2017).

Through local manipulation of the endogenous levels of *N. attenuata* CKs, by using transgenic plants with a dexamethasone (DEX) inducible IPT (i-ov*ipt*), we previously observed increased damage by *T. notatus* in tissues richer in CKs. Individual DEX treated leaves of field grown plants suffered from more damage than mock-treated leaves. This led to the hypothesis that increased CK levels promote better nutritional quality, which in turn attracts *T. notatus* feeding (Schäfer *et al.* 2013). Infestation with *T. notatus* surprisingly does not decrease plant fitness (Kessler and Baldwin 2004), despite damaging large parts of the photosynthetically active leaf-

surface during their feeding. Tissues around *T. notatus* feeding sites have increased rates of photosynthesis per chlorophyll content that may compensate for the damage from herbivore feeding. The increase in photosynthesis results from an active ingredient of the oral secretion of *T. notatus,* which has not yet been identified (Halitschke *et al.* 2011).

Here, we report that *T. notatus* adults and nymphs contain high concentrations of two types of CKs. When confined to feeding on single *N. attenuata* leaves, concentrations of CKs increase in attacked leaves throughout the feeding period, with consequences for nutrients and CK-related transcripts. Using ^{15}N-labeled tracers, we test the hypothesis that *T. notatus* transfer CKs to the leaves during feeding. Finally, we analyzed how changes in CK-metabolism in plants affect the feeding preferences. We conclude that CK-dependent manipulation of plant-metabolism is not only a strategy used by gall-forming insects or leaf-miners but is a more general strategy also employed by free-living insects.

RESULTS:

Tupiocoris notatus *feeding induces the JA pathway and associated defenses in* Nicotiana attenuata

To characterize the defensive response of *N. attenuata* to mirid attack, we analyzed jasmonate hormones and defense metabolites that are known to be induced by *M. sexta* as well as *T. notatus* feeding (Kessler and Baldwin 2004). Continuous feeding by *T. notatus* (Fig. 1 A) causes severe damage on *N. attenuata* leaves (Fig 1 B) and induces defense reactions in attacked leaves (Fig. 1). Three days of *T. notatus* feeding induced levels of the defense metabolites nicotine (Wilcoxon-Mann-Whitney test (WMW): $p=0.022$), caffeoylputrescine (CP; WMW: $p=0.001$) and trypsin proteinase activity (TPI; $p<0.001$). *T. notatus* feeding also elevated the levels of jasmonic acid (JA;WMW: $p<0.001$), as well as its precursor *cis*-(+)-12-oxophytodienoic acid (OPDA; WMW: $p<0.001$) and its bioactive isoleucine conjugate (JA-Ile; WMW: $p<0.001$). Interestingly, there was also a significant increase in salicylic acid (SA; WMW: $p<0.001$) but no influence on abscisic acid (ABA; WMW: $p=0.620$) (Fig.1 I, J). After six days of feeding, JA and JA-Ile accumulated and remained at high levels regardless of whether insects were allowed to feed on whole plants or caged on only one leaf (Fig. 1 S1).

T. notatus *feeding reduces protein contents and photosynthesis rates, but elevates starch and sugar, with mild effects on chlorophyll content*

Feeding of *M. sexta* elicits downregulation of photosynthesis in unattacked tissue (Halitschke, et al. 2011, Barron-Gafford *et al.* 2012) and a reduction in sugars and starch (Machado *et al.* 2013). As it has been shown before that feeding of *T. notatus* is increasing photosynthesis in unattacked tissue, we wanted to see how continuous feeding of *T. notatus* over several days affects the nutritional quality of the attacked leaves. We analyzed protein, sugar and starch levels as well as photosynthesis and chlorophyll content in leaves over a period of 144 h. Surprisingly, even leaves heavily damaged did not show significant decrease in these nutrient levels when mirids were only feeding on one leaf of the plant. We restricted their movement by small plastic cages surrounding the leaf (Fig. 2). Although protein levels decreased with time (Two-Way-ANOVA (TWA): $P=0.005$), mirid feeding did not have a significant influence (TWA: $P=0.125$; Fig. 2 B). Mirid feeding tended to increase levels of starch compared to mock-treated leaves. This increase was significant after 144 h (t-test with Bonferroni correction (tt): $P=0.004$; Fig. 2 C). Mirid feeding showed no significant change in sucrose levels but attacked leaves tended to have slightly higher levels (TWA: $P=0.056$; Fig. 2 D). Pairwise t-tests with Bonferroni corrections showed that sucrose levels were higher after 96 h (tt: $P=0.017$) and 144 h (tt:

P=0.045). Mirid feeding had no significant influence on glucose (TWA: P=0.708; Fig. 2 E) or fructose levels (TWA: P = 0.476; Fig. 2 F).

Although we did not see large changes in carbohydrate levels, photosynthesis was significantly reduced in attacked leaves (TWA: P < 0.001; Fig. 2 S1 B). Levels of photosynthesis were significantly decreased after 48 h (tt: P = 0.020), 72 h (tt: P = 0.0132), 120 h (tt: P = 0.031) and 144 h (tt: P = 0.0046). Mirid feeding also tended to have an influence on chlorophyll content (TWA: P = 0.056). After 96 h chlorophyll content was slightly higher in mirid-attacked leaves (tt: P = 0.048).

While levels of nutrients were not much influenced by mirid feeding if only one leaf was attacked, larger changes in nutrient levels in the plant became visible when whole plants were infested with mirids (Fig. 2 S2). This became especially evident for protein levels. Protein levels decreased after mirid feeding (TWA: P < 0.001; Fig. 2 S2 B) and were significantly lower after 120 h of mirid feeding (tt: P = 0.002). When whole plants were attacked, levels of starch (TWA: P = 0.533; Fig. 2 S2 C), sucrose (TWA: P = 0.0891; Fig. 2 S2 D), glucose (TWA: P = 0.9233; Fig. 2 S2 E) and fructose (TWA: P = 0.535; Fig. 2 S2 F) were not affected by mirid feeding when analyzing the whole time series of 144 h. Nonetheless, comparisons at single time points revealed increased levels of starch after 96 h (tt: P = 0.024), sucrose after 72 h mirid feeding (WMW: P = 0.029), glucose levels after 48 h of feeding (tt: P = 0.033) and fructose after 48 h (WMW: P = 0.029).

Both, chlorophyll content (TWA: P < 0.001) and photosynthesis rates (TWA: P < 0.001; Fig. 2 S3) significantly decreased after *T. notatus* whole-plant attack. Wilcoxon tests revealed significantly lower photosynthesis activity in response to mirid feeding at all time points except 24 h where it was only a tendency (P = 0.057) and a lower chlorophyll content from 48 to 120 h (Welch t-tests).

T. notatus *attack increases the abundance of cytokinin levels and transcripts responsible for cytokinin degradation*

Cytokinins (CKs) are known to regulate source sink relationships and stabilize nutrient levels in tissues fed on by endophytic insects. As we did not see a strong decrease in nutrients after mirid feeding, we analyzed transcripts involved in CK metabolism. Mirid feeding significantly increases the accumulation of *NaCKX5* transcripts (TWA: P < 0.001), coding for a CK oxidase/dehydrogenase responsible for CK degradation (Fig. 3 A). Welch-t-tests revealed significantly increased transcript levels after 1 h (tt: P = 0.043), 72 h (tt: P = 0.019) and 96 h (tt: P = 0.04706) mirid attack. Transcript levels of *NaZOG2*, which codes for a CK glucosyltransferase responsible for CK inactivation (TWA: P < 0.001; Fig. 3 S1 B), as well as transcripts of *NaLOG4* (TWA: P < 0.001; Fig. 3 S1 C), which is involved in CK biosynthesis were also increased after

mirid feeding. Transcript levels of the isopentenyltransferase *NaIPT5,* which catalyzes the rate limiting step of CK biosynthesis, were reduced after mirid feeding (TWA: $P < 0.001$; Fig. 3 S1 D). Nevertheless, feeding of *T. notatus* did not change levels of the CK response regulator *NaRRA5* (Fig. 3 S1 A).

We then measured analyzed the CK levels in the attacked leaves. Even though transcript levels of enzymes responsible for CK degradation increased and levels of a gene for CK biosynthesis decreased, we saw no decrease, but an increase of CKs over time when whole plants were infested with *T. notatus* (sum of free bases and ribosides; TWA: $P = 0.004$; Fig. 3 B).

When looking at single CKs (Fig. 3 C, Fig. 3 S2), we saw changes in different CKs depending on time and type of induction by mirid feeding. Levels of *cis*-zeatin (*c*Z; TWA: $P < 0.001$; Fig. 3 S2 A), *cis*-zeatin riboside (*c*ZR; TWA: $P = 0.0210$; Fig. 3 S2 D), *trans*-zeatin (*t*Z; TWA: $P = 0.0294$; Fig. 3 S2 B) and *trans*-zeatin riboside (*t*ZR; TWA: $P < 0.001$; Fig. 3 S2 E) were significantly higher after *T. notatus* attack. Levels of isopentenyladenine (IP) remained unaffected by mirid feeding (TWA: $P = 0.142$; Fig. 3 S2 C) and levels of isopentenyladenosine (IPR) decreased in attacked leaves (TWA: $P < 0.001$; Fig. 3 S2 F). This decrease was significant in the first 48 h after the initiation of mirid attack and disappeared at later time points.

If mirids were only allowed to feed on a single leaf, we could not see any changes in levels of summed CKs over the whole time series (TWA: $P = 0.169$; Fig. 3 S3 B). Nevertheless, Bonferroni corrected t-tests revealed increased levels after 144 h of feeding (tt: $P = 0.026$). We found significantly increased levels of *c*Z (TWA: $P < 0.001$; Fig. 3 S3 C) and decreased levels of *c*ZR (TWA: $P = 0.005$; Fig. 3 S3 F). IP levels were significantly higher after mirid feeding (TWA: $P = 0.011$; Fig. 3 S3 C) although pairwise comparisons for each time point did not reveal significant changes at any given time point, while *t*Z, *t*ZR and IPR remained unaffected by mirid feeding if only one leaf was attacked.

T. notatus *contains high levels of IP*

We found that mirid attack increases the levels of *c*Z and *c*ZR. This fits to the observation of Schäfer, et al. (2015c) and Brütting, et al. (2017) that herbivory, wounding, as well as JAs can promote the accumulation of *c*Z-type CKs over a longer period of time. However, mirid feeding and the associated JA accumulation did not decrease IP levels as it would be indicated by the same publications (Schäfer, et al. 2015c, Brütting, et al. 2017). To find a possible explanation as to why CKs and in particular IP do not decrease in mirid attacked leaves even though degradation and inactivation processes seem to be activated, we analyzed CK levels in *T. notatus* insects as a possible external source of CKs. Surprisingly, we found very high levels of IP and high levels of IPR in extracts from the insect bodies (Fig. 3 C). While concentrations of IPR were comparable to those in leaves, around 1 pmol per g fresh mass (FM), levels of IP exceeded concentrations in the

leaves by a factor of 10 to 1600. While levels in leaves usually were in a range of 0.01 to 0.1 pmol g FM^{-1}, levels in insects were between 1 and 5 pmol g FM^{-1} but reached levels up to 16 pmol g FM^{-1}. Insects collected from their natural environment at our field site in Utah, USA, also contained high amounts of IP. In a pooled sample we measured 18.26 pmol IP per g FM of insects.

Mirids contained high CK levels in their body independently from their sex, developmental stage or their food source (Fig. 3 S4). IP concentration in nymphs was about half as high as in adult males (Tukey HSD after one way ANOVA: $P = 0.003$) and females ($P < 0.001$) but still several times higher than in leaves. Sex of the insect did not have any effect on the IP levels ($P = 0.257$; Fig. 3 S4 A). IPR levels were not affected by developmental stage or sex.

To exclude that *T. notatus* accumulates IP from the plant tissue, we reared the insects for 5 days either on artificial diet (containing no CKs) or on plants. Insects raised on artificial diet had IP levels in their body that were not different from levels in insects raised on plants (Fig. 3 S4 B; tt: $P = 0.341$) and IPR levels were also unchanged (tt: $P = 0.6695$)

T. notatus *transfers IP into the plant via its oral secretions*

To test whether CKs can be transferred to the plant we conducted ^{15}N-isotope labeling experiments (Fig. 4, Fig. 4 S1, S2, S3). We grew plants in hydroponic culture with ^{15}N labeled KNO_3 as the only source of nitrogen. We furthermore created a stock of *T. notatus* insects that were ^{15}N labeled by raising them for a whole generation on the ^{15}N labeled plants. We then performed two different experiments to trace back the origin of CKs in *T. notatus* attacked leaves: we either used ^{14}N plants that we exposed to ^{15}N labeled insects or we used ^{15}N labeled plants and exposed them to ^{14}N insects. CKs are adenine derivatives that contain 5 nitrogen atoms. Therefore, CKs produced by ^{15}N labeled plants or insects are labeled 5 times with ^{15}N and can therefore easily be distinguished from ^{14}N CKs by mass spectrometry.

In the first approach, we used a low-infestation setup by placing 20 ^{15}N labeled *T. notatus* adults in a small cage on the leaf of a ^{14}N labeled plant for 5 days (Fig. 4 S1, S3). After 4 days of continuous feeding we found detectable amounts of ^{15}N labeled IP and IPR in the leaves. While IPR was barely detectable in attacked leaves (Fig. 4 S3), we found around 2.35 fmol [$^{15}N_5$]-IP per g FM in the attacked leaves (Fig. 4 S1). This means about 3.3 % of the total IP in the leaves was [$^{15}N_5$]-IP. The labeled IP was most likely originating from the insects, as a natural occurrence of IP with 5 ^{15}N is stochastically nearly impossible. With a natural abundance of below 0.4 % an IP with five ^{15}N would occur with a chance of about once in a trillion. If calculated back, one mirid feeding on one leaf for 5 days accounts for a transfer of at least 0.12 fmol IP per g FM (Table S1) not taking any CK degradation or conversion to other CK forms into account.

In the reverse experiment, we used ^{15}N plants grown on ^{15}N labeled hydroponic culture and insects raised on non-labeled ^{14}N plants (Fig. 4). We placed the ^{15}N labeled plants in the cages where *T. notatus* was reared on ^{14}N plants. The plants were switched to another cage once per day to ensure that the plants were mainly attacked by fresh ^{14}N insects and to prevent a potential accumulation of ^{15}N in the ^{14}N insects. We found [$^{14}N_5$]-IP in the leaves of ^{15}N plants after 24 h of feeding by ^{14}N *T. notatus.* After 5 days, an average of around 48 % of IP was ^{14}N labeled and therefore originating from the insects feeding on the leaf. In this stronger induction setup, IPR could also be detected to be transferred from the insect to the plant. We found [$^{14}N_5$]-IPR in leaves of ^{15}N plants already after 24 h which increased to a share of 19 % after 5 d (Fig. 4 S2).

To find out how IP and IPR were transferred to the leaf, we analyzed CK content in oral secretions and frass of *T. notatus*, which we considered the most likely means of transfer. Mirids were fed on sugar solution without CKs. The solution was covered with parafilm, allowing penetration of the film by the insects' stylet but preventing an evaporation of the liquid and a transfer of frass into the liquid. We measured CKs in the liquid, which should contain substances that are transferred by the oral secretions, as well as in the surface wash, which contains excretions by the insects (Fig. 5, Fig. 5 S1). We found high amounts of IP mainly in the oral secretions (i.e. the sugar solution mirids were feeding on) and only in much lower amounts in the frass of the mirids (i.e. the surface wash; Fig. 5). IPR was found in oral secretions and in frass in similar amounts (Fig. 5 S1).

Altered CK metabolism in N. attenuata *affects its interaction with* T. notatus

In nature, *T. notatus* feeds on young plant tissue, such as younger stem leaves and young growing leaves. This was observed in damage distributions on whole plants in the natural environment (Fig. 6 S1 A) as well as in two-choice assays (Fig. 6 S1 B; P= 0.013). Those young leaves preferred by *T. notatus* are usually rich in CKs (Brütting, et al. 2017). To see how CK metabolism affects the interaction of *N. attenuata* with *T. notatus*, we used transgenic plants altered either in CK production (i-ov*ipt*) or in CK perception (ir*c2/3*). Transgenic i-ov*ipt* plants contain a dexamethasone (DEX) inducible promotor system coupled to an IPT gene that allow a chemically inducible induction of CK overproduction. ir*chk2/3* plants are silenced for two of three CK receptors.

Our previous study has shown that *T. notatus* prefers leaves of i-ov*ipt* plants which have been treated with DEX and therefore have higher levels of CKs (P = 0.01595; Fig. 6 A; Schäfer, et al. (2013). If *T. notatus* is given the choice between empty vector (EV) and ir*chk2/3* plants, mirids show a strong preference towards EV plants, as shown in lower damage levels on ir*chk2/3* plants (P < 0.001; Fig. 6 B). Furthermore, we found differences in the reaction of the plants to the

damage caused by *T. notatus*. Mirid attack caused necrotic lesions in ir*chk2/3* plants, whereas this did not happen in WT, EV or i-ov*ipt* plants (Fig. 6 C).

To find a possible explanation for the feeding preferences of *T. notatus*, we measured nutrient levels in unattacked ir*chk2/3* and DEX-induced i-ov*ipt* plants and compared it to EV plants (Fig. 7). Starch and sucrose did not differ between the lines (Fig. 7 C, F). However i-ov*ipt* plants had higher concentrations of protein, free amino acids, glucose and fructose than ir*chk2/3* plants. Also, i-ov*ipt* tended to have higher nutrient levels than EV plants but only contained significantly higher amounts of glucose (Fig. 7 D). ir*chk2/3* plants tended to have lower nutrient levels but they only contained significantly lower concentrations of fructose (Fig. 7 E).

DISCUSSION

Endophytic insects have been long known for their ability to manipulate plants via phytohormones like cytokinins (CKs). The common opinion is that CK-dependent manipulation of plant metabolism by insects is a specialized mechanism that evolved during the intimate relationship between endophytic specialists like gall-formers and leaf-miners. In our study we provide evidence that the free living mirid bug *T. notatus* is capable of transferring CKs to its host plant *Nicotiana attenuata* and possibly manipulates the host plant's metabolism for its own benefit. This strategy, not known for free living insects so far, could indicate that CK-mediated manipulation of plant metabolism by insects could be a mechanism far more widespread than previously thought.

Mirid feeding does not strongly decrease levels of nutrients in attacked plants

Earlier studies have shown that colonization by *Tupiocoris notatus* does not decrease plants' overall fitness in nature (Kessler and Baldwin 2004). Commonly, herbivore feeding decreases plant fitness by damaging or removing photosynthetically active tissue and inducing costly defense reactions (van Dam and Baldwin 1998, Huot *et al.* 2014). It could be shown that the loss of photosynthetically active tissue caused by *T. notatus* feeding could be compensated by an increase of photosynthesis rates in undamaged tissue and therefore could provide an explanation for the insignificant effect on plant fitness (Voelckel and Baldwin 2003, Halitschke, et al. 2011). This suggests either a tolerance response by the plant or an active manipulation by the insect. Herbivore attack, including attack of *T. notatus* leads to increases in levels of jasmonates, especially jasmonic acid (JA) and its isoleucine conjugate (JA-Ile) (Baldwin *et al.* 1994, Krumm *et al.* 1995, Baldwin 1996, Kang *et al.* 2006, Erb *et al.* 2012, Campos *et al.* 2014). Herbivore attack and JA accumulation are known to decrease photosynthesis and levels of nutrients in affected tissue (Herms and Mattson 1992, Baldwin 1998, Barron-Gafford, et al. 2012, Machado, et al. 2013, Attaran *et al.* 2014). These reductions are part of senescence processes activated by JA (Satler and Thimann 1981, Ueda and Kato 1981, Ueda *et al.* 1981).

We also found that levels of SA, JA, JA-Ile and JA-dependent defenses increased after mirid attack (Fig. 1). Nevertheless, we could not observe a significant reduction of nutrients in leaves attacked by *T. notatus* except whole plants were severely attacked. We found levels of protein, starch, glucose, fructose and sucrose to be relatively stable after mirid attack (Fig. 2, 2 S2). This already suggests some other factor influencing the nutrient balance in attacked leaves. Previously it could be demonstrated that mirid feeding cause an increase of photosynthesis in unattacked tissue surrounding their feeding sites probably by an "active ingredient" in their oral secretions (Halitschke, et al. 2011). However we could not detect an increase of photosynthesis but an overall decrease in photosynthesis in attacked leaves (Fig. 2 S1, 2 S3) This discrepancy

could possibly be explained, as we had a more severe attack than in previous studies and we did not normalize to unattacked leaf-tissue but did include the whole leaf surface including damaged and undamaged area. The decrease in photosynthetic assimilation in response to herbivory has recently been shown to be caused by early JA signaling responses, namely OPDA (Meza-Canales *et al.* 2017). OPDA, which is also increased by *T. notatus* feeding (Fig. 1) has been shown to reduce stomatal conductance which leads to the reduced rates of photosynthesis.

In either case it seems unlikely that a stable level of nutrients in the leaves is due to an increase in photosynthesis and anabolism in attacked leaves. We think that the stable levels of nutrients are rather due to an inhibition of senescence or even a transport of nutrients to the attacked leaves than to an increased production of proteins and sugars at the site of attack. Similar cases are known from endophytic insects, which can increase sink strength, inhibit senescence, and increase nutrient transport to their feeding sites (Engelbrecht 1968, Hartley 1998, Harris, et al. 2006, Saltzmann, et al. 2008, Body, et al. 2013, Giron, et al. 2016, Zhang, et al. 2016). We found that protein levels only drop in infested leaves if whole plants, and not only single leaves were infested (Fig. 2, 2 S2). It is possible that the feeding activity of *T. notatus* inhibits senescence or elevates sink strength in infested leaves. If only one leaf is infested, it is possible that allocation of nutrients to the infested tissue occurs as a consequence of sink generation by the insect feeding. From leaf-miners it is known that increase in photosynthesis in attacked tissue comes at the cost of increased senescence in the rest of the plant (Behr, et al. 2010). If all leaves are induced equally, the effect of a sink at infested tissue is likely to disappear due to a lack of a gradient in sink strength. Furthermore, the plant suffers from much greater tissue loss if the whole plant is infested, which might limit the capability of the plant to compensate for tissue damage at a particular infested leaf.

To determine whether a sink is created, further experiments like ^{14}C pulse labeling of source leaves (techniques reviewed e.g. in Epron *et al.* 2012) and ^{15}N pulse labeling (Ullmann-Zeunert *et al.* 2012) would be necessary to trace fluxes of nutrients and defense metabolites upon herbivore attack. Previous studies with endophytes demonstrated the importance of CKs in the insect-triggered generation of sinks (Engelbrecht 1968, Engelbrecht, et al. 1969, Elzen 1983, Body, et al. 2013, Kudoyarova *et al.* 2014, Giron, et al. 2016, Zhang, et al. 2016). Furthermore we found mirids to prefer leaves with higher CK content (Fig. 6, 6 S1; Schäfer, et al. 2013). We therefore focused our investigation on CK levels and CK metabolism in the plant after attack, as well as into CK levels in insects and their oral secretions.

Cytokinin levels increase after mirid feeding

It is known from endophytic insects that CK levels increase around the feeding sites, namely the galls and green islands (reviewed in Giron, et al. 2016). We measured levels of CKs in

attacked tissue and found an increase of several CKs after feeding of *T. notatus* (Fig. 3, 3 S2, 3 S3). The greatest differences were found in *c*Z levels and *c*ZR levels, when whole plants were attacked. An increase of *c*Z and *c*ZR could be a general response to the wounding of the leaves and JA increase (Schäfer *et al.* 2015a, Schäfer, et al. 2015c, Brütting, et al. 2017). Other CK levels did not show differences (IP, *t*ZR). From previous studies, we know that JA increase (by application of Methyl-jasmonate) decreases levels of IPR and IP (Schäfer, et al. 2015c, Brütting, et al. 2017).

Surprisingly we found levels of transcripts for CK degradation and inactivation increasing and of the CK biosynthesis gene *NaIPT5* decreasing (Fig. 3, 3 S1), which conflicts with the observation that CKs do not decline. Previous studies showed a similar decrease of *NaIPT5*, and an increase of *NaCKX5, NaZOG2* and *NaLOG4* after wounding (Schäfer, et al. 2015c), which suggests that the transcriptional response could be a more general reaction to wounding. Unlike the results of methyl jasmonate application (Schäfer, et al. 2015c, Brütting, et al. 2017), we did not find a decrease in IP, and no long term decrease in IPR, although JAs were accumulating more and more in mirid infested leaves. There are several possible reasons for that discrepancy: There could be increased biosynthesis of CKs in attacked leaves, but because we observe reduced transcript levels of an IPT gene, this seems unlikely. Admittedly, we cannot exclude that there are other IPTs in attacked leaves that are upregulated upon mirid attack. Also, it is possible that precursors of CKs are converted to active forms to compensate inactivation and degradation. Enzymes of the LONELY GUY (LOG) group convert CK phosphoribosides to free bases and are therefore responsible for their activation (Kuroha *et al.* 2009). As *NaLOG4* transcripts increase after mirid feeding, it is possible that an increased conversion of phosphoribosides to ribosides can account for the stable levels of CKs. Nevertheless, this seems unlikely without elevated biosynthesis of CK phosphoribosides. A broader analysis of CK-dependent transcripts and CK glycosides after mirid feeding would help to gain deeper insight into CK biosynthesis and activation. Another possible explanation would be a flux from other plant parts, for example roots, to the infested tissue to compensate for the CK loss by degradation and inactivation. To test this hypothesis, isotopic labeling experiments would be required to trace CK flux through the plant.

Lastly, there could be an external source of CKs compensating for the CK loss. CK application can also trigger transcriptional changes of CK biosynthesis and degradation enzymes as has been observed after mirid feeding (Schäfer, et al. 2015c). We know from endophytic insects that insects themselves might be a source of CKs in leaf-galls and green islands (Engelbrecht, et al. 1969, Matsui, et al. 1975, Mapes and Davies 2001, Giron, et al. 2016) and so, we considered *T. notatus* as a possible source of CKs in attacked leaves.

Mirids contain CKs in their body and in their oral secretions

We determined levels of CKs in insects as well as their oral secretions (OS) and their frass. We found extraordinary high concentration of the active CK IP in the insects, in the OS and in traces in the frass of *T. notatus* (Fig. 3, 3 S4, 5). Abundance of high concentrations of IP has been reported before in several cases of endophytic insects like leaf miners and gall-formers (Mapes and Davies 2001, Body, et al. 2013). We also detected IPR in the insects, their OS and frass (Fig. 3, 3 S4, 5 S1), but concentrations were similar as in plant tissue and much lower as concentrations of IP. We therefore hypothesize that IPR might play a minor role and possibly only be a precursor or a side-product of IP.

This raises the question: What is the source of IP in the insects? There are two possible explanations: Either it is accumulated from their food source or it is synthesized by the insects or their symbionts. An accumulation of IP from the food source seems rather unlikely. We did only find IP and, in lower concentrations, IPR but no other active CKs in insects, which means that the accumulation should be very specific for IP. Furthermore we can detect comparable levels of IP also in insects reared on artificial diet for 5 days (Fig. 3 S4). Based on our IP measurements in insects reared on artificial diet each mirid contains about 0.85 fmol IP. If calculated back from the measurement of IP in OS, each *T. notatus* insect excreted about 0.33 fmol IP within one day, which accounts for about 39 % of the IP found in the insects. Considering the high amount of IP excreted each day it seems unlikely that IP levels in insects reared on artificial diet without an IP source remain at a level comparable to insects fed on plants. For the less abundant IPR the effect was even stronger (approximately 0.13 fmol IPR in each insect vs. 0.07 fmol excreted by these insects within a day) and additionally some IP and IPR was excreted by frass (approximately 0.01 fmol IP and 0.09 fmol IPR per insect within a day). Even if this is just a rough calculation of an exemplary data set, it strongly suggests that 5 days artificial diet feeding should heavily deplete the CK pool of mirids if they would not be able to gather more in a plant feeding-independent way. Therefore this supports the hypothesis that mirids are directly or indirectly capable to produce the CKs IP and IPR.

Although it is possible that insects themselves are able to produce IP for example via a tRNA derived biosynthesis of IP (Persson *et al.* 1994, Kaminek 2015), the common opinion considers endosymbiontic bacteria as the most likely producers of IP in insects. Antibiotic feeding experiments have revealed that endosymbionts like *Wolbachia* are the most likely producers of CKs in the leaf-miners (Kaiser, et al. 2010, Frago *et al.* 2012, Body, et al. 2013, Giron *et al.* 2013, Giron and Glevarec 2014).

So far the data we have is neither sufficient to confirm nor to disprove that endosymbionts are responsible for IP production in *T. notatus*. Recent investigations on the microbial community did not provide an obvious candidate for IP production, as microbial communities differed between different samples and between field and lab collected insects (Crava *et al.* 2016, Adam, et al. 2017). We also find IP in insects collected in the natural habitat, which indicates that the IP

data are reliable also under natural conditions. We are therefore searching for a symbiotic organism that is present in animal samples from the lab and from nature, which we could not identify so far. Using an RNAseq approach sequencing the transcriptome of *T. notatus* we revealed 125 hits for sequences from bacteria including 11 hits for *Wolbachia* (Crava, et al. 2016). But also *Wolbachia* could only be found in one out of nine samples using a pyrosequencing approach and PCR of 16S rRNA amplicons (Adam, et al. 2017). This indicates that maybe another organism is responsible for the production of IP. The fact that we did not find a candidate yet might be due to the fact that a bacterium not characterized so far as CK producer is synthesizing the IP, the bacterium we are looking for is underrepresented in a sample of the whole body of *T. notatus* or that organisms other than bacteria are responsible for the IP biosynthesis. Some fungi are also capable of producing IP (Jameson 2000, Walters *et al.* 2008, Giron, et al. 2013). It is also possible that fungi are associated to *T. notatus* and are responsible for the IP biosynthesis.

To elucidate the origin of IP in the insects further experimental steps will be necessary. First the localization of highest concentrations of IP would need to be specified. IP labeling with antibodies in slices of insects or measurement of IP in single insect organs like labial glands or midgut could be used to identify location of highest IP concentration. An RNAseq or metagenomics approach could then identify possible candidates for IP production at the site of highest IP concentration in the insect. Future work could then target those candidate organisms and cure insects from endosymbionts to find out its function in insect plant interactions.

Mirids are able to transfer CKs to the leaves of the hostplant with their oral secretions

Independently from its origin, we were able to show with ^{15}N labeling experiments that *T. notatus* is able to transfer IP and IPR to its hostplant (Fig. 4, 4 S1, 4 S2, 4 S3). After 6 d of mirid attack, almost half of the IP in attacked leaves could be traced back to insects, as it was ^{14}N IP in ^{15}N-labeled plants. Other explanations for high levels of unlabeled IP, like contaminations of plants, of medium or during measurement are very unlikely for several reasons: 1) We find transferred IP in two independent experiments, where ^{14}N and ^{15}N insects and plants were reversed. 2) The share of insect derived IP is substantial and increases over time and does not occur in unattacked plants. 3) We only find hints for transfer for exactly those two CKs, which are also present in the insects, namely IP and IPR. Also the possibility that we are detecting natural isotopes seems statistically almost impossible due to their mass difference of 5 and the big proportion of IP we can detect.

As we found IP and IPR in the OS (Fig. 5, 5 S1), we consider OS the most likely way of transfer of IP and IPR to the leaves. Although mirid excretions might contribute to that transfer in

a minor extend. As CKs can also be incorporated via the leaf-surface, this also seems to be a functional way of transfer in addition to the transfer via OS.

Changes in the CK metabolism of the hostplant alters feeding preferences of the mirids

In endophytic insects CKs are considered one of the key factors influencing the performance of leaf miners and gall formers on their hostplant as they increase the quality of their food (Body, et al. 2013, Giron, et al. 2013, Giron, et al. 2016). Whether a transfer of IP has a similar function for free living mirids as it has for endophytes is a question that remains not fully answered. As nutrient levels do not drop in leaves attacked by *T. notatus* (Fig. 2, 2 S2), we can at least suggest, that IP may play a role in the stabilization of nutrients by possibly delaying senescence processes.

Choice assays provide a hint that CKs play an important role in regulating plant features that effect feeding preferences and performance of insects on the plant. *T. notatus* is attracted to tissue with higher CK levels. They are attracted, either if CK levels are naturally higher (Fig. 6 S1), like in young plant tissues or if CKs were artificially increased using transgenic plants (Fig. 6; Schäfer, et al. 2013). When CK perception is impaired in transgenic ir*chk2/3* plants, mirids preferred WT or EV plants over the transgenic plants as displayed by different damage levels (Fig. 6).

This particular preference for high CK levels and against ir*chk2/3* plants could either be a direct effect of CKs or – more likely – an indirect effect of CK-related processes. A direct attraction to CKs is possible but not very likely, although a direct influence of CKs on insects has been discussed (Robischon 2015). To our knowledge there is so far no evidence toward a direct sensation of CKs by insects; also CK levels are not reduced in ir*chk2/3* plants (Schäfer, et al. 2015c). More likely, insects and in particular *T. notatus* are attracted by some metabolites associated with high CK levels. Those metabolites could be for example volatiles or other specialized metabolites. *T. notatus* is attracted to quercetin (Roda *et al.* 2003). If quercetin is associated to high CK levels is not known so far and could be a target of future research. In previous research we found at least other closely related phenolic compounds being influenced by CKs (Schäfer, et al. 2015c, Brütting, et al. 2017). Or it could simply be an attraction to high nutrient levels, which correlate to young, CK rich tissues. High levels of nutrients seem to be the most likely explanation to us and they could be detected from *T. notatus* by probing the tissue. Higher protein levels in EV compared to ir*chk2/3* (Fig. 7) could explain the feeding preferences. Nonetheless we can summarize that mirids are attracted to traits associated with CKs. If CK metabolism or signaling is altered, it affects the interaction with the plant. More CKs and a functioning CK perception seems to be attractive for *T. notatus* and could possibly be beneficial for the insects.

By injecting IP in the leaves, *T. notatus* could eventually stabilize the quality of its food source. Especially it could be a way to keep the high levels of CKs and the associated high levels of nutrients over a prolonged period of time, if feeding continues for several days on the same plant or even the same leaf.

If the injection of IP also provides a beneficial effect for the plant compared to damage without IP injection remains elusive so far. We saw evidence that an altered CK metabolism increases the damage rate (i-ov*ipt*) and a non-fully functioning perception of CKs (ir*chk2/3*) alters the response to mirid attack. We see necrotic lesions in attacked leaves of ir*chk2/3* plants (Fig. 6). This is a hint for higher stress levels and a non-functional response to mirid feeding. *N. attenuata* seems to require a functioning CK-signaling and perception for a fully functioning tolerance to stresses like herbivore attack. To see how a functioning CK signaling affects the effects of *T. notatus* feeding on the fitness of *N. attenuata* plant fitness could be determined in WT and ir*chk2/3* plants with and without damage by *T. notatus* by comparing influence of feeding on seed-production.

Manipulation of the host plant by a free living insect? An evolutionary more ancient trait?

Summarizing, our results provide evidence that a free living insect could be capable of manipulating its host plant metabolism for its own benefit by injecting CKs into the plant. CK-mediated plant manipulation strategies have so far only been known from endophytic insects (Giron, et al. 2016). The common theory is that endophytic insects that are so tightly bound to their hostplant and so lowly mobile had to evolve mechanisms to increase the food quality locally to be able to sustain in the host plant.

Insects that are known for their CK-dependent manipulation of hostplants share similar endophytic lifestyles. Phylogenetically they are not very closely related to each other. There are known examples of leaf mining insects from the orders of Lepidopterans, Coleopterans (Buprestidae) and Dipterans (Agromyzidea). Although not all leaf miners cause green islands and are known to manipulate the hostplant using CKs, it is known for some lepidopterans like *Phyllonorycter blancardella* (Giron, et al. 2007, Kaiser, et al. 2010, Body, et al. 2013) or *Stigmella argentipedella* (Engelbrecht, et al. 1969) and could at least be assumed for other lepidopterans causing green islands like *Ectoedemia argyropeza*. From some gall-midges like *Bruggmannia* (Diptera: Cecidomyiidae) it is known that they are also capable of producing green islands (Fernandes, et al. 2008). Leaf-Gall forming insects can be found in Hymenopterans like gall-wasps, Dipterans like gall-midges and gall-flies, Hemipterans like psyllids, as well as gall-aphids. A role of CKs in the formation of galls (see also Elzen 1983) has been shown for Dipterans like *Eurosta solidaginis* (Mapes and Davies 2001) or *Rhopalomyia yomo-gicola*

(Tanaka, et al. 2013), Hymenopterans like the gall wasp *Dryocosmus kuriphilus* (Matsui, et al. 1975) or sawflies of the genus *Pontania* (Yamaguchi, et al. 2012) and Hemipterans like *Pachypsylla celtidis* (Straka, et al. 2010) or the gall-aphid *Tetraneura nigriabdominalis* (Takei *et al.* 2015). The fact that insects from different orders have developed a similar mechanism either suggests an evolutionary ancient manifestation or a convergent evolutionary trait.

In both cases it is likely, that CK-dependent plant manipulation has not only evolved in those very special cases. IP has been found in many organisms other than plants including fungi (Chanclud *et al.* 2016), bacteria (Costacurta and Vanderleyden 1995) and animals like nematodes (Siddique *et al.* 2015). It is thought that IP and IPR derived from tRNA might be a source of IP shared by almost all organisms (for review see Persson, et al. 1994). Especially, as it has been shown in previous studies that CKs in endophytic insects seems to be produced by endosymbiotic bacteria (Kaiser, et al. 2010, Giron and Glevarec 2014), it seems likely that the potential for CK secretion is common amongst many insects, as likely all insects may have endosymbiotic bacteria. A broader analysis of insects from different orders regarding their ability to secrete CKs could further illuminate this interesting field of plant-herbivore research and show if *T. notatus* is an exception among freely moving insects, or if this is a more general phenomenon.

Free living phytophagous insects have been considered so far as not being in need of the possibility to manipulate their food plant in this way, as they are able to choose the best feeding spots. Now, we can suggest that at least some insect species might combine the benefits of the two different lifestyles: The ability to move, hide, choose the best feeding spot and being able to manipulate the hostplant for their own benefit.

MATERIALS AND METHODS

Plant cultivation and transgenic plants

We used 31st inbred generation of *Nicotiana attenuata* (TORR. ex S. Wats.) originating from a population at the Great Basin desert (Washington County, Utah, USA) as wildtype (WT) plants. Transgenic plants were generated from WT *N. attenuata* as described by (Krügel *et al.* 2002) by Agrobacterium mediated transformation. Empty vector plants (EV) were used instead of WT in experiments, where we used transgenic plants.

In our experiments we used two different types of previously described transgenic plants:

The first transgenic line, ir*chk2/3* has a construct silencing two of the three known cytokinin (CK) receptor homologs (CHASE DOMAIN CONTAINING HISTIDINE KINASE 2 and 3; NaCHK2 and NaCHK3) at a silencing efficiency of about 50 % (Schäfer *et al.* 2015b). We used line A-12-356 for experiments.

The second transgenic line, i-ov*ipt* contains a gene encoding for the rate limiting step of CK biosynthesis, the isopentenyltransferase (IPT) from *Agrobacterium tumefaciens* (*Tumor morphology root; Tmr*). The IPT gene is controlled by the pOp6/LhGR expression system, which allows transcriptional regulation by the application of dexamethasone (DEX; Schäfer, et al. 2013). Application of DEX to the leaves of the plant induces the transcription of *IPT* which increased CK levels locally. We used line number A-11-92 x A-11-61.

DEX was solved in lanolin paste with 1% DMSO at a final concentration of 5 µM. For control treatments we used 1% DMSO in lanolin. The lanolin paste was applied to the petioles of the leaves 24 h prior to following treatment (Schäfer, et al. 2015b).

Plant cultivation was performed as described by Krügel, et al. (2002) with the modifications described by Brütting, et al. (2017): Ten days after germination plants were transplanted to TEKU pots containing soil, and maintained under greenhouse conditions (27°C; ca. 60% RH, 18:8 light:dark regime). Soil growth conditions have been previously described (Krügel, et al. 2002).

Hydroponic plants have been grown as described by Ullmann-Zeunert, et al. (2012).

To prevent spreading of *Tupiocoris notatus* in our greenhouse facilities, we transferred the plants right before start of flowering, when the plants were about 25 cm elongated, to a separate greenhouse designated for *T. notatus* experiments for treatment with insects. In both greenhouses we kept plants at comparable growth conditions. After transferring the plants to the second greenhouse, we waited at least 2 days before we started the experiments to allow them to acclimatize to the new environment.

Insect colony

We used a lab colony of *T. notatus* (DISTANT, 1893; Fig. 1B) originating from insects caught in the Great Basin desert (Washington County, Utah, USA; Kessler and Baldwin (2001). Lab colonies are regularly refreshed by insects caught in nature. Lab colonies are kept in cages made of acrylic glas (2 * 1 * 1 m) with a fine mesh for air circulation. The cages are placed in the same greenhouse where the experiments with plants were done under the same light and temperature regime. We feed insects with hydroponically grown *N. attenuata* plants. Fresh plants are provided weekly and old plants are kept in the cages for several weeks to allow neonates to hatch from eggs laid in the food-plants. We collected insects from the cage for experiments using an insect exhauster. For separation between adults and nymphs, insects were stunned with CO_2.

Herbivory treatment

We used two different ways of herbivory treatment of *N. attenuata* plants. Either we exposed only one leaf to *T. notatus* or the whole plant.

To treat one leaf we enclosed twenty *T. notatus* adults on the first stem leaf with a round plastic clip-cage (7 cm dimeter, 5 cm height). Clip-cages had holes covered with a fine mesh for air ventilation. Only one clip-cage per plant was applied. Control plants received empty clip-cages to distinguish from potential cage effects. Before sampling, mirid mortality was scored, and samples with more than 50% mortality were discarded. Control and damaged leaf lamina were collected at seven time-points (0, 24, 48, 72, 96, 120 and 144 h), snap frozen in liquid nitrogen and kept at -80°C until analysis.

To treat whole plants, plants were placed in the mirid rearing cage and control plants were placed in a cage without mirids. Damaged lamina of first stem leaf was sampled at the same time-points than the experiment described before or at time points given in the description of the according experiment. Both experiments were started in the morning (09:00 – 12:00).

Measurement of caffeoylputrescine and nicotine

Caffeoylputrescine and nicotine were determined using UHPLC-ToF-MS by analyzing extracted ion chromatograms as described in Schäfer, et al. (2015b) and (Brütting, et al. 2017).

80 % MeOH (v/v) was used for extraction of approximately 100 mg of frozen and ground leaf material from each sample. Values are presented as peak area * g FM^{-1}.

Trypsin Proteinase inhibitor (TPI) assay

TPI activity was determined using a radial diffusion assay (Jongsma *et al.* 1994) with approximately 50 mg of frozen and ground leaf-material. TPI-activity was normalized to leaf

protein content. The protein content of the extracts used for the TPI assay was determined with the Bradford-assay (Bradford 1976).

Measurement of jasmonic acid (JA), jasmonoyl-isoleucine conjugate (JA-Ile), cis-(+)-12-oxophytodienic acid (OPDA), salicylic acid (SA) and abscisic acid (ABA)

JA, JA-Ile, OPDA and SA were extracted and analyzed as described by Kallenbach *et al.* (2010). ABA was extracted in the same way and analyzed as described in Dinh *et al.* (2013).

Quantification of protein levels

Protein levels were determined using a Bradford assay (Bradford 1976) on a 96-well microtiter plate. We used around 50 mg of ground plant tissue and extracted it with 0.5 ml of 0.1 M TRIS HCL buffer (pH 7.6).

Measurement of starch, glucose, fructose and sucrose with a hexokinase assay

Glucose, fructose, sucrose and starch were determined in control and mirid-damaged leaf lamina following the protocol described by Machado, et al. (2013). Briefly, for soluble sugars 100 mg plant tissue was extracted first with 80% (v/v) ethanol and later twice with 50% (v/v) ethanol, each by incubation for 20 min at 80°C. Supernatants from all extractions were pooled together, and sucrose, glucose and fructose were quantified enzymatically as described by Velterop and Vos (2001). The remaining pellets were used for an enzymatic determination of starch content (Smith and Zeeman 2006).

Quantification of free amino acids

Free amino-acids were extracted from leaf material with acidified MeOH [MeOH:H_2O:HCOOH 15:4:1 (v/v/v)] and analyzed by LC-MS/MS (Bruker EVOQ Elite, www.bruker.com), as described by Schäfer *et al.* (2016).

Photosynthesis measurement

Photosynthesis rate was measured using a LI-COR LI-6400/XT portable photosynthesis measurement system. We measured photosynthesis rate at control leaves and leaves damaged by *T. notatus*. Leaves with clip-cage were analyzed in the covered area.

Chlorophyll measurement

Chlorophyll was quantified using a Minolta SPAD Chlorophyll meter 502. Chlorophyll content is displayed in arbitrary SPAD-units. We measured chlorophyll content at 3 different random spots of each analyzed leaf and used the average value as its chlorophyll content. Leaves with clip-cage were analyzed in the covered area.

Quantification of cytokinin related transcripts with qPCR

RNA was extracted with TRIzol (Invitrogene), according to the manufacturer instructions. cDNA was synthesized by reverse transcription using oligo(dT) primer and RevertAid reverse transcriptase (Invitrogen). qPCR was performed using actin as standard on a Stratagene Mx3005P qPCR machine using a SYBR Green reaction mix (Eurogentec; qPCR Core kit for SYBR Green I No ROX). The primer sequences are provided in Table S2.

Cytokinin measurement

CK-measurement was done as described by Schäfer, et al. (2016). In brief, CKs were extracted from 100 mg of fresh ground leaf material or around 10 mg of insects (around 20 adults) using acidified methanol and purified on reversed phase and cation exchange solid-phase extraction columns. The measurements were done via liquid chromatography coupled to a triple quadrupole MS (LC-MS/MS; Bruker EVOQ Elite, www.bruker.com) equipped with a heated electrospray ionization source. The method was extended for detection of ^{15}N labeled CKs. The parent → product ion transitions for ^{15}N labeled CKs are listed in Table S3. Chromatograms of IP, $[D_6]$-IP, $[^{15}N_5]$-IP as well as IPR, $[D_6]$-IPR and $[^{15}N_5]$-IPR are shown in Figure 7 S4 and 7 S5.

Rearing T. notatus *on artificial diet*

For the artificial diet we dissolved amino acids ($_L$-alanine, 50 mg; $_L$-arginine, 30 mg; $_L$-cysteine, 20 mg; glycine, 20 mg; $_L$-histidine, 30 mg; $_L$-leucine, 30 mg; $_L$-lysine, 20 mg; $_L$-phenylalanine, 30 mg; $_L$-proline, 80 mg; $_L$-serine, 100 mg; $_L$-tryptophan, 500 mg; $_L$-tyrosine, 10 mg; $_L$-valine, 40 mg; $_L$-asparagine, 200 mg; $_L$-aspartic acid, 200 mg; $_L$-glutamine, 500 mg; $_L$-glutamic acid, 300 mg; $_L$-isoleucine, 20 mg; $_L$-methionine, 10 mg; $_L$-threonine, 100 mg), sugars (glucose, 400 mg; fructose, 150 mg; sucrose, 800 mg) and vitamins (Vanderzant Vitamine mix, 650 mg) in 40 mL water and sterile filtrated it. Additionally, we prepared an agar solution (1 g Agar-Agar in 60 mL water) that was sterilized by autoclaving. After cooling down the liquid agar solution to approximately 60°C in a water bath we added the amino acid/sugar/vitamin solution and aliquoted each 400 µL of the diet under sterile conditions in single 0.5 mL reaction tubes where it solidified. The tubes were stored in the fridge until use.

For the experiment mirids were placed in plastic boxes (10 * 6 * 6 cm), covered with paper tissue and sealed with a perforated lid. 15 to 20 mirids were placed in each box and a tube of the described artificial diet was offered as sole food and water source. To prevent the diet to get moldy it was replaced each day with a new tube. The boxes were kept at a shaded place under greenhouse conditions described above. After 5 days the surviving mirids were collected and shock frozen in liquid nitrogen and stored at -80°C until extraction.

^{15}N labeling of plants and insects to track transfer of cytokinins

N. attenuata plants with more than 98% total nitrogen content represented by ^{15}N were obtained following the protocol described by Ullmann-Zeunert, et al. (2012). Briefly, twelve days after germination, plants were transferred into 50 ml hydroponic culture single pots containing only $Ca(^{15}NO_3)_2$ as nitrogen source. Ten days later, they were moved to 1 L hydroponic culture pots with the same $^{15}NO_3$- concentration in the form of $K^{15}NO_3$. Once per week, the plants were fertilized with 1 mM $K^{15}NO3$ and the pots were filled up to 1 L with water.

To generate ^{15}N labeled *T. notatus* we reared them for a generation on ^{15}N-labelled *N. attenuata* plants. Two-hundred adult females were transferred to a 47.5x47.5x93 cm insect cage with four early elongated ^{15}N-labelled *N. attenuata* plants. Females were allowed to lay eggs for four days and were then removed. ^{15}N-labelled plants were fertilized once a week (as described above), and after three weeks two fresh ^{15}N-labelled plants were added into the insect cage. One week after the first adults emerged, the ^{15}N labeled mirids were collected and used for the cytokinin transfer experiment.

Cytokinin transfer experiment

We performed studies of transferring of cytokinins from mirids to *N. attenuata* plants in two ways. In the first experiment, twenty ^{15}N-labelled *T. notatus* adults were quickly anesthetized with CO_2 prior to be clip-caged on the first stem leaf. Only one clip-cage per plant was applied. We collected leaf lamina corresponding to the area included in the clip-cage and thus damaged by mirids at different time-points: 0, 3, 6, 24, 48, 72, 96 and 120 h, and froze it in liquid nitrogen. We conserved samples at -80°C until analysis.

In the second experimental setup we directly placed five ^{15}N-labelled *N. attenuata* plants in the mirid rearing cage. We sampled the first stem leaf at different time-points: 0, 3, 6, 24, 48, 72, 96 and 120 h. We transferred plants once per day to a different cage to ensure that mirids do not accumulate ^{15}N metabolites. We separated the leaf lamina from the mid-rib and froze it in liquid nitrogen. We conserved the samples at -80°C until analysis.

Determination of CKs in oral secretions and frass

The collection of mirid oral secretions was done similar as described by Halitschke, et al. (2011) with some modifications. In brief, we placed mirids in plastic boxes (10 * 6 * 6), covered with paper tissue and sealed with a perforated lid. We placed 15 to 20 mirids in each box and offered an upside-down lid of a scintillation vial filled to the top with sugar water (~3 mL, 40 mM glucose) as sole food and water source. To separate oral secretions from frass and to prevent insects from drowning, we covered the lids with a thin layer of Parafilm. After 24h we collected the lids, removed the sugar water with a syringe and washed off the frass spots on the parafilm with MeOH. As control we used sugar water containing lids that were kept in boxes without mirids under the same conditions. We pooled frass samples and sugar water samples originating from approximately 100 mirids. We evaporated the sugar solution in a freeze dryer overnight. We extracted and analyzed the CKs as described for plant and insect tissues (We used extraction buffer to dissolve the evaporated sugar solution).

Damage distribution under field conditions in WT plants

Damaged area on different leaf types was estimated in % of the total leaf surface. We estimated the damaged proportion in 3 different leaf types (See Fig. 6 S1 A): Rosette leaf, the first 3 stem leaves and all younger stem leaves and side branches.

Choice assays under field conditions

We collected insect from their natural environment at our field station in Utah, USA. 10-15 *T. notatus* insects were placed in a plastic cup. The cup was connected to two other plastic cups (Fig. 6 S1 B), one with a fully grown stem leaf inside and the other with a young, growing tissue (apical meristem and small growing leaves). Plant material was attached to a 2 ml plastic tube filled with water to prevent it from drying out. We gave insects one night (12 h) to choose one of the two cups. In the morning we counted the number of mirids in each cup.

Choice assays between EV and irchk2/3 plants

We placed WT and ir*chk2/3* plants in a big cage in our greenhouse (3 * 4 * 1.6 m). The cage was covered with a fine mesh to prevent insects from escaping. We released about 500 *T. notatus* in the cage and estimated the damage on each plant (as described above) after 10 days of exposition to *T. notatus.*

*Choice assays using i-ov*ipt *plants*

Data from choice assays were taken from the dataset published in (Schäfer, et al. 2013). i-ov*ipt* plants were either treated with LAN (control) or with DEX as described above. We treated the first 10 stem leaves of a flowering plant. We placed each one DEX and one LAN treated plant in one 47.5x47.5x93 cm insect cage and added about 100 *T. notatus*. We estimated damaged area on leaves after 10 days. We calculated an average damage level from all 10 treated leaves from each plant, which was counted as one replicate.

Chemicals

All used chemicals were obtained from Sigma-Aldrich (http://www.sigmaaldrich.com/), Merck (http://www.merck.com/), Roth (http://www.carlroth.com/) or VWR (http://www.vwr.com), if not mentioned otherwise in the text. CK standards were obtained from Olchemim (http://www.olchemim.cz), DEX from Enzo Life Sciences (http://www.enzolifesciences.com/), HCOOH for ultra-performance LC from Fisher Scientific (http://www.fisher.co.uk/), otherwise from Riedel-de Haën (http://www.riedeldehaen.com/) and GB5 from Duchefa (http://www.duchefa-biochemie.nl/).

Statistical analysis

Data were analyzed using R 3.3.1 (2016-06-21; http://www.r-project.org). Relevant tests and number of replicates are mentioned in the figure legends. We used two-way ANOVAs (TWA) to analyze effect of mirid feeding, time after start of mirid attack and interaction of both factors in Figures 1 S1, 2, 2S1, 2 S2, 2 S3, 3 A and B, 3 S1, 3 S2, 3 S3. In all experiments, where clip-cages were used, we only used data from control clip-cages and clip-cages with mirids for analysis. Additionally, we carried out Welch t-tests or Wilcoxon–Mann–Whitney tests (if not homoscedastic) between control and *T. notatus* attacked samples. These tests were done for each time point of induction separately. The comparisons of the clip-cage experiments were Bonferroni corrected.

Data in Figure 1 and 3 C have been analyzed with a Wilcoxon–Mann–Whitney test. Data in Figure 3 S4A, 6 S1, 7A have been analyzed with a one-way ANOVA followed by a Tukey's HSD *post hoc* test. Data in Figure 3 S4 B, 6 and 6 S1 B have been analyzed by Welch t-test. If necessary, data were transformed to fit requirements of the particular test (homoscedasticity, normality)

Error bars in the figures represent standard errors. Differences were considered significant if $p < 0.05$.

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FIGURES

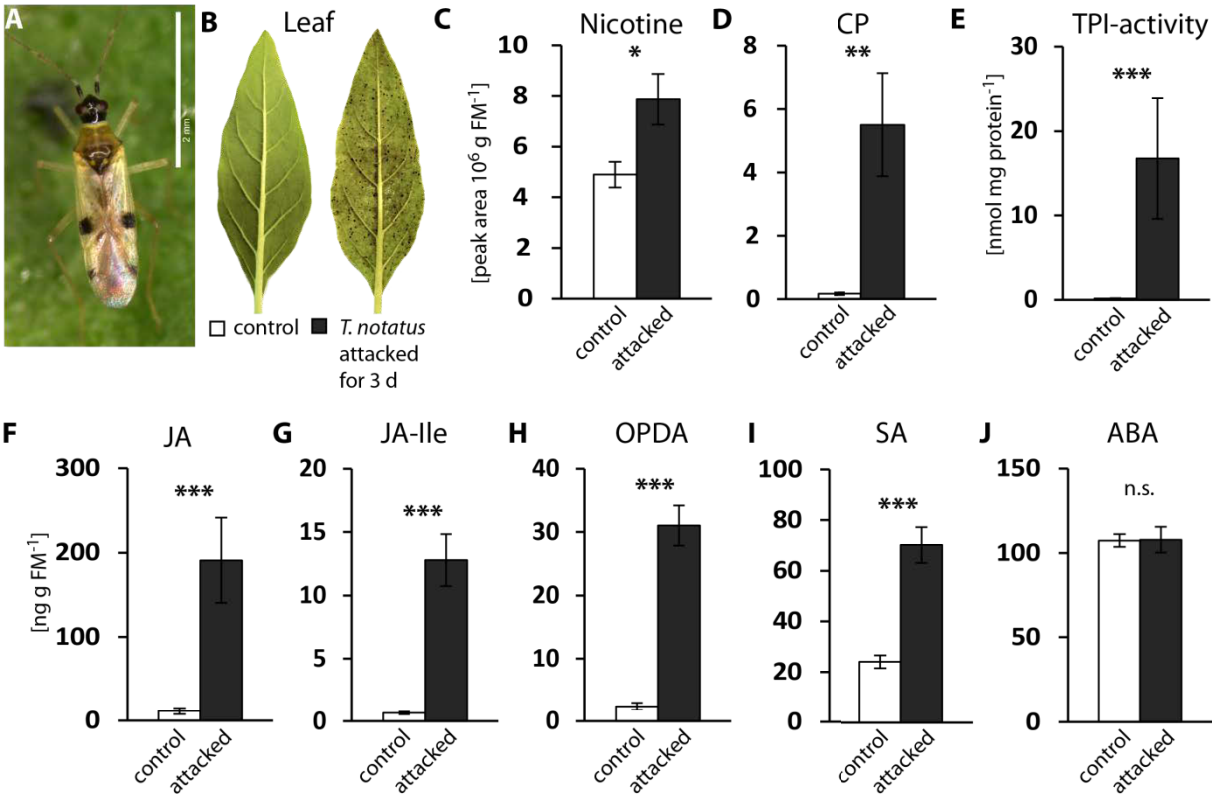

Figure 1: *Tupiocoris notatus* feeding induces JA-dependent defense reactions in *Nicotiana attenuata*.

A Control leaf of *N. atteunata* and a leaf after 3 d attack by *T. notatus*. **B** *T. notatus* adult. **C-J** Defense metabolites and stress related hormone levels induced by 3 d *T. notatus* attack (dark columns) and in control leaves (white)*:* **C** nicotine, **D** caffeoylputrescine (CP), **E** trypsin proteinase inhibitor (TPI) activity, **F** jasmonic acid (JA), **G** jasmonic acid-isoleucine conjugate (JA-Ile), **H** *cis*-(+)-12-oxophytodienoic acid (OPDA), **I** salicylic acid (SA) and **J** abscicic acid (ABA). Wilcoxon-Mann-Whitney test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, n.s.: not significant. Error bars depict standard errors. N≥6. FM, fresh mass.

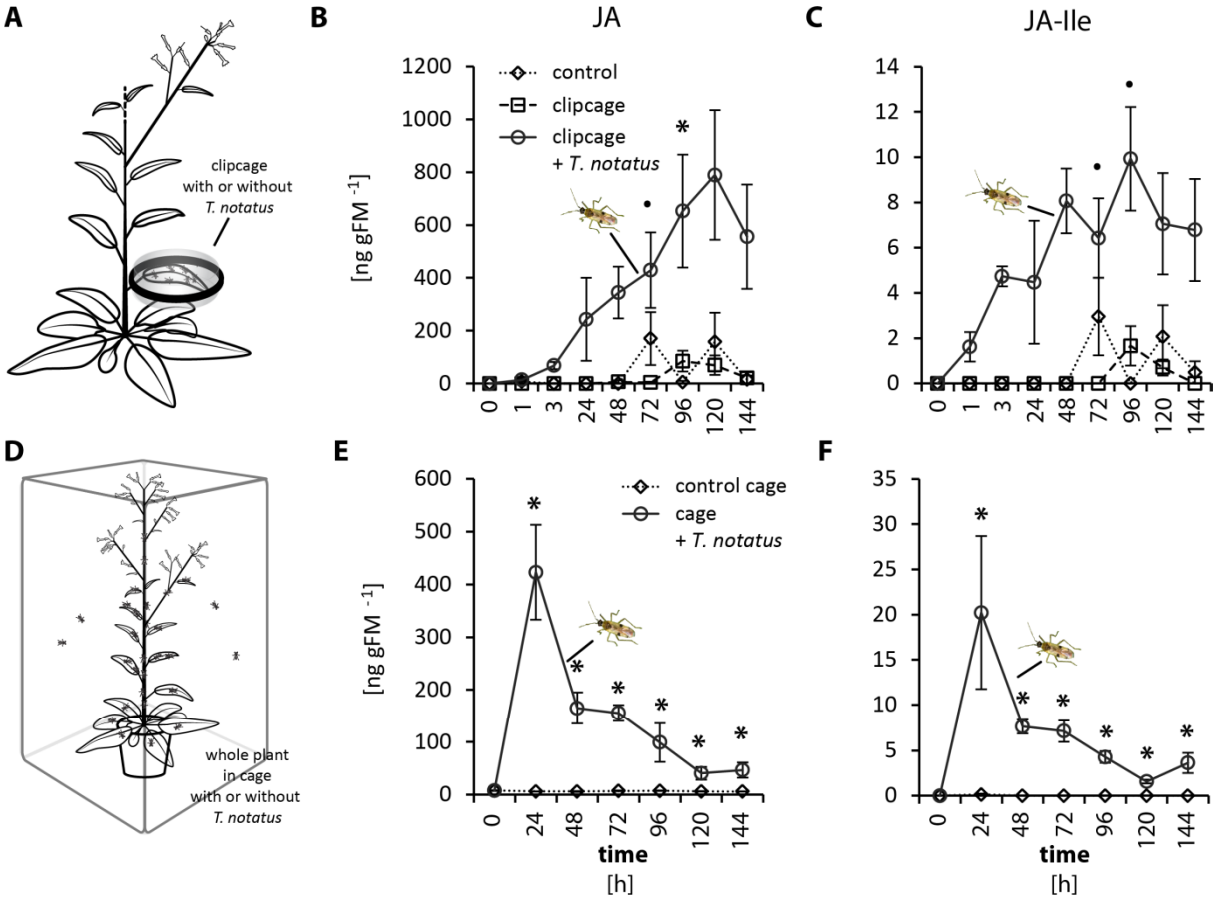

Figure 1 S1: *Tupiocoris notatus* feeding increases levels of JA and JA-Ile.

A Experimental setup corresponding to **B** and **C**. On each plant we caged one leaf in a plastic clipcage with (clipcage + *T. notatus*) or without (clipcage) 20 *T. notatus.* Additionally, we collected untreated control leaves (control, dotted line). **B** Jasmonic acid (JA) and **C** jasmonic acid-isoleucine conjugate (JA-Ile) were monitored over 144 h. Wilcoxon rank sum test with Bonferroni correction between clipcage and clipcage + *T. notatus* (B,C) for each time point: • $P < 0.1$, * $P < 0.05$. Error bars depict standard errors. $N \geq 3$. **D** Experimental setup corresponding to **E** and **F**. A whole plant was caged in an insect cage with (cage + *T. notatus*) or without (control cage) *T. notatus* adults. **E** JA and **F** JA-Ile kinetics were monitored over 144 h. Wilcoxon–Mann–Whitney test between control and *T. notatus* attacked leaves (E, F) for each time point: * $P < 0.05$. Error bars depict standard errors. N=4. FM, fresh mass.

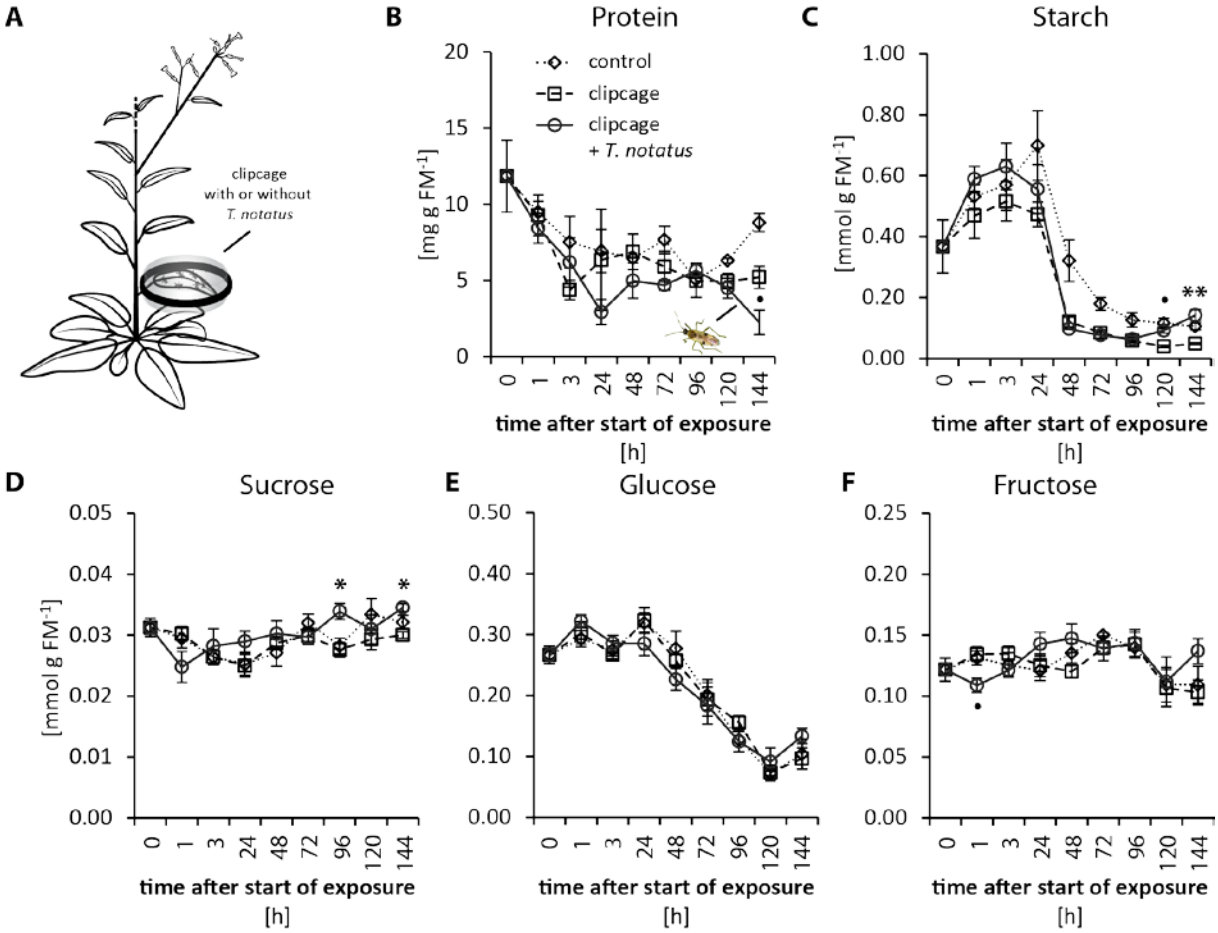

Figure 2: *Tupiocoris notatus* feeding is not significantly changing nutrient levels.

A Experimental setup: On each plant we caged one leaf in a plastic clipcage with 20 *T. notatus* (clipcage + *T. notatus*; solid line) or without (clipcage, dashed line). Additionally, we collected untreated control leaves (control, dotted line). **B** Protein, **C** starch, **D** sucrose, **E** glucose and **F** fructose were analyzed in a time-kinetic from 1 – 144 h. Pairwise t-test (B, C, D and F) or Wilcoxon rank sum test (E) with Bonferroni correction between clipcage and clipcage + *T. notatus* for each timepoint: • $P < 0.1$, * $P < 0.05$, ** $P < 0.01$. Error bars depict standard errors. N ≥ 3. FM, fresh mass.

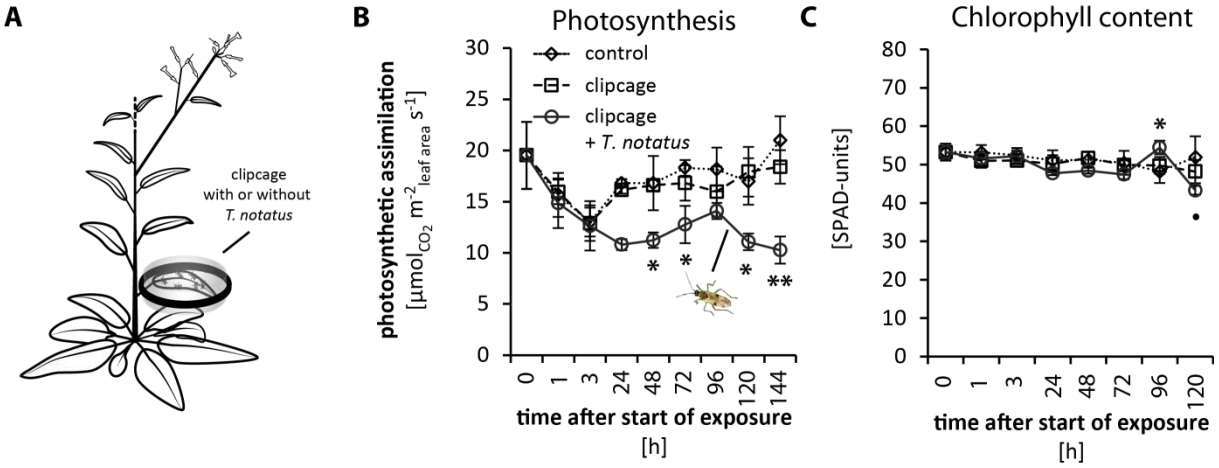

Figure 2 S1*:* *Tupiocoris notatus* feeding is decreasing photosynthetic activity while not influencing chlorophyll content.

A Experimental setup: On each plant we caged one leaf in a plastic clipcage with 20 *T. notatus* (clipcage + *T. notatus*; solid line) or without (clipcage, dashed line). Additionally, we collected untreated control leaves (control, dotted line). **B** Photosynthetic assimilation and **C** chlorophyll content in a time-kinetic from 1 – 144 h (120 h). Pairwise t-test with Bonferroni correction between clipcage and clipcage + *T. notatus*: • $P < 0.1$, * $P < 0.05$, ** $P < 0.01$. Error bars depict standard errors. $N \geq 3$.

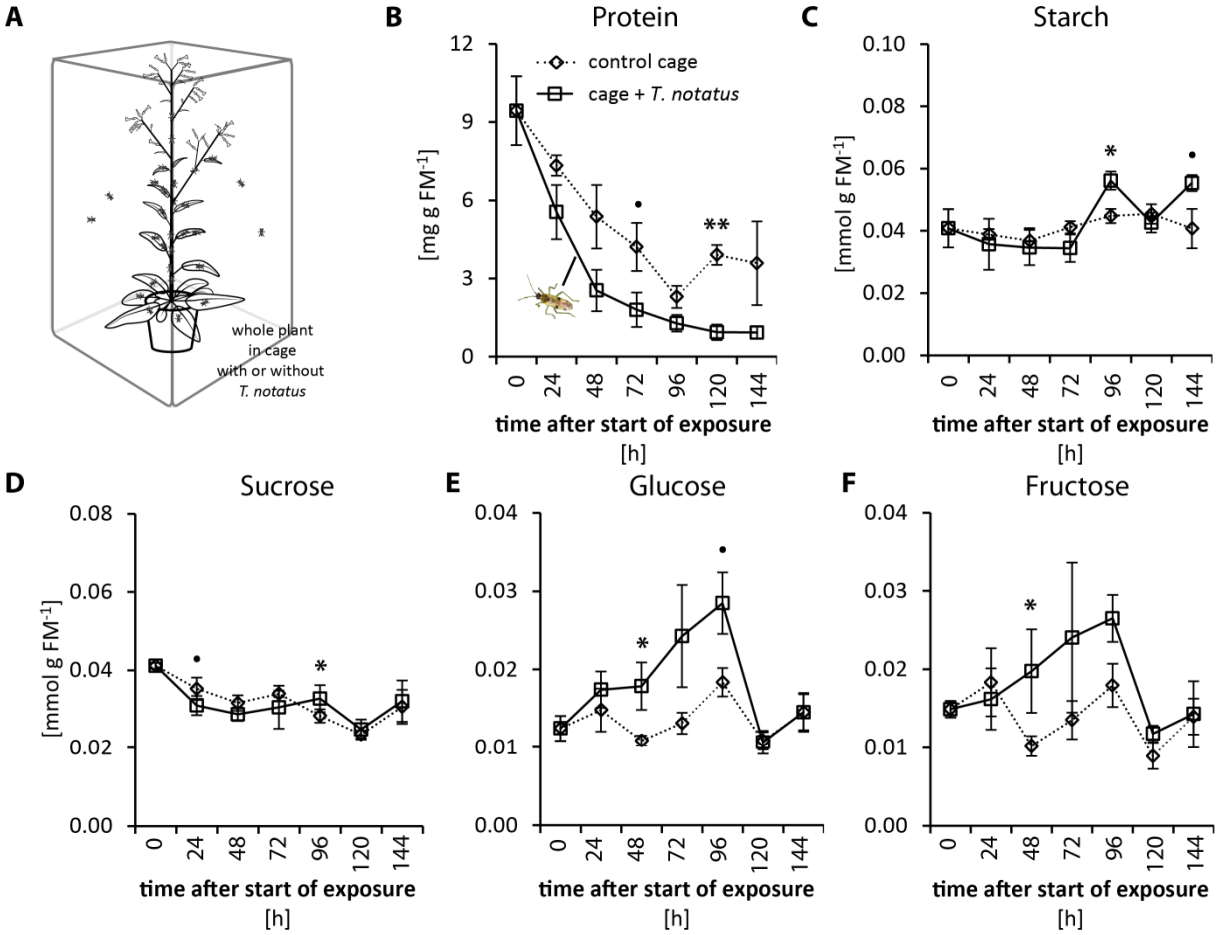

Figure 2 S2: *Tupiocoris notatus* feeding on whole plants is affecting nutrient levels in leaves of Nicotiana attenuata.

A Experimental setup: whole plants were caged with *T. notatus* (cage + *T. notatus* attacked; solid line) or without (control cage, dotted line). **B** Protein, **C** starch, **D** sucrose, **E** glucose and F fructose were monitored in a time-kinetic from 24 – 144 h. Welch t-test (B, C and E) or Wilcoxon–Mann–Whitney test (D, F) between control and *T. notatus* attacked: • $P < 0.1$, * $P < 0.05$, ** $P < 0.01$. Error bars depict standard errors. $N \geq 4$ (3:B, D). FM, fresh mass.

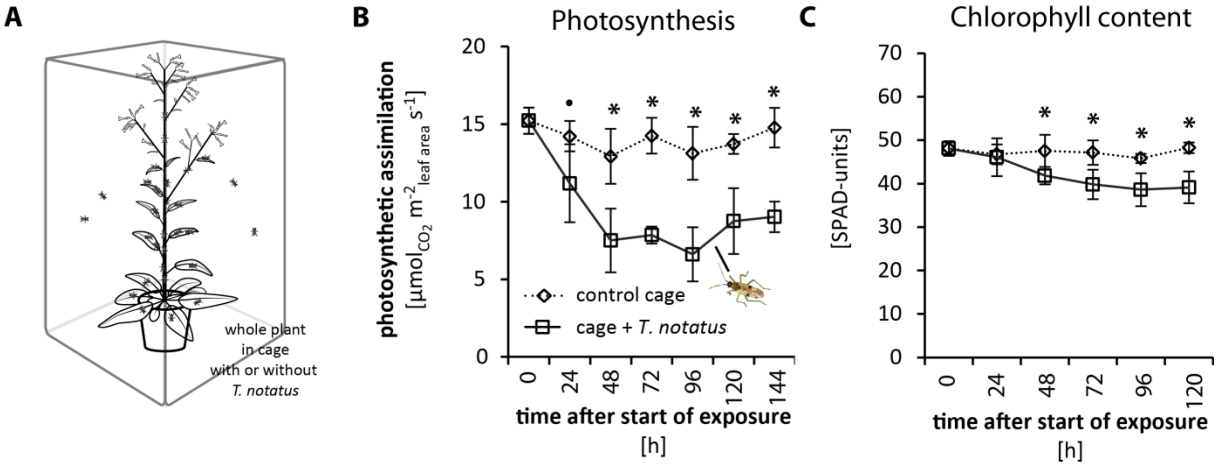

Figure 2 S3: *Tupiocoris notatus* feeding is decreasing photosynthetic activity and chlorophyll content in leaves of *Nicotiana attenuata*..

A Experimental setup: whole plants were caged with *T. notatus* (cage +*T. notatus*; solid line) or without (control cage, dotted line). **B** Photosynthetic assimilation and **C** chlorophyll content were monitored in a time-kinetic from 24 – 144 h (120 h). Welch t-test (C) or Wilcoxon–Mann–Whitney test (B) between control and *T. notatus* attacked: • $P < 0.1$, * $P < 0.05$. Error bars depict standard errors. N = 4.

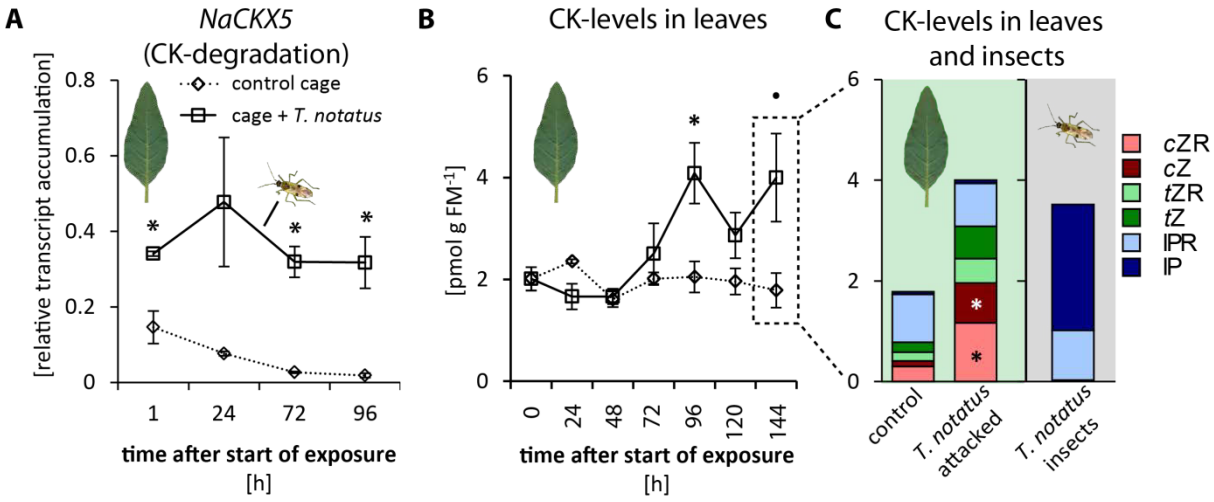

Figure 3: *Tupiocoris notatus* is influencing *Nicotiana attenuata's* cytokinin (CK) metabolism and contains high amounts of CKs in its body.

A Transcript accumulation of *NaCKX5*: cytokinin oxidase/dehydrogenase 5 (inactivation of CKs by oxidation) and **B** CK levels in leaves: sum of *cis*-zeatin (*c*Z), *trans*-zeatin (*t*Z), N6-isopentenyladenine (IP) and their ribosides (*c*ZR, *t*ZR, IPR) in leaves exposed to *T. notatus* feeding (cage + *T. notatus*, solid line) and control leaves (control cage, dotted line) at different time points after start of exposure. **C** Single CK types in leaves after 144 h of exposure to *T. notatus* and in the insects themselves. Wilcoxon-Mann-Whitney test between control and attacked leaves at single time points: • $P < 0.1$, * $P < 0.05$. Error bars depict standard errors. A: $N \geq 2$; B, C: $N = 4$. FM, fresh mass.

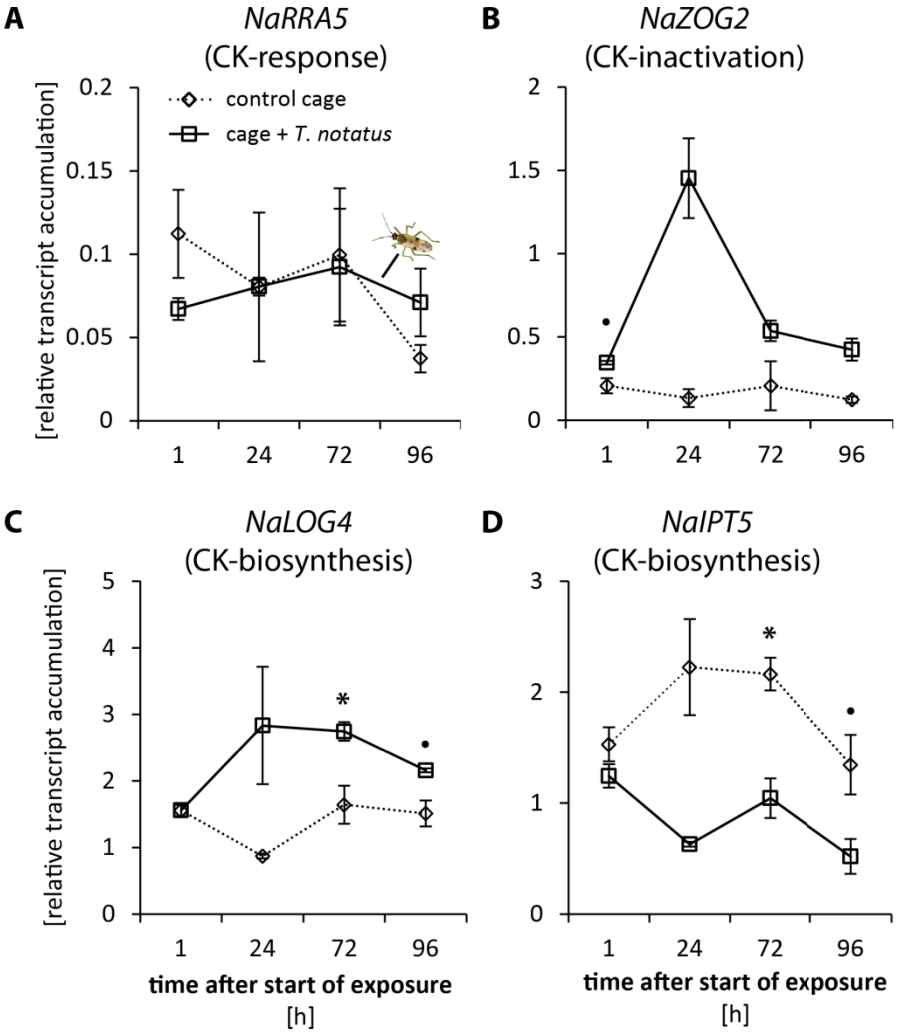

Figure 3 S1: *Tupiocoris notatus* feeding is influencing transcript levels of cytokinin inactivation and cytokinin biosynthesis genes in *Nicotiana attenuata*.

Relative transcript accumulation (*NaActin* as reference gene) in leaves infested with *T. notatus* (cage + *T. notatus*, solid line) and control leaves (control cage, dotted line) at different time points after start of exposure. **A** *NaRRA5*: CK response regulator 5. **B** *NaZOG2*: Zeatin-O-glucosyltransferase 2. **C** *NaLOG4*: Cytokinin riboside 5'-monophosphate phosphoribohydrolase LOG (LONELY GUY) 4. **D** *NaIPT5*: Isopentenyltransferase 5. Welch-Two-Sample-t-test control and attacked leaves at single time points: • $P < 0.1$, * $P < 0.05$. Error bars depict standard errors. $N \geq 2$.

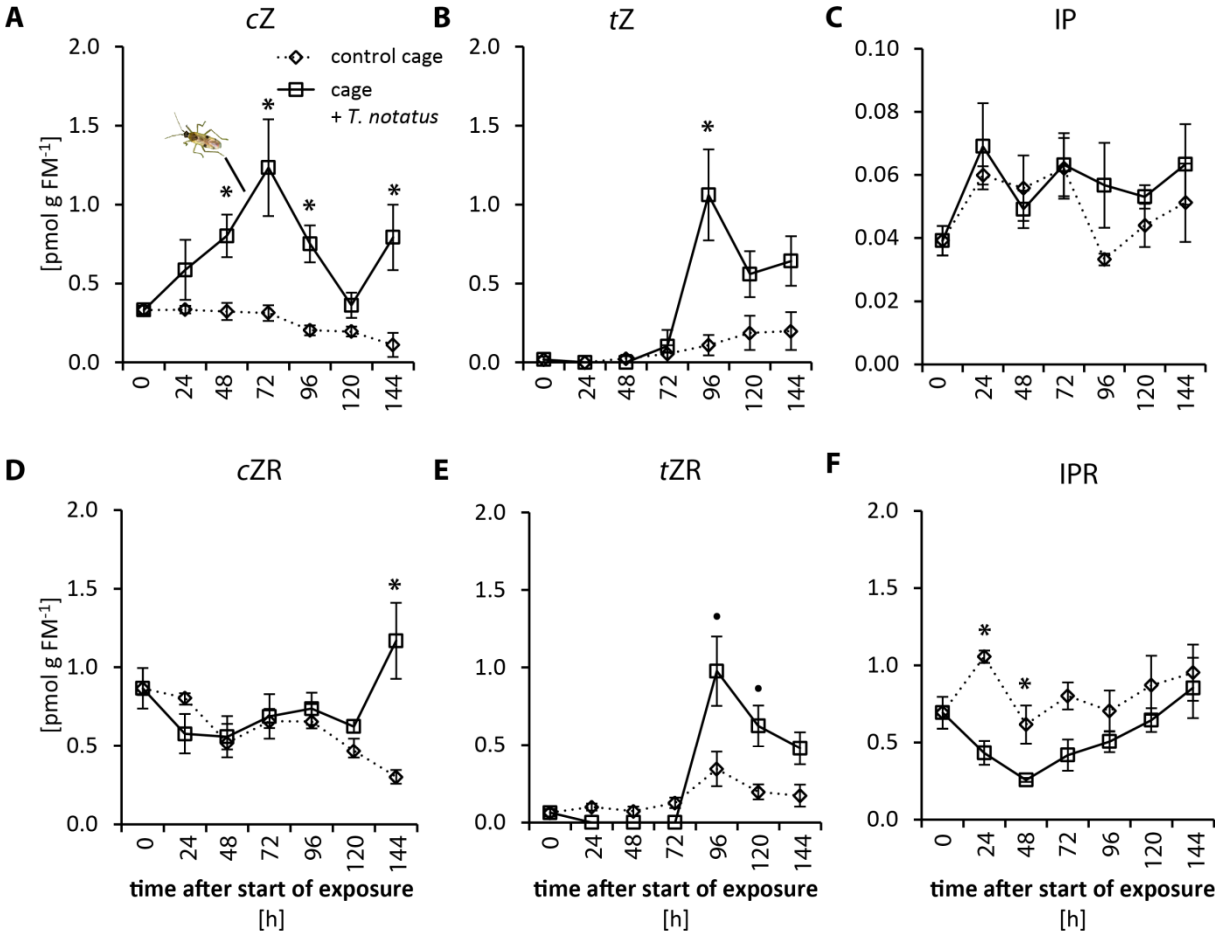

Figure 3 S2: *Tupiocoris notatus* feeding affects cytokinin (CKs) levels in *Nicotiana attenuata* leaves.

CK levels in leaves infested with *T. notatus* (cage + *T. notatus*, solid line) and control leaves (control cage, dotted line) at different time points after start of exposure. **A** *cis*-Zeatin (*c*Z), **B** *trans*-zeatin (*t*Z), **C** *N6*-isopentenyladenine (IP) and their ribosides **D** *c*ZR, **E** *t*ZR, **F** IPR. Wilcoxon-Mann-Whitney test between control and attacked leaves at single time points: • $P < 0.1$, * $P < 0.05$. Error bars depict standard errors. N = 4. FM, fresh mass.

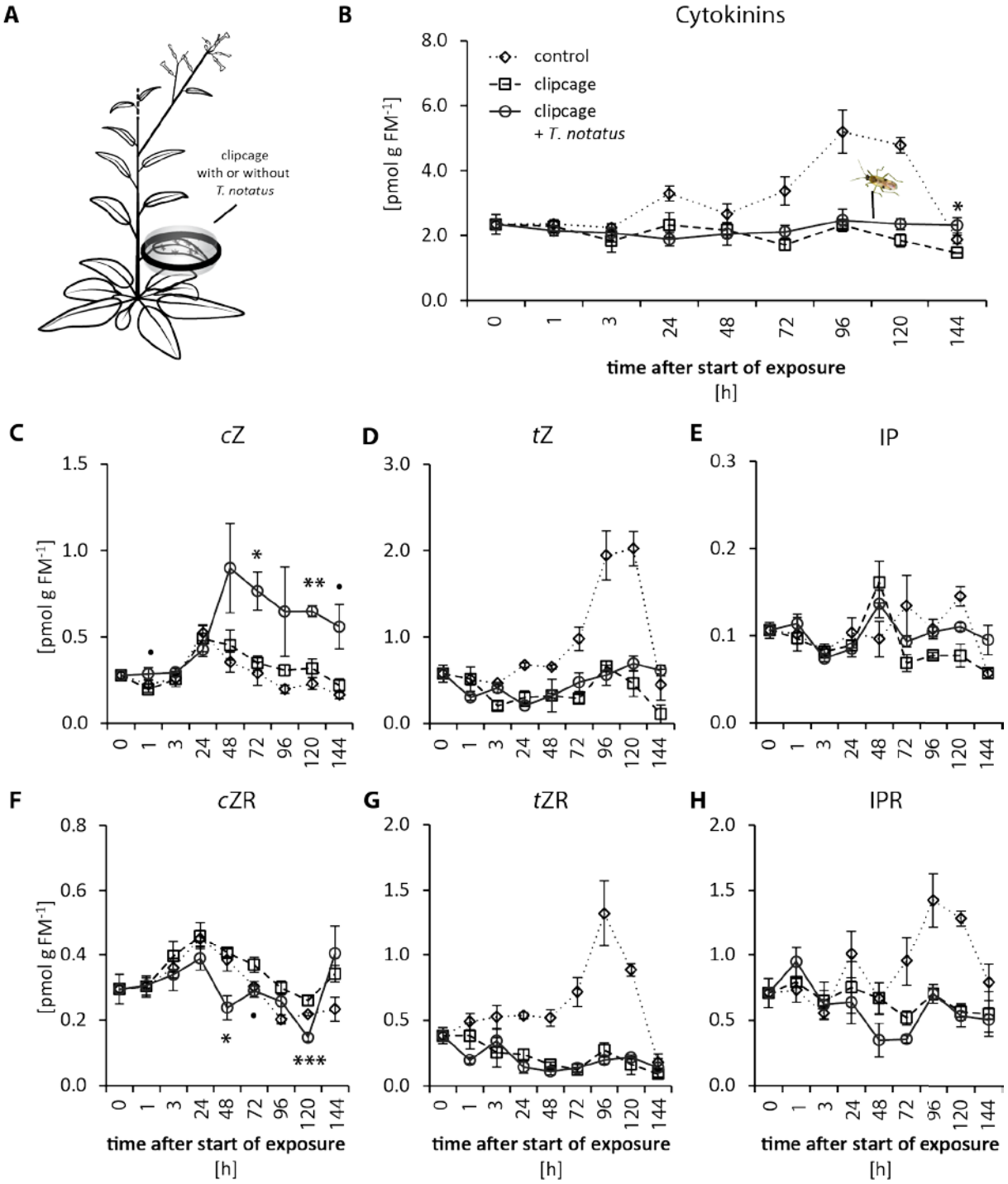

Figure 3 S3: Influence of *Tupiocoris notatus* single-leaf feeding on cytokin levels.

A Experimental setup: one leaf is caged with plastic clipcage with 20 *T. notatus* (clipcage + *T. notatus*; solid line) or without (clipcage, dashed line). Additionally, we collected untreated control leaves (control, dotted line). **B – H** CK values in leaves at different time-points after start of exposure: **B** sum of *cis*-zeatin (*c*Z), *trans*-zeatin (*t*Z), N6-isopentenyladenine (IP) and their ribosides (*c*ZR, *t*ZR, IPR). **C** *c*Z, **D** *t*Z, **E** IP, **F** *c*ZR, **G** *t*ZR and **H** IPR. Pairwise t-tests with

Bonferroni correction between clipcage and clipcage + *T. notatus* for each timepoint: • $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Error bars depict standard error. $N \geq 3$. FM, fresh mass.

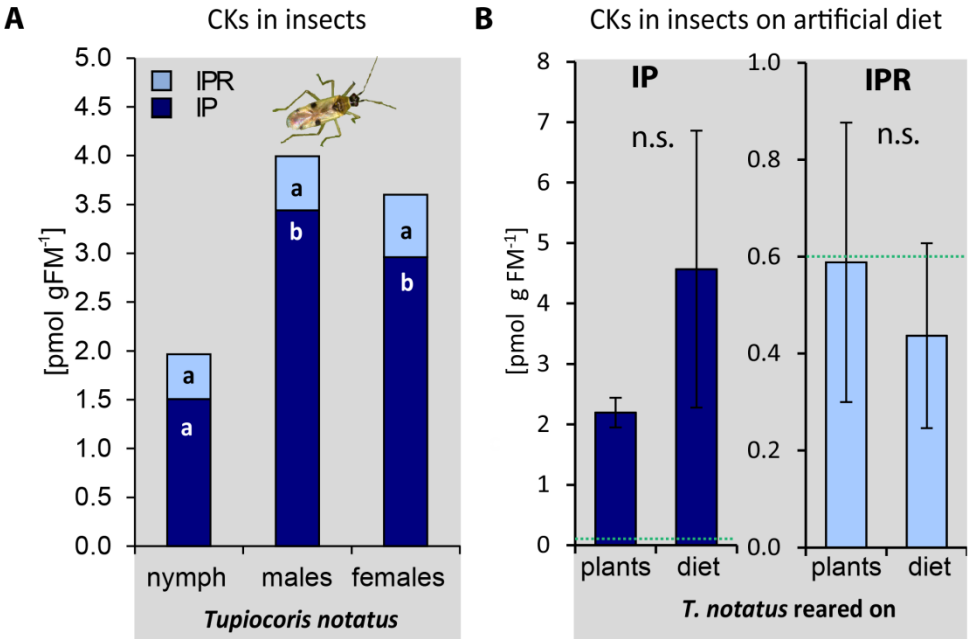

Figure 3 S4: *Tupiocoris notatus* contains high amounts of *N6*-isopentenyladenine (IP) in its body independent from its sex or food source.

A IP and IPR (*N6*-isopentenyladenosine) in nymphs, males and females of *T. notatus*. One-Way-ANOVA with Tukey HSD posthoc test. N ≥ 3. **B** IP and IPR in *T. notatus* adults reared on plants or on artificial diet. Green dotted lines present representative levels of IP and IPR in leaf tissue. Welch-t-test. N.s: not significant. N ≤ 7. Error bars depict standard errors. FM, fresh mass.

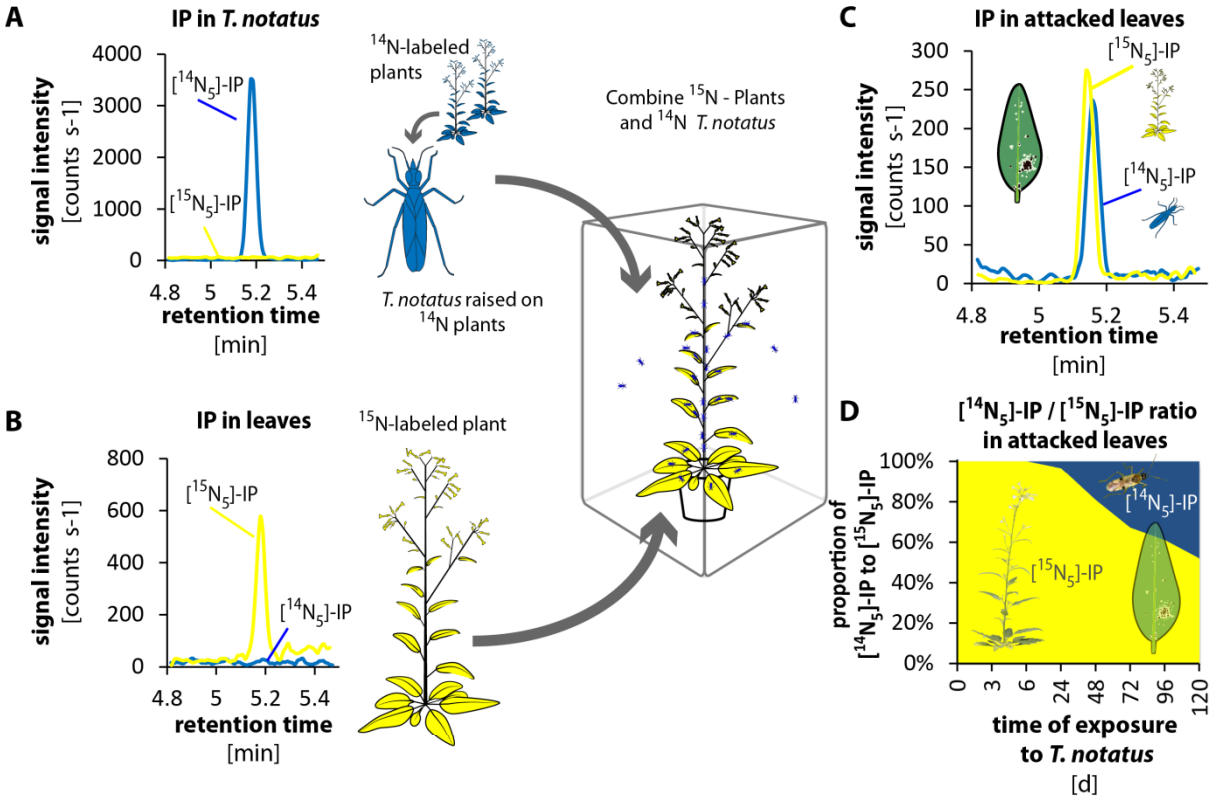

Figure 4: *Tupiocoris notatus* transfers IP to leaves of its hostplant.

A and **B** Experimental setup and chromatograms of IP: **A** *T. notatus* raised on hydroponic plants grown on a ^{14}N containing N-source have only [$^{14}N_5$]-IP in their body. **B** Plants raised on a hydroponic medium containing only a ^{15}N containing N-source have only [$^{15}N_5$]-IP in leaves. ^{15}N labeled plants and ^{14}N labeled insects were placed in the same cage for 5 days. Ratio of [$^{14}N_5$]-IP (originating from insects, blue) and [$^{15}N_5$]-IP (from hostplant, yellow) were determined in attacked leaves. **C** Chromatogram of [$^{14}N_5$]-IP and [$^{15}N_5$]-IP in the leaves of 5d attacked plants. **D** Ratio of [$^{14}N_5$]-IP and [$^{15}N_5$]-IP at different time-points after start of exposure to *T. notatus*. N ≥ 3.

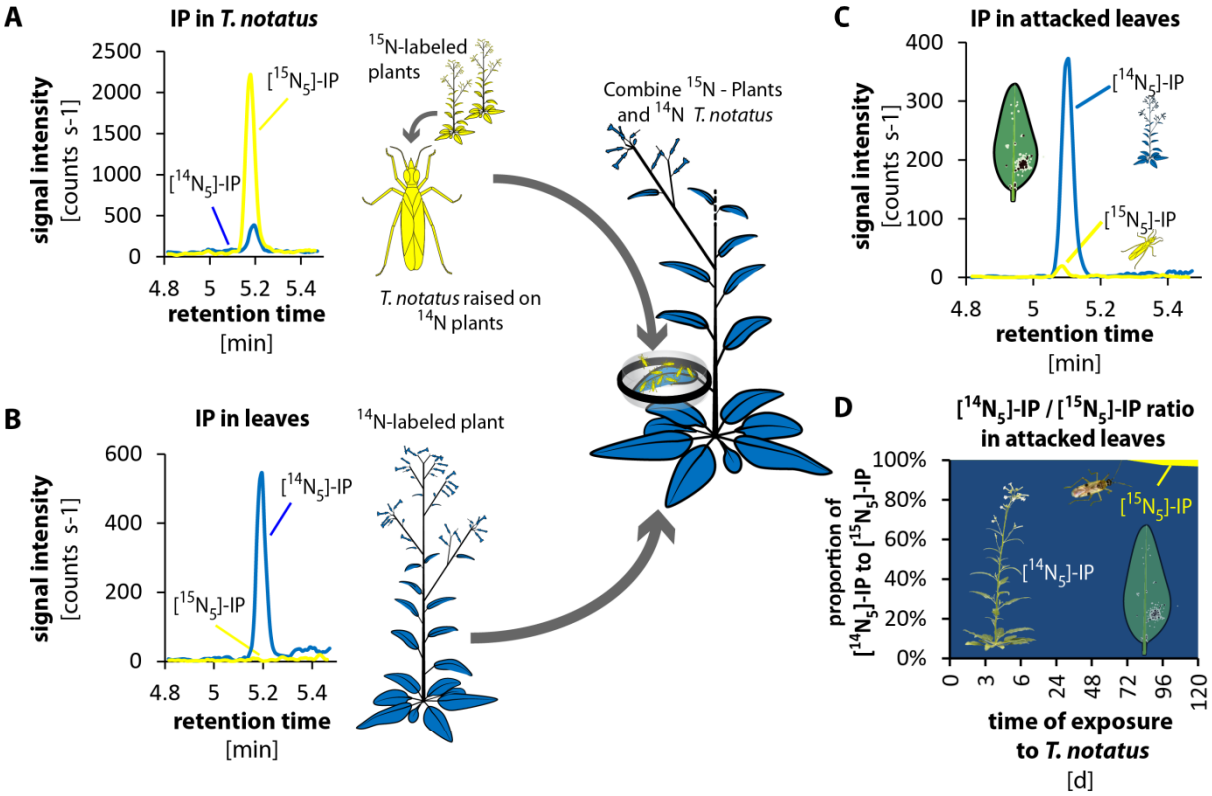

Figure 4 S1: Few individuals of *Tupiocoris notatus* transfer detectable amounts of IP to leaves of its host plant.

A and **B** Experimental setup and chromatograms of IP: **A** *T. notatus* raised on hydroponic plants grown on a ^{15}N containing N-source have only [$^{15}N_5$]-IP in their body. **B** Plants raised on a hydroponic medium containing only ^{14}N containing N-source have only [$^{14}N_5$]-IP in leaves. 20 ^{15}N labeled insects were placed in small cages on one leaf of ^{14}N labeled plants for 5 days. Ratio of [$^{15}N_5$]-IP (originating from insects, yellow) and [$^{14}N_5$]-IP (from hostplant, blue) were determined in attacked leaves. **C** Chromatogram of [$^{15}N_5$]-IP and [$^{14}N_5$]-IP in the leaves that were 5d attacked. **D** Ratio of [$^{15}N_5$]-IP and [$^{14}N_5$]-IP at different time-points after start of exposure to *T. notatus*. $N \geq 3$.

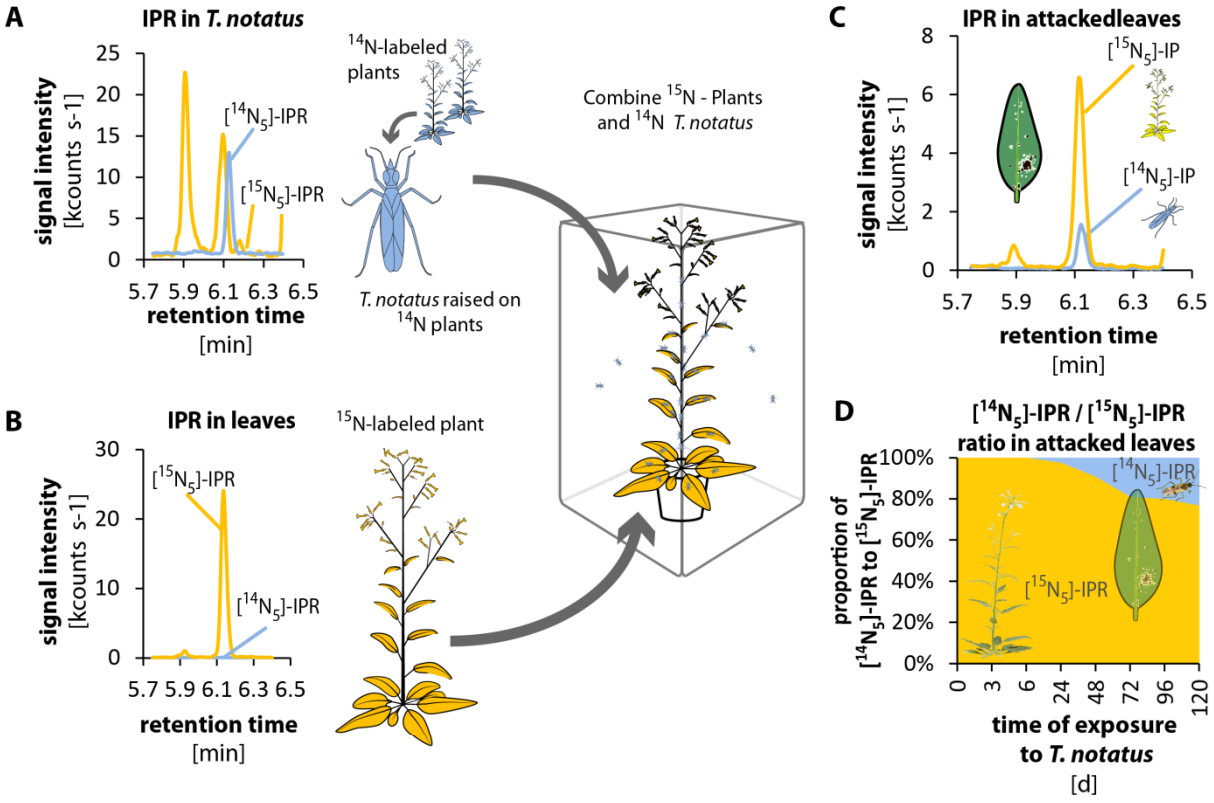

Figure 4 S2: *Tupiocoris notatus* transfers IPR to leaves of its hostplant**.**

A and **B** Experimental setup and chromatograms of IPR: **A** *T. notatus* raised on hydroponic plants grown on a ^{14}N containing N-source have only [$^{14}N_5$]-IPR in their body. **B** Plants raised on a hydroponic medium containing only a ^{15}N containing N-source have only [$^{15}N_5$]-IPR in leaves. ^{15}N labeled plants and ^{14}N labeled insects were placed in the same cage for 5 days. Ratio of [$^{14}N_5$]-IPR (originating from insects, blue) and [$^{15}N_5$]-IPR (from hostplant, yellow) were determined in attacked leaves. **C** Chromatogram of [$^{14}N_5$]-IPR and [$^{15}N_5$]-IPR in the leaves of 5d attacked plants. **D** Ratio of [$^{14}N_5$]-IPR and [$^{15}N_5$]-IPR at different time-points after start of exposure to *T. notatus*. $N \geq 3$.

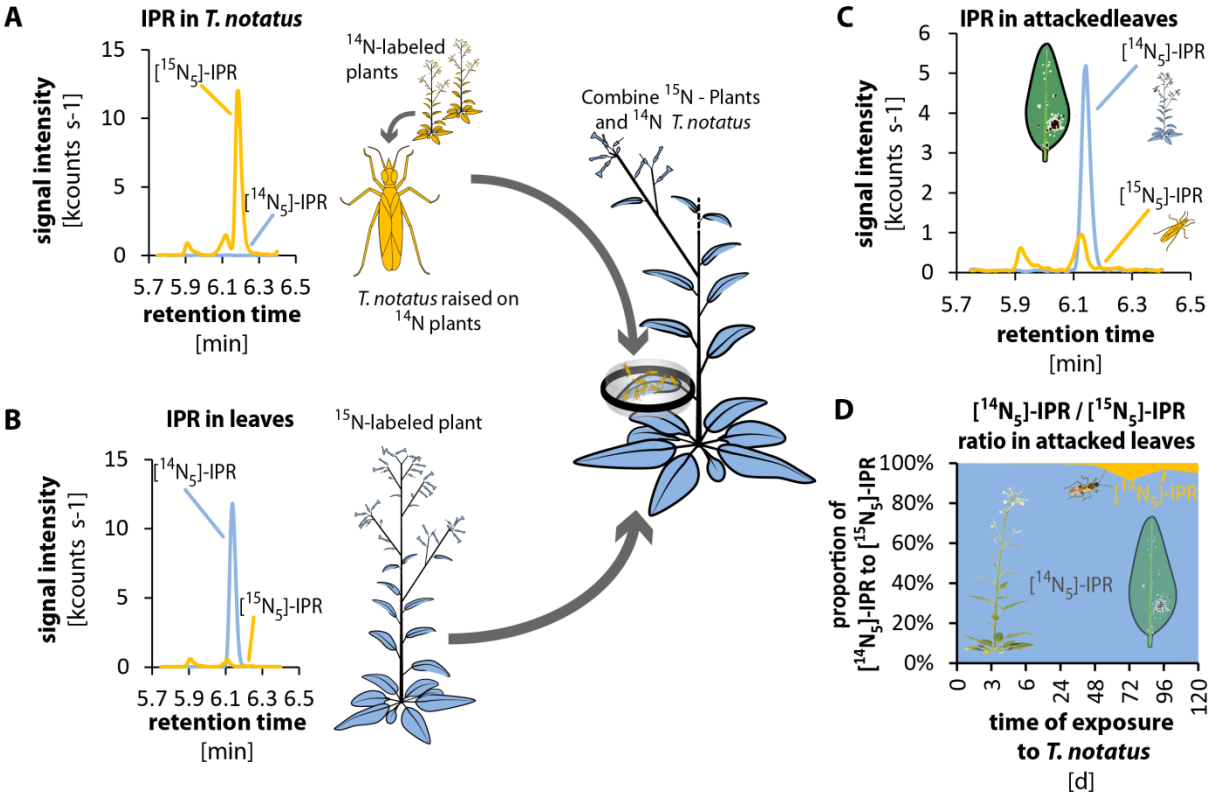

Figure 4 S3: Few individuals of *Tupiocoris notatus* transfer barely detectable amounts of IPR to leaves of its host plant.

A and **B** Experimental setup and chromatograms of IP: **A** *T. notatus* raised on hydroponic plants grown on a ^{15}N containing N-source have only [$^{15}N_5$]-IPR in their body. **B** Plants raised on a hydroponic medium containing only ^{14}N containing N-source have only [$^{14}N_5$]-IPR in leaves. 20 ^{15}N labeled insects were placed in small cages on one leaf of ^{14}N labeled plants for 5 days. Ratio of [$^{15}N_5$]-IPR (originating from insects, yellow) and [$^{14}N_5$]-IPR (from hostplant, blue) were determined in attacked leaves. **C** Chromatogram of [$^{15}N_5$]-IPR and [$^{14}N_5$]-IPR in the leaves that were 5d attacked. **D** Ratio of [$^{15}N_5$]-IPR and [$^{14}N_5$]-IPR at different time-points after start of exposure to *T. notatus*. $N \geq 3$.

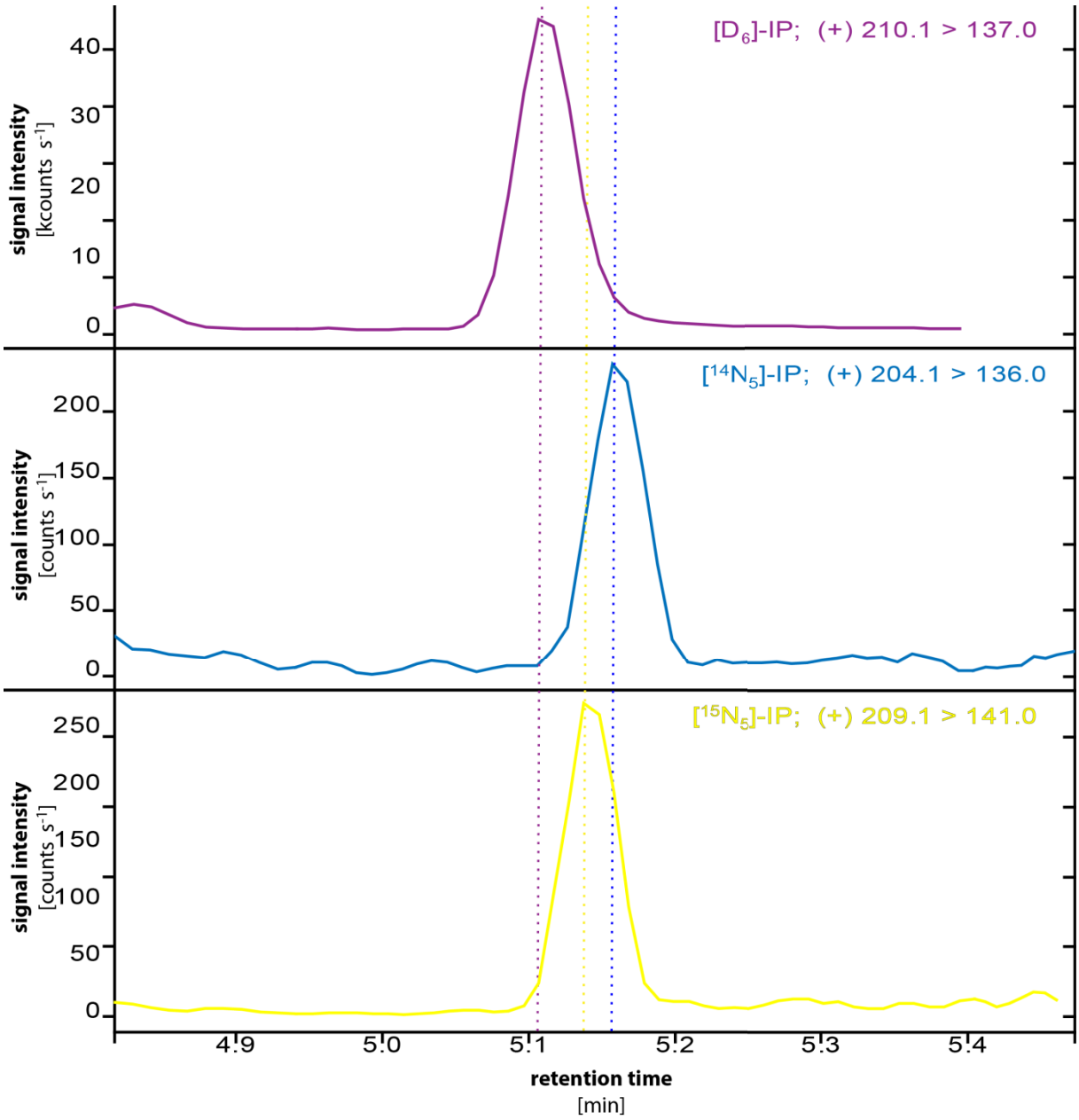

Figure 4 S4: Chromatograms of IP, $[D_6]$-IP, $[^{15}N_5]$-IP.

Dashed lines show the retention-time shifts between $[^{14}N_5]$-IP , $[D_6]$-IP (internal standard) and $[^{15}N_5]$-IP. Color coding is the same as in the according chromatograms. The parental → daughter ion transitions that have been monitored are given in the top right of each chromatogram.

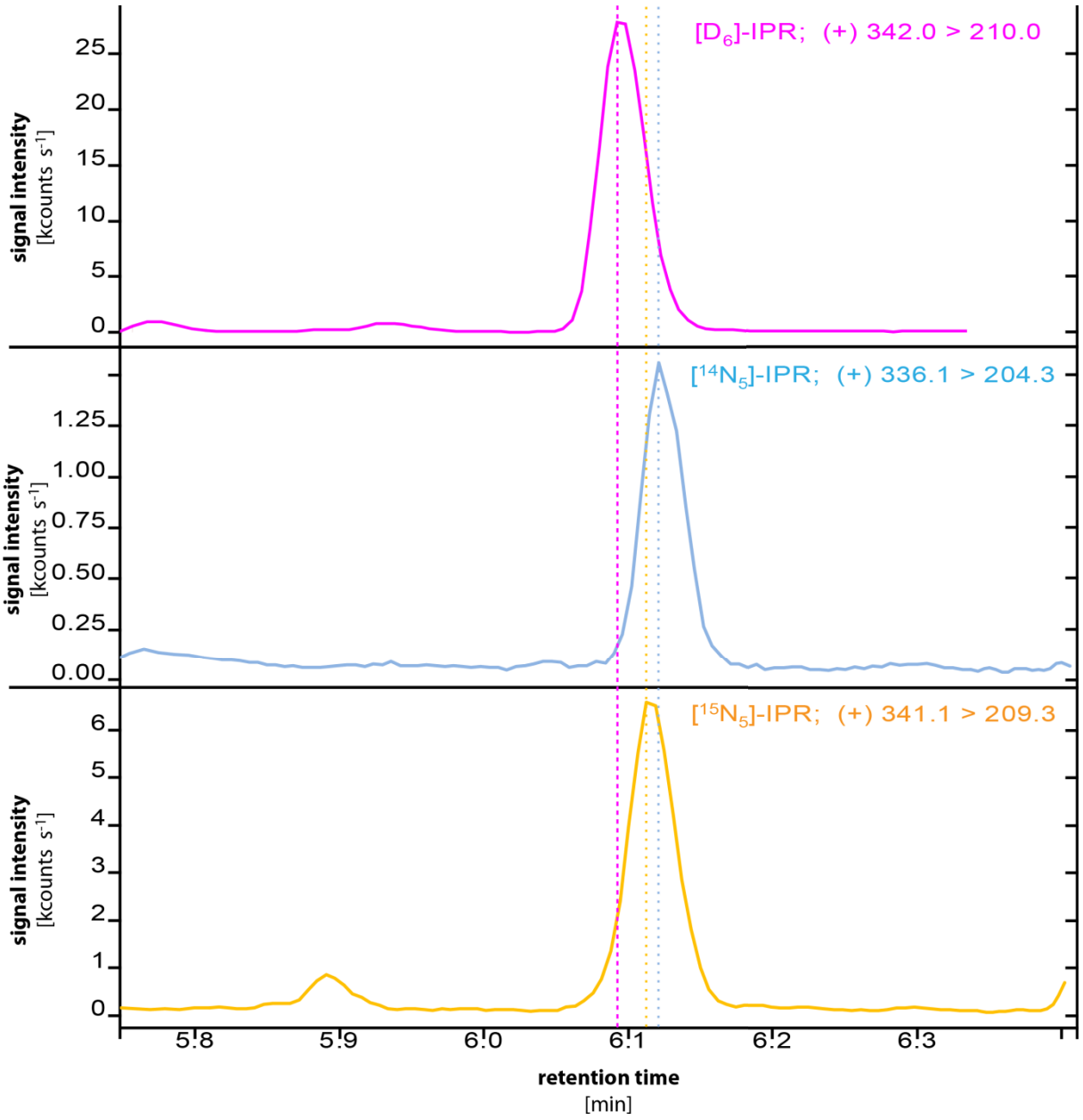

Figure 4 S5: Chromatograms of IPR, [D_6]-IPR and [$^{15}N_5$]-IPR

Dashed lines show the retention-time shifts between unlabeled IP, [D_6]-IP (internal standard) and [$^{15}N_5$]-IP Color coding is the same as in the according chromatograms. The parental → daughter ion transitions that have been monitored are given in the top right of each chromatogram.

Table S1: Calculation of the minimum amount of IP transferred by a single mirid in Experiment 2 and estimation of the amount of mirids necessary to transfer the measured amount of IP in Experiment 1

After 120 h constant feeding	**Experiment 2**		**Experiment 1**	
	Transferred [$^{15}N_5$]-IP/IPR: fmol / g FM leaf	**fmol IP/mirid**	**Transferred [$^{14}N_5$]-IP/IPR: fmol / g FM leaf**	**estimated number of mirids on one leaf:**
IP	2.3505	0.117523758	15.641	133.0905159
IPR	34.8575	1.74287662	135.36	77.66555141

Experiment 2: Each 20 ^{15}N labeled mirids feeding on single leaves of ^{14}N labeled plants for 5 days.

Experiment 1: ^{15}N labeled plants exposed to an unknown amount of ^{14}N labeled mirids.

FM, fresh mass.

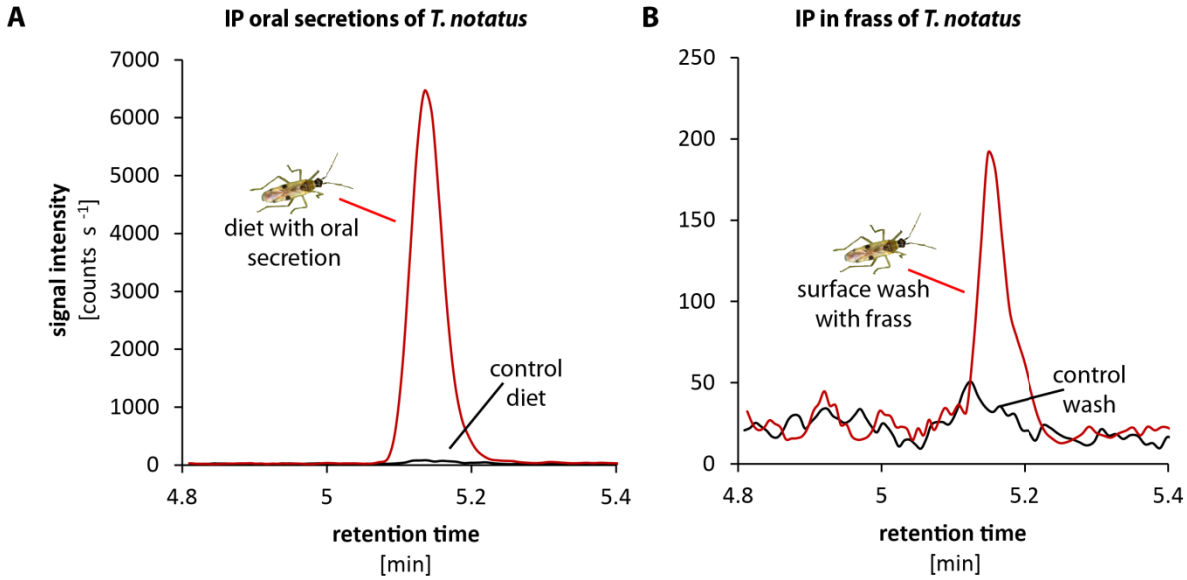

Figure 5: *Tupiocoris notatus* contains IP in its saliva and in small amounts in its frass.

Chromatograms showing the signal intensity of a MS/MS- trace for IP (204.1 → 136.0). **A** IP signal of pure sugar solution (black line) or sugar solution that has been used as diet for *T. notatus* for 5 days (red line). The sugar solution was covered with a thin layer of parafilm that allows piercing and feeding on the solution but prevents contamination with *T. notatus* frass. **B** Chromatogram of the surface wash of the parafilm covering the sugar solution after *T. notatus* feeding (red line, covered with visible frass spots) or without (control wash, black line).

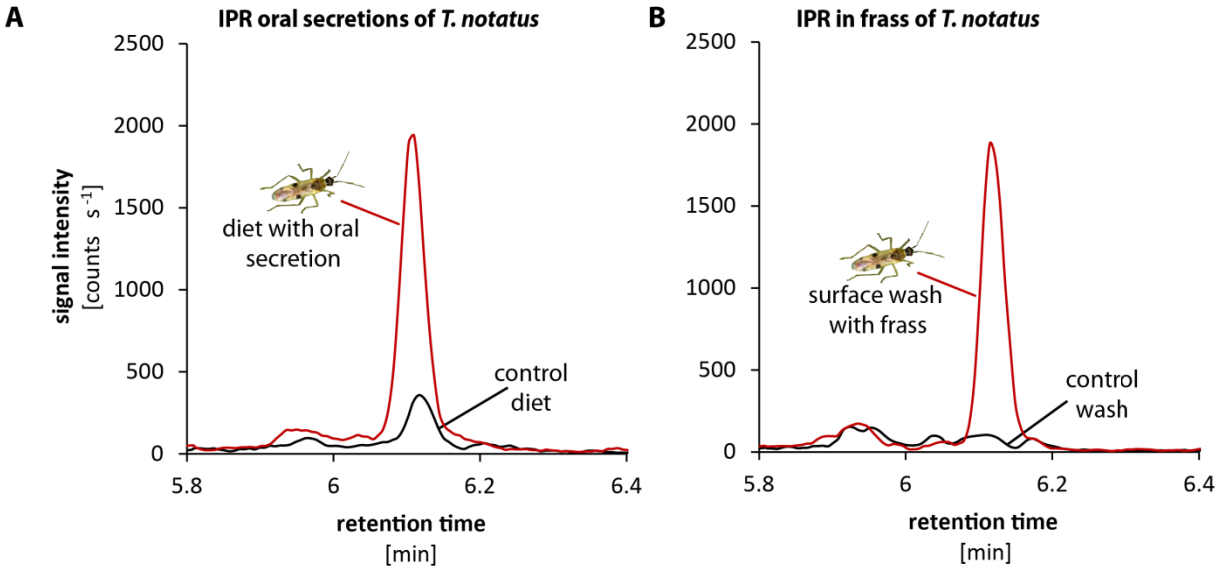

Figure 5 S1: *Tupiocoris notatus* contains IPR in its saliva and in its frass.

Chromatograms showing the signal intensity of a MS/MS- trace for IPR (336.1 → 204.1). **A** IPR signal of pure sugar solution (black line) or sugar solution that has been used as diet for *T. notatus* for 5 days (red line). The sugar solution was covered with a thin layer of parafilm that allows piercing and feeding on the solution but prevents contamination with *T. notatus* frass. **B** Chromatogram of the surface wash of the parafilm covering the sugar solution after *T. notatus* feeding (red line, covered with visible frass spots) or without (control wash, black line).

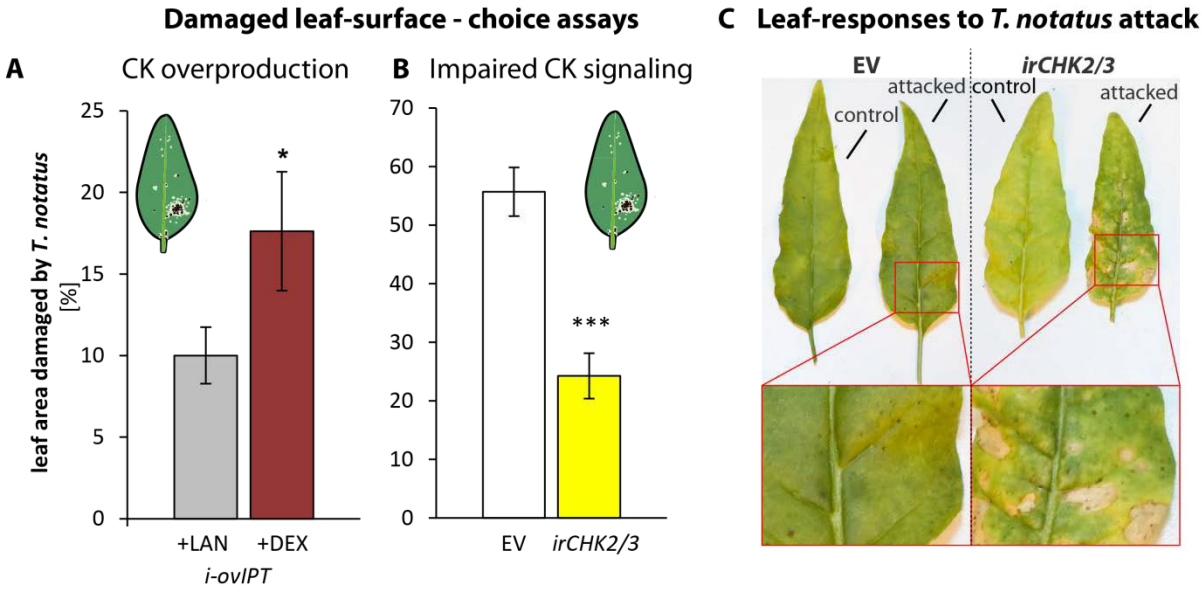

Figure 6: Cytokinin-regulated traits mediate *Tupiocoris notatus* feeding preferences and alter leaf responses to feeding.

A and **B**: Surface damage on *N. attenuata* plants after 10 d of *T. notatus* feeding. **A** *T. notatus* could choose between dexamethasone-inducible isopentenyltransferase-overexpressing plants (i-ov*ipt*) treated with dexamethasone-containing lanolin paste (+DEX) or lanolin paste without dexamethasone as control (+LAN; figure based on data from Schäfer *et al*. 2013). N = 7, Welch t-test: * $p < 0.05$. **B** Choice between empty vector (EV) and ir*chk2/3* plants silenced in the two cytokinin receptor genes *NaCHK2* and *NaCHK3* (ir*chk2/3*). N = 6, Welch t-test: *** $p < 0.001$. Error bars depict standard errors. **C** Exemplary pictures of leaves of EV or ir*chk2/3* plants with or without *T. notatus* damage. Magnifications show necrotic lesions occurring only in ir*chk2/3* plants after several days of mirid feeding.

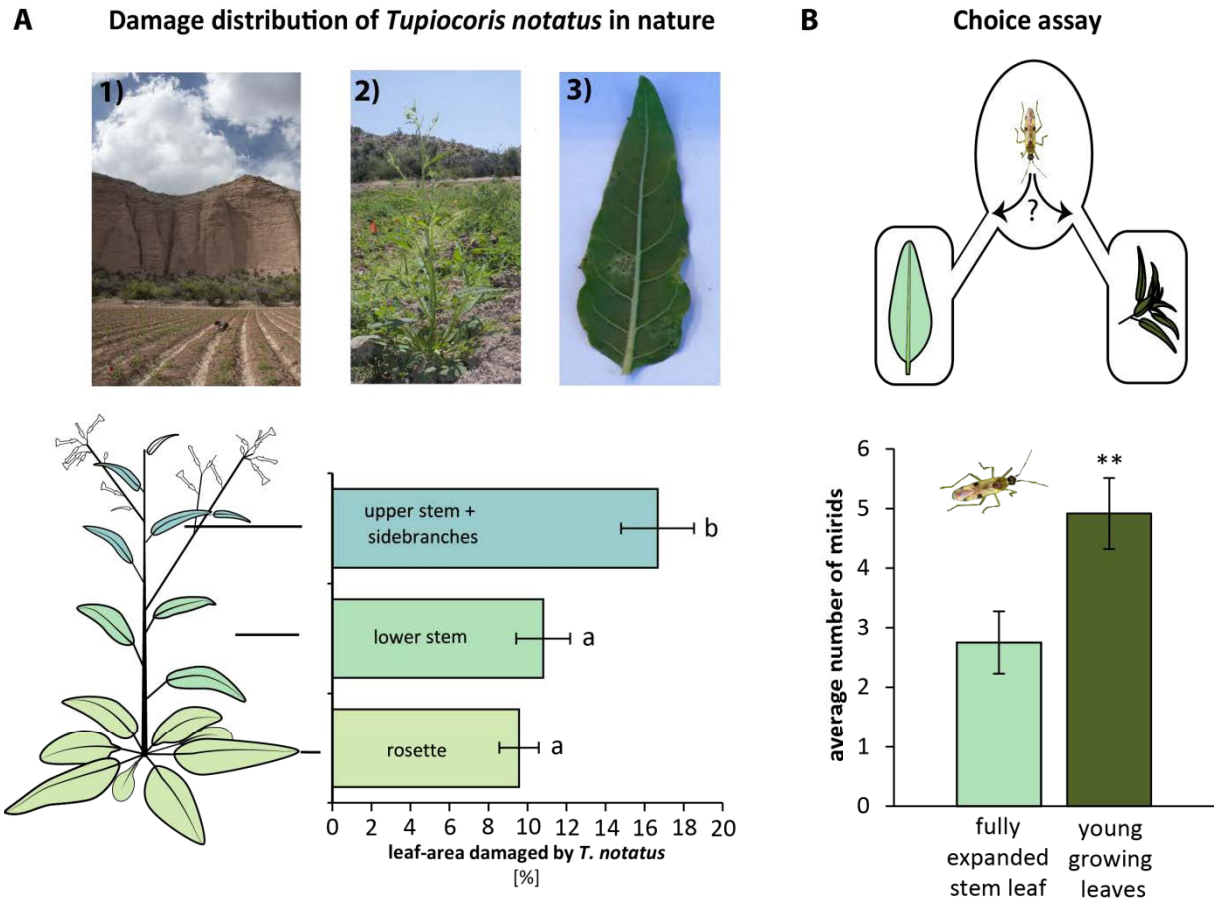

Figure 6 S1: *Tupiocoris notatus* prefers to feed on young leaves.

A Distribution of *T. notatus* damage in flowering *Nicotiana attenuata* plants under field conditions. Picture 1) field plot at Lytle Preserve, Utah, 2) a growing *N. attenuata* plant in the field, 3) a typically damaged leaf. Below: Damaged leaf-area in %. Leaves were grouped into rosette leaves, lower stem leaves and upper stem leaves and side branches as indicated in the schematic drawing on the left. N=21, One-Way-ANOVA, Tukey HSD posthoc test, different letters indicate significant differences ($p < 0.05$), error bars depict standard errors. **B** Choice assay: 10 mirids were placed in an arena with two tubes connected to either a fully grown leaf or a young growing leaves on a stem tip. Number of mirids on each side was counted after 12 hours. N=12, Welch t-test **: $p < 0.01$, error bars depict standard errors.

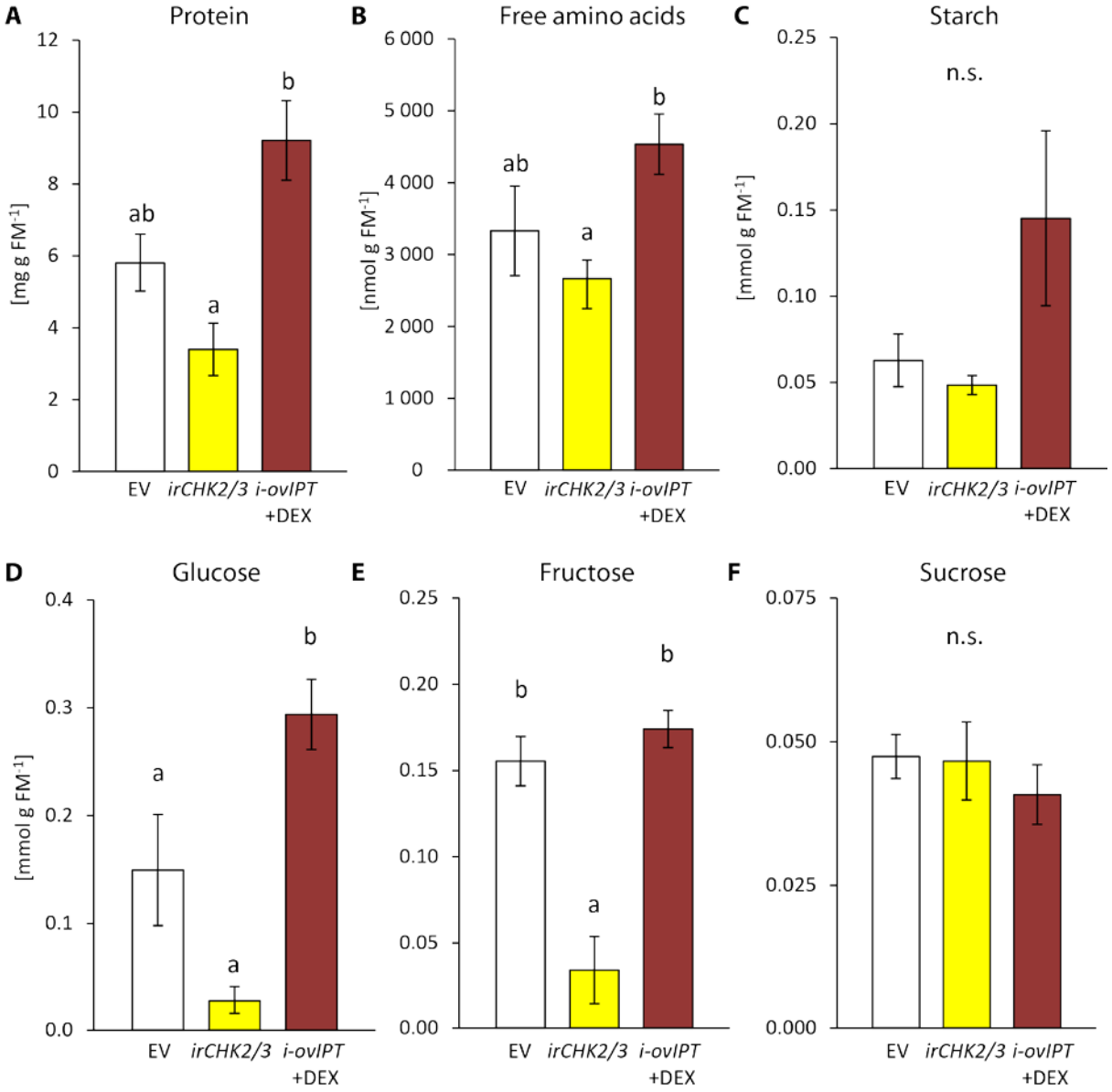

Figure 7: Transgenic *Nicotiana attenuata* plants altered in their cytokinin metabolism are changed in their nutrient content.

We compared empty vector (EV) plants, plants silenced in the two cytokinin receptor genes *NaCHK2* and *NaCHK3* (ir*chk2/3*) and dexamethasone-inducible isopentenyltransferase-overexpressing plants (i-ov*ipt*) treated with dexamethasone-containing lanolin paste (DEX) leading to increased CK levels. Concentrations were determined in untreated rosette leaves of *N. attenuata:* **A** Protein, **B** free amino acids, **C** starch, **D** glucose, **E** fructose and **F** sucrose. One-way ANOVA and Tukey HSD *post hoc* test. Different letters indicate significant differences. Starch data was log-transformed. N = 4. Error bars depict standard errors. FM, fresh mass.

Table S2: Sequences of primers used for qPCR

Gene	**Forward primer**	**Reverse primer**
NaActin	5'ggtcgtaccaccggtattgtg3'	5'gtcaagacggagaatggcatg3'
NaCKX5	5'ttgtcggcttattgtaaccgtcg3'	5'gttaagaactgccatcggctc3'
NaRRA5	5'agatgagttgcatgttcttgctgt3'	5'tcaatccccacagaggtcttct3'
NaZOG2	5'agtcatgcaagtcaatttaagagctc3'	5'aggaaatttgggaagaaggtgtaag3'
NaLOG4	5'ctcagctcacaaagtcttcacg3'	5'ccattaagccaacacttccacc3'
NaIPT5	5'tcagccacttattaatttccgagag3'	5'ttggctagatcaatggatagtctag3'

Table S3. Multi-reaction-monitoring settings for the quantification of [$^{14}N_5$]-, [$^{15}N_5$]- and deuterated cytokinins in positive ionization mode.

Analyte	RT [min]	Q1 [m/z] →	Q3 [m/z][a,b]	CE [V][a]	Standard[c]
[$^{15}N_5$]-IP	5.12	(+)209.10 →	141.00	-14	D_6-IP
[$^{15}N_5$]-IPR	6.14	(+)341.10 →	209.30	-12	D_6-IPR
		(+)341.10 →	141.00	-28	
*c*Z	2.53	(+)220.20 →	136.30	-16	D_5-*t*Z
		(+)220.20 →	148.30	-16	
*c*ZR	4.45	(+)352.20 →	220.30	-16	D_5-*t*ZR
		(+)352.20 →	136.00	-25	
IP	5.18	(+)204.10 →	136.00	-14	D_6-IP
IPR	6.10	(+)336.10 →	204.30	-12	D_6-IPR
		(+)336.10 →	136.50	-28	
*t*Z	2.25	(+)220.20 →	136.30	-16	D_5-*t*Z
		(+)220.20 →	148.30	-16	
*t*ZR	4.04	(+)352.20 →	220.30	-16	D_5-*t*ZR
		(+)352.20 →	136.00	-25	
D_6-IP	5.11	(+)210.10 →	137.00	-14	
D_6-IPR	6.04	(+)342.00 →	210.00	-12	
		(+)342.00 →	136.50	-28	
D_5-*t*Z	2.22	(+)225.20 →	136.60	-16	
D_5-*t*ZR	3.98	(+)357.20 →	225.50	-16	

RT: retention time

CE: collision energy

[a] Qualifiers are depicted in grey

[b] Resolution: Q1: 0.7, Q3: 2

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4 General Discussion

CKs are long known regulators of growth and development and also their involvement in responses to abiotic and biotic stresses has been discovered long time ago (reviewed in Argueso, et al. 2009, Werner and Schmülling 2009). When I started my PhD, the role of CKs in plant-herbivore interaction has only attracted minor attention compared to the role of CKs in response to abiotic stresses (e.g. Rivero *et al.* 2007, Werner, et al. 2010) or its role in tolerance to pathogens (Choi, et al. 2010, Grosskinsky, et al. 2011, Argueso, et al. 2012, Grosskinsky, et al. 2016). Still, an involvement of CKs in manifestation of endophytes in plants was hypothesized just a few years after the discovery of CKs (Matsubar.S and Nakahira 1967, Engelbrecht 1968, Engelbrecht, et al. 1969). Due to the early discovery of CKs as important factors in the infestation processes of leaf-miners and gall-insects, research in the CK-plant-herbivory interactions has mainly focused on these prominent examples (reviewed in Giron, et al. 2016).

Phytohormones have so far never been found to be functionally isolated, but to act in concert with other phytohormones and signaling cascades (e.g. Robert-Seilaniantz, et al. 2011, Durbak, et al. 2012). Phytohormone signaling in response to herbivory is no exception from that rule (Erb, et al. 2012). The JA pathway has been identified as the core part of the herbivore triggered signaling (Koo and Howe 2009, Ballare 2011, Wasternack and Hause 2013, Campos *et al.* 2014). Nevertheless, many interactions of that pathway with other phytohormones have been described, such as with SA (Smith *et al.* 2009), ABA (Hou *et al.* 2010) or ethylene (Diezel *et al.* 2011a). Backed up by first findings that CK primes plant responses to herbivory (Dervinis, et al. 2010), that CK overexpression increased the resistance of *Nicotiana tabacum* to *M. sexta* (Smigocki, et al. 1993), and that CK levels can interfere with JA levels after wounding (Sano *et al.* 1996), it has been hypothesized that CKs also play an important role in a plant's interaction not only with endophytic insects, but also with other herbivores (Erb, et al. 2012). Due to their role in plant-development, it has particularly been hypothesized that they could be responsible for developmental regulation of plant defenses. This developmental regulation has been described in many cases before and predicted by theories like the OD theory (Meldau, et al. 2012).

In our model plant *N. attenuata*, CK-related transcripts were amongst the heavily regulated transcripts after simulated herbivore treatment (Hui *et al.* 2003, Gilardoni *et al.* 2010), making an involvement in responses to herbivores likely. Due to that growing evidence, I decided to study the role of CKs in interactions of plants with freely moving herbivores from different feeding guilds on *N. attenuata.*

By the end of my thesis, I could demonstrate that CK levels and signaling are influenced by attack of free living herbivores from different feeding guilds on *N. attenuata* and *A. thaliana* (**manuscript I and VI**). I showed that manipulations in CK levels or signaling influence anti-

herbivore defense responses (**manuscript II, III and V**) and that CK manipulations are sufficient to alter developmental gradients of defense metabolites (**manuscript III**), which are all at least partially controlled by the transcription factor NaMyb8 (**manuscript IV**). Finally, I also found that not only endophytic insects, but also a free living insect species is capable of manipulating their host plants by injection of CKs (**manuscript VI**).

4.1 Wounding and herbivory from different herbivores affects CK signaling

It has been long known that endophytic insects are able to interfere with the plant CK metabolism and signaling (Matsubar.S and Nakahira 1967, Engelbrecht 1968). The formation of leaf-galls and green islands are examples that can be found in almost any plant physiology textbook. In those prominent cases the changes in the CK pathway, as well as in CK levels are thought to be caused by an external transmission of CKs by the insect or its associated microbes (Mapes and Davies 2001, Giron *et al.* 2013, Giron and Glevarec 2014, Giron, et al. 2016). But only little was known about the interference of feeding of free living herbivores with the CK pathway. In **manuscript I** we were exploring the changes in CK levels, as well as in CK-related genes in *N. attenuata* after wounding and the attack of the free living Lepidopteran specialist *M. sexta.* Coinciding with previous literature (Conrad and Kohn 1975, Mitchell and Vanstaden 1983, Crane and Ross 1986) we found an increase of CKs, in particular *c*ZR and IPR, in leaves after wounding. Furthermore we found several transcripts of the CK pathway changed after wounding. These responses in CK levels and transcript changes were even amplified after application of oral secretions (OS) of *M. sexta* or its elicitor FACs to wounded tissue. Previous microarray analyses already provided hints that CK related transcripts might be affected by wounding and application of FACs (Hui, et al. 2003, Gilardoni, et al. 2010). In addition, I show in **manuscript III** that levels of *c*ZR in leaves are still increased after several days of herbivory, whereas levels of IPR are not. Regarding the fast responses after wounding and OS application, we found similar increases of cZR and IPR also in *A. thaliana* leaves after wounding and application of OS from *Schistocerca gregaria*, suggesting that this is a more common response to feeding of chewing herbivores.

To expand our studies about responses of the CK pathway to herbivory, I decided to extend the study in **manuscript VI** to *T. notatus,* a piercing-sucking herbivore from a different feeding guild and a different insect order. *T. notatus*, which has a completely different feeding behavior, caused changes in CK levels and CK transcripts similar to those caused by *M. sexta* or *S. gregaria* feeding or simulated feeding. After several days of *T. notatus* feeding we found levels of *c*ZR and *c*Z increased, while IPR levels where unaffected. The increase in *c*ZR is common for all three model systems and could be a general wound- or stress response. In our review , we discussed this potential function of *c*Z type CKs (Schäfer, et al. 2015).

Not only changes in CK levels, but also transcriptional changes after the attack of these two different herbivores, *M. sexta* and *T. notatus*, on *N. attenuata* were similar. In both cases, transcript levels of the CK biosynthesis gene *NaIPT5* are lower, and transcript levels of *NaCKX5,* which codes for a CK-oxidase and *NaZOG2,* which codes for a CK inactivating glucosyltransferase*,* are higher after herbivore attack. These similarities suggest that the influence of herbivore feeding on the CK pathway might be a more general response to herbivory in *N. attenuata*. Further studies with different plant and insect species could illuminate the prevalence of these responses within the plant kingdom.

Although the responses to herbivory seemed to be similar amongst different plant and herbivore species, the underlying mechanistically connection between responses to herbivory and the CK pathway could not be fully elucidated in our study. The JA-pathway has been identified as the control center of anti-herbivore responses in plants (Koo and Howe 2009, Ballare 2011, Wasternack and Hause 2013, Campos, et al. 2014). Therefore the conclusion that the increase of JA and downstream effects could cause these effects on the CK pathway seems obvious. Indeed we found an effect of MeJA treatment on several CKs, where *c*ZR seems to be positively influenced by herbivory and IPR negatively (**manuscript I**). On the other hand, transgenic plants with impaired JA production or JA perception still partially showed the responses in CK metabolism after herbivory. This indicates a CK response independently from JA and a HAMP-specific regulation of CK metabolism somewhere upstream of JA, possibly additionally to a JA mediated regulation. Another possible explanation for increases of CKs might be the application of those CKs through the OS or excretions of the feeding insect as it was already suggested for endophytic insects (e.g. Engelbrecht, et al. 1969, Matsui, et al. 1975, Elzen 1983, Mapes and Davies 2001, Giron *et al.* 2007, Kaiser, et al. 2010, Giron, et al. 2016). In **manuscript VI**, I could measure high levels of the CK IP in the OS of *T. notatus* and could prove with ^{15}N labeling experiments that they are able to transfer it to the host-plant. To my knowledge, this behavior was formerly not observed amongst free living insects. After five days of feeding almost half of the IP in the attacked leaves of a heavily attacked plant originated from the insects. Nevertheless, the absolute levels of IP did not change after attack. The type of CK injected (mainly IP) and the types of CKs increasing in the attacked leaves (mainly *c*Z and *c*ZR) are not the same. This makes it unlikely that increases in CKs in the leaf tissue are directly caused by injections of CKs from the insect. More likely the injection causes CK-related processes that lead to the observed changes in the CK pathway. The injected IP in the leaves could possibly be degraded, or transported away from the feeding site to maintain a physiological level of CKs in attacked leaves. Levels of transcripts support the conclusion that the plant reacts to increased CK levels after herbivory with a decrease of CK biosynthesis and an increase of CK inactivation and degradation probably as kind of a countermeasure activated by some feedback regulation. We could also see similar changes in transcripts after CK application to the leaves (supplemental figure S 11 of **manuscript**

I). If *M. sexta* is also capable of transferring CKs to its hostplant remains elusive until measurements of CKs in *M. sexta* oral secretions have been carried out. The increased levels of *cZ* and *cZR* after *M. sexta* attack are possibly a more general response to wounding and the increase in JA.

This still leaves the open question about the biological function of an activation of the CK pathway. An activation of CK triggered responses after herbivory could have several different conceivable functions. CKs are known for its stimulation of cell division (Letham 1963, Werner and Schmülling 2009). This could favor the healing of wounded plant tissue after herbivore attack, as it was already proposed long ago (Conrad and Kohn 1975). Another possible advantage of the activation of the CK pathway after herbivore attack could be an influence on the resource allocation in the plant. CKs are known to be able to create a sink at sites of their highest concentration (Roitsch and Ehness 2000, Arnold *et al.* 2004). An attraction of nutrient to wounded tissue (Quilliam *et al.* 2006) could therefore promote the biosynthesis of defense metabolites. The fact that we also see changes in unattacked systemic tissue, could provide a biological implication to the priming effect of CKs that has been described before (Dervinis, et al. 2010). If CK levels increase in unattacked systemic tissue after wounding, the CK signal could possibly prime the so far unattacked leaf for an imminent attack by the herbivore.

4.2 Cytokinin levels and cytokinin signaling is modulating anti-herbivore defenses

Besides the possible role in wound healing, resource acquisition for defense metabolites, caused by herbivory triggered changes in the CK pathway, suggests that CK and CK signaling might influence a plants defense reactions to herbivory. The rare studies that have been published before I started my PhD, provided first evidence of a positive influence of CKs on defense reactions (Dervinis, et al. 2010), JA accumulation (Sano, et al. 1996) or on resistance to insects (Smigocki, et al. 1993, Smigocki, et al. 2000). In these studies an influence on defense compounds has been suggested but not fully validated. Nonetheless, other studies have shown that CKs can upregulate specialized plant metabolites (Hino *et al.* 1982, Ozeki and Komamine 1986, Grosskinsky, et al. 2011). Based on these studies we examined in **manuscript II**, how CK manipulations affect the defense reactions of *N. attenuata*.

A major problem in CK manipulation has always been the production of severe side-effects on growth and development of the plant. Constitutive expression of CK-biosynthesis genes like IPTs can cause severe developmental implications (Klee, et al. 1987). To circumvent these problems, two major strategies have been used in the past: Either CKs have been externally applied, for example by spraying (e. g. Sano, et al. 1996), or IPTs have been expressed under the control of developmentally or stress regulated promoters (e. g. Smigocki, et al. 1993, Gan and Amasino 1995, Qin, et al. 2011). External CK application is certainly the simpler method, but

carries problems of CK localization, dosing and CK transport away from treated tissue as well as side effects of the spraying procedure itself.

In my manuscripts, I used five different methods to manipulate CK levels and CK signaling to minimize effects on the development of the plant and maximize the confirmation of CK effects on defense. To increase CK levels, we sprayed *tZ* on the leaves (**manuscript II**), we used a senescence activated promoter system (*SAG-IPT4*), which lead to plants that only overproduce IP-type CKs in senescing tissues (**manuscript III**) and we used a DEX-inducible promoter system (i-ov*ipt*), which allowed us to locally increase the *tZ*-type CK levels (**manuscript II, III, V** and **VI**). Especially the DEX-inducible system (described in **manuscript V**) allowed us to trigger CK overproduction in a locally and temporally highly controlled way. To impair the CK perception we first used virus-induced gene silencing (VIGS) of different combinations of the three known receptor homologs for CKs in *N. attenuata* (**manuscript II;** description of the receptors in **manuscript I**). Then we used stable transient co-silencing of two of these receptors (ir*chk2/3*; **manuscript II and VI**).

Independently from the method we used, we always triggered an increase herbivory induced defenses like TPI and CP if we increased CK levels in the leaves. If the two receptors CHK2 and CHK3 were silenced together either by VIGS or transiently in stable lines, we saw lower levels of herbivory induced defenses. This confirmed the positive influence of CKs on the accumulation of plant defense metabolites, as this was the opposite effect on herbivore induced defenses compared to increased CK levels.

All defenses that we found to be influenced by CK manipulations were defenses that were inducible by herbivore feeding. Affected defenses were TPIs and PAs like CP, dicaffeoylspermidine (DCS) and other PAs. The basal levels of those defenses seemed to be unaffected by CK manipulations, which suggests again an involvement of CKs in HAMP triggered herbivore defenses. In **manuscript IV** we provide evidence, that the R2/R3 MYB transcriptional activator NaMyb8, which was thought to be a specific regulator of PAs (Kaur, et al. 2010) is regulating also other defenses influenced by CKs, especially TPI but also a threonine deaminase (TD). As several studies have found an interaction between CKs and MYB transcription factors (Sardesai *et al.* 2013, Schmidt *et al.* 2013, Bar *et al.* 2016), this seems to be an interesting target of future research. Transcript levels of *NaMyb8* were also affected by CK manipulations (**manuscript II and VI**); nevertheless the influence on this transcription factor, as well as on the transcript levels of biosynthetic genes was not as strong as on the defenses and sometimes even not significant. This suggests for example additional posttranscriptional or posttranslational mechanisms that are influenced by CK signaling. Another possible mechanism is the idea that the influence of CKs is also due to a regulation of precursors. CKs are for example increasing photosynthetic activity (Jordi, et al. 2000) and create a sink

strength (Roitsch and Ehness 2000). Therefore CKs could favor the production of defense metabolites by the promotion of increased levels of precursors of the defense metabolites, such as amino acids like phenylalanine or arginine which are important for PA biosynthesis (Kaur, et al. 2010).

As also JA levels are changed by CK manipulations, it is likely that CKs also interfere with the defense pathway upstream of JA, as already suggested before (Dervinis, et al. 2010). Surprisingly, JA-Ile, which is thought to be the main active regulator of JA-dependent responses (Kang *et al.* 2006), is not affected by CK treatment (**manuscript II**). CKs might promote other active jasmonates that we did not measure but can also play a role in plant defense processes (Stintzi *et al.* 2001, van Doorn *et al.* 2011). It is likely that CKs interact with the pathway at multiple points, upstream and downstream of JA, as well as indirectly via precursor availability. Similar effects have also been reported in SA-dependent defense reactions to pathogens before (Choi, et al. 2010). Furthermore an interaction with other phytohormones seems likely. CK-promoted elevation of the defensive compound scopoletin for example has been shown to be indirectly regulated by ABA in tobacco (Grosskinsky *et al.* 2014). Another example is GA, which is attenuating JA responses (Hou, et al. 2010) and can be suppressed by CKs (Jasinski *et al.* 2005, Fleishon *et al.* 2011). GA-CK interaction in plant-herbivore responses might therefore provide also a possible future target of research.

In all my studies, I did not see an effect of CK manipulations on the accumulation of nicotine. In contrast to defenses like CP, other PAs or TPI, nicotine is already produced in high amounts without induction by herbivores. It is possible that CKs only affect plant defenses, which are inducible. In **manuscript III**, I show that nicotine levels are not inducible under the conditions and with the treatments I used, although an inducibility of nicotine has been demonstrated before (Baldwin 1996). However, it also has been described before that induction of nicotine gets lost in plants in pots, like they are used in greenhouse experiments (Baldwin 1988). As nicotine is produced mainly in the roots (Iljin 1958), this effect may be caused by the restricted growth potential of the root system in pots. It is possible that nicotine levels respond to CK manipulations, if the plants are not pot-bound and nicotine levels are inducible. Studies in the natural environment or in much bigger pots could clarify this hypothesis.

4.3 Cytokinins are controlling the developmental regulation of anti-herbivore defenses

The question about the evolutionary advantage of a connection between a growth hormone and the defense pathway turns out to be very fascinating. CKs are associated to young growing tissue, i.e. cell-division and growth (Hewett and Wareing 1973; **manuscript III**). This means that high CK levels are normally associated to metabolically active tissue, which provides

a higher future productivity to a plant than senescing or old tissue, which has lower levels of CKs. For a plants fitness, meaning its seed production, young active leaf tissue is more valuable than old tissue (Harper 1989). Therefore, CK levels might be indirectly associated to the fitness value of a particular tissue. Protecting the tissue that provides a bigger fitness value for the plant more than tissue with a lower value, is predicted by the OD theory (McKey 1974, Stamp 2003). Therefore, we wondered if the distribution patterns predicted by the OD theory might be influenced by the CK content of a certain tissue. We hypothesized that the observed patterns of defenses might be a reflection of the distribution pattern of CKs in the plant.

In earlier studies, it has been shown that defenses follow distributions predicted by the OD theory with high levels of defense in young tissue and low levels in old tissue (e.g. James 1950, Mothes 1955, Ohnmeiss and Baldwin 2000, Gutbrodt *et al.* 2011, Massad *et al.* 2014). Additionally, other studies have shown that CK levels are high in young, growing tissue, but low in senescing tissue (Hewett and Wareing 1973, Ori, et al. 1999). But so far, a connection between CKs and developmental defense patterns has only been hypothesized (Meldau, et al. 2012).

In **manuscript III**, we analyzed levels of defenses and CKs simultaneously in leaves of different ages in *N. attenuata* and could find a clear positive correlation between inducible defenses and CKs. Especially defenses were correlating with *t*Z- and IP-type CKs. The defenses that followed OD predictions and correlated with CK levels were again those inducible defenses that we showed to be influenced by CK manipulations in **manuscripts II** and **V**. Namely those defenses were CP, DCS and TPI transcripts. Furthermore in **manuscript IV** we show another potential defensive TD to follow the same gradual distribution in the plant. In a preliminary experiment, we could show that spraying *t*ZR on plants increased the levels of TD transcripts after wounding (figure 5). Further experiments must proof, if also other CK manipulations affect TD transcript levels, but it seems likely that also developmental TD distribution is dependent on CK levels. In case of CP, DCS and TPI, we show in **manuscript III** that a change in the developmental distribution of CK levels also changed levels of the defenses following the predicted OD patterns. Using the DEX inducible i-ov*ipt* plants it was possible to manipulate within plant distributions of CKs and therefore defenses, whereas the *SAG-IPT4* plants allowed to prevent a decrease of CKs and defenses in aging leaves. In the past a rigorous test of the OD theory has often been confounded by the lack of possible manipulations of developmental distribution patterns of defenses. As I now have identified that CK manipulations are sufficient to manipulate defense distributions, CKs could be used as a potential tool in the future research on ontogenic defense regulation.

All the defenses we found following the OD gradients, and being influenced by CK manipulations, were dependent on the expression of the R2/R3 MYB transcription factor NaMyb8. The transcripts of *NaMyb8* itself did also follow OD distributions and were affected by the CK manipulations. One of the major defensive compounds, nicotine that was not affected by

CK manipulations, also did not show a gradient following OD predictions, and is also independent of NaMyb8 (Kaur, et al. 2010). Therefore, we consider NaMyb8 an important regulator in the CK dependent regulation of developmental gradients of defenses predicted by the OD theory. Further experiments using NaMyb8 silenced transgenic lines, as in **manuscript IV** could help to find out, if NaMyb8 is necessary for the CK influence on developmental regulation of inducible defenses. As mentioned before, it is of course possible and also likely that this influence is due to several direct and indirect influences of CKs on the herbivore defense pathway.

Clearly, I could demonstrate in **manuscript III**, that a manipulation of the distribution of CKs is sufficient to manipulate the distribution of defenses in the plant. Therefore, we consider CKs as key players in the developmental regulation of defense metabolites that underlie the OD theory. The fact that a growth hormone is involved in the regulation of OD gradients could also be a link to connect OD theory with other defense theories, like the GDBH.

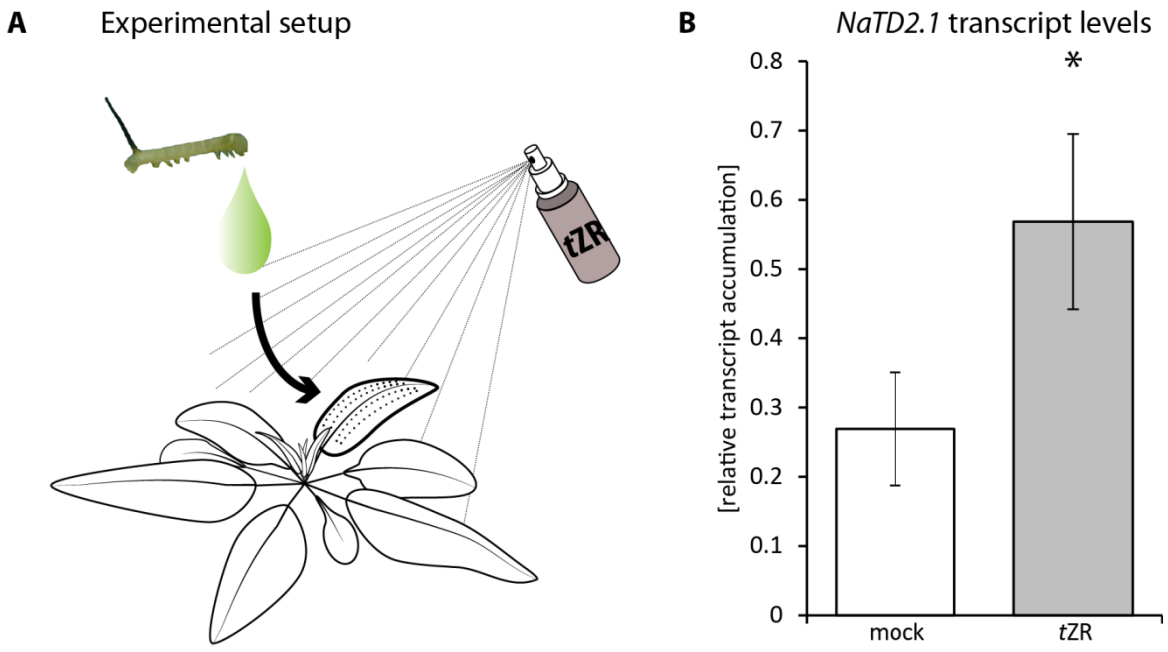

Figure 5: Spraying of the CK *t*ZR increases transcript levels of an herbivory-induced threonine deaminase (*NaTD2.1*)

A One rosette leaf was induced by wounding and application of oral secretions. Whole plants were either sprayed with 5 µM *t*ZR (*t*ZR) or 0 µM tZR (mock) two times per day for two days. **B** Relative transcript accumulation of threonine deaminase *NaTD2.1* in treated leaves. T-test: * $P < 0.05$. N = 5. Methods are described in the appendix.

4.4 The drawbacks of staying "forever young"- the suboptimal defense

Due to the capacity of CKs to delay senescence (Richmond and Lang 1957, Gan and Amasino 1995, Ori, et al. 1999), to increase drought tolerance (Rivero, et al. 2007) or their potential for biocontrol of pathogens (Grosskinsky, et al. 2016), CK manipulations have been target of plant breeders all over the world. Often, creating plants with enhanced CK levels and delayed senescence have been discussed as a way to increase yield and stress tolerance of certain crops (Ma 2008, Zalabak *et al.* 2013, Koprna *et al.* 2016). Some studies (nicely summarized in Koprna, et al. 2016) already showed that CK overproduction increases yield especially under abiotic stress conditions like salinity or drought for example in peanut (Qin, et al. 2011), tomato (Ghanem *et al.* 2011) or rice (Peleg *et al.* 2011).

Looking at my results, the positive influence on defense metabolites might seem to be a way to create plants more resistant to insect herbivore attack. This hypothesis comes at the presumption that more defenses always mean more protection against herbivores. Although, studies have demonstrated that less defenses mean a higher susceptibility to herbivores (Steppuhn, et al. 2004, Zavala and Baldwin 2004, Kaur, et al. 2010), the reverse presumption that genetically increasing defenses always means better protection can be doubted to be universal for several reasons: 1) manipulations of plant metabolism practically always affects other non-target metabolic processes and 2) because defense production comes at a cost and 3) toxic defense metabolites may not be effective enough against highly specialized insects and may even be toxic for the plant's metabolism. Therefore to make an assumption, of whether an increase of defenses by increases in CKs is an effective protection against herbivores or not, requires at least three further explorations: 1) The broad analysis of metabolic changes that occur upon a certain manipulation, to assess possible side effects; 2) efficacy trials to assess the performance of herbivores on the manipulated plants, which are ideally performed in the natural environment. 3) Furthermore the yield, i.e. the fitness of a manipulated plant, would need to be assessed as the final measure of a potential benefit of a trait is the production of offspring. Only, if the CK-induced increase in defenses brings an overall fitness benefit under given circumstances, the trait can be assessed as beneficial. In our case with *N. attenuata* we did not yet perform such a broad analysis. Nevertheless, we performed first studies, which question the effectiveness of CK manipulations to improve resistance against herbivores.

In **manuscript V**, I could show that an increase of CKs in certain plant parts, or even single leaves, using the DEX inducible i-ov*ipt* plants, increased the attractiveness of the tissue to the highly specialized herbivore *T. notatus.* Even though we could show that an increased level of CKs increased the levels of defense metabolites. A reduced attractiveness for *T. notatus* of plants silenced for the CK receptors in **manuscript VI** matches to the hypothesis, that enhanced CK levels and CK-dependent processes benefit *T. notatus* on *N. attenuata.* In preliminary tests, we

also could demonstrate that *M. sexta* performs much better on CK overproducing *SAG-IPT4* plants in the greenhouse (figure 6). Previous studies have reported an opposite effect of CK overexpression (Smigocki, et al. 1993, Smigocki, et al. 2000, Dervinis, et al. 2010). Dervinis and colleagues performed experiments with Poplar and an application of 6-benzylaminopurine, whereas Smigocki *et al.* used *Nicotiana* and tomato species and transgenic IPT expression with the promoter of a PI gene. This highlights the dependency on plant and insect species and the type of manipulation on the effects of CK manipulations on biotic interactions.

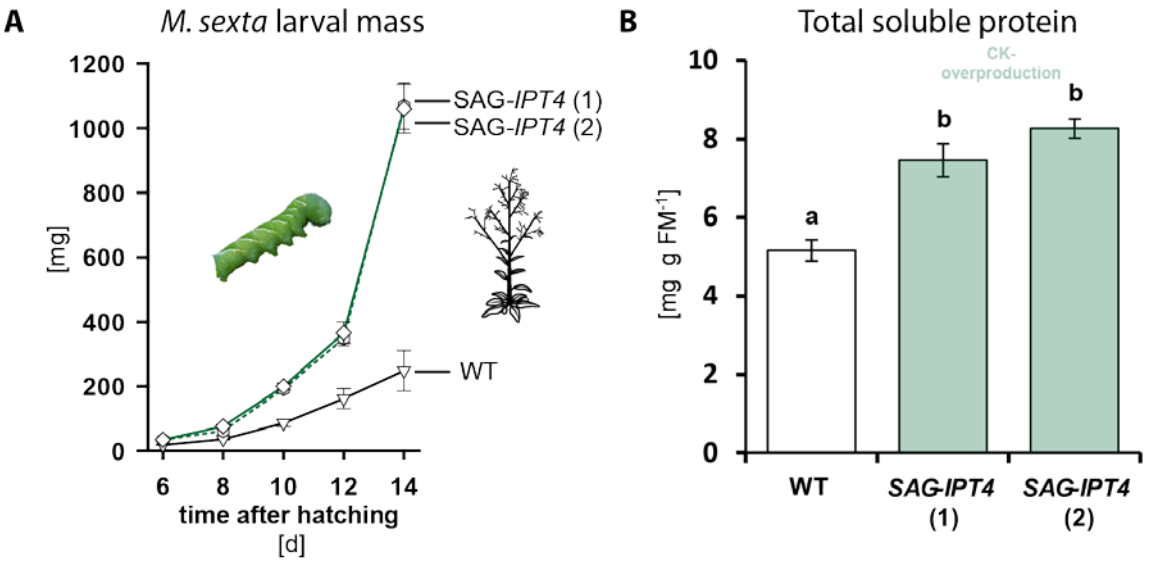

Figure 6: *M. sexta* larvae show increased larval growth on CK overproducing *SAG-IPT4* plants with higher protein levels.

A *M. sexta* larval growth on flowering wildtype (WT) plants and two independently transformed *SAG-IPT4* plants with increased CK levels. T-test with Bonferroni correction for comparisons between WT and both lines at each timepoint: all comparisons $P < 0.001$. $N = 15 – 49$. **B** Concentrations of free soluble protein in rosette leaves of flowering WT and *SAG-IPT4* plants. One-way-ANOVA with TukeyHSD posthoc test: $P < 0.05$. N = 10.FM, fresh mass. Methods are described in the appendix.

Obviously, the increased levels of CKs do not enhance the protection against specialized insect herbivores in our case, although we tested this with two different manipulation strategies. All defense strategies of a plant and its defense regulation are the consequence of a long evolutionary history. All traits that evolved in the past provided a benefit at some point of evolution. It can be assumed that the current defense strategy has been the optimal strategy in the past, which is already included in the term “optimal defense” in the OD theory. Our preliminary results support the idea that an increase of CK levels does not create an even “more optimal” defended plant but rather comprises the optimal defense strategy and causes a “suboptimal defense”.

The big question is why the increase of defense metabolites through enhanced CK levels does not improve the protection against herbivores. The major reason probably lies somewhere in

the side-effects of CKs on other metabolic processes. CKs are known to create a sink strength (Roitsch and Ehness 2000, Mok and Mok 2001), increase photosynthetic activity (Jordi, et al. 2000), delay senescence (Richmond and Lang 1957, Gan and Amasino 1995, Ori, et al. 1999), and therefore, high levels of CKs are often associated to high levels of nutrients. It is known that nutrient content of a plant tissue can strongly influence the performance of herbivores on the plant (Mattson 1980). It can be assumed that increased levels of CKs not only increase levels of defenses, but also nutrients in *N. attenuata.* Indeed, in **manuscript VI** and in preliminary studies (figure 6), I could measure higher levels of nutrients like soluble proteins in leaves with enhanced CK levels (i-ov*ipt* plants and *SAG-IPT4* plants). Those higher levels could account for the higher larval mass of *M. sexta* on *SAG-IPT4* plants and the preference of *T. notatus* for plants with enhanced CK levels. Especially for highly specialized insects like *M. sexta* and *T. notatus* the levels of defense metabolites may only play a minor role in the plants defense strategy. *M. sexta* has been suggested to be able to metabolize nicotine (Kumar, et al. 2014), and we could show that also *T. notatus* is able to express transcripts related to detoxification enzymes (Crava, et al. 2016). Although detoxification of toxins demands energy and defenses in the plant like nicotine, CP or TPI, are known to limit growth of *M. sexta* (Steppuhn, et al. 2004, Zavala and Baldwin 2004, Kaur, et al. 2010), this drawback might be overcompensated by the higher nutritional value of the leaves with enhanced CK levels. Also it cannot be excluded, that other defensive compounds that we do not know so far might be decreased by higher CK levels. Therefore, it would be interesting to evaluate the performance of non-specialized insects on CK overproducing plants. It is possible that in those cases the higher levels of toxic compounds might not be compensated by the higher levels of nutrients, due to the higher sensitivity to defense compounds by generalist herbivores.

If similar effects would occur in crops, the higher vulnerability of plants with higher CK levels to specialized herbivores might make a higher investment in plant protection and pesticides necessary and therefore might reduce the economic yield. Furthermore a faster and better development of herbivores on CK-overproducing plants might favor a higher reproductive rate, higher survival and faster population growth of the herbivores. In combination with prolonged growth periods of CK-overproducing plants with delayed senescence, this might even favor more reproductive cycles of the pest, which additionally increases the herbivore pressure.

To defend against highly specialized herbivores, the plant might also need to rely on defense strategies other than toxin production. One of them could be the attraction of predators through HIPVs (Kessler and Baldwin 2001, Schuman, et al. 2012, Schuman and

Baldwin 2016). In **manuscript II** we could show that particular green leaf volatile (GLV) esters are decreased by CK increase. Therefore, it seems likely that GLV-mediated indirect defense is also decreased upon CK increase, which could provide another disadvantage for the plant in natural environments. The ecological consequence of the diminished GLV production remains to be tested for example by egg-predation essays in the field (e.g. described in Schuman, et al. 2012).

Another possible strategy to react to herbivore attack is tolerance. This means an allocation of resources away from the attacked tissue to other leaves or storage- and reproductive organs to minimize the loss of resources (Schwachtje *et al.* 2006, Frost and Hunter 2008, Schultz *et al.* 2013). In that way, a plant might additionally be able to starve an herbivore by resource mobilization and slow down its growth. Possibly these reactions could also be interpreted as a form of stress-induced senescence reaction. In the past, studies could demonstrate that simulated herbivory advances autumn phenology in *Acer rubrum* (Forkner 2014), that JA can induce senescence processes (He *et al.* 2002, Shan *et al.* 2011) and in **manuscript III**, I could show that the senescence activated SAG12 promoter from *Arabidopsis* is activated by herbivore feeding. This supports the idea that herbivory induced senescence is a part of a plants defense response. Especially this process might be very important in the protection against specialized herbivores. As CKs are able to inhibit senescence (Richmond and Lang 1957, Gan and Amasino 1995, Ori, et al. 1999, Guo and Gan 2011), they possibly also inhibit senescence-like processes that occur after herbivory. The senescence inhibition caused by CK overproduction might therefore account for the herbivore phenotype.

Plant breeding has often aimed for an inhibited senescence in order to increase yield by a longer growth period. Due to the complexity of their effects, CKs have not yet found their way into large scale agricultural application (Koprna, et al. 2016). In the creation of “forever young” plants in agriculture, the inhibition of herbivory- or other stress-induced senescence processes might be a big drawback of that breeding approach and requires to be considered. In an ideal situation the delay of senescence might occur for higher yield, but in a natural environment with all kinds of abiotic and biotic stresses, this might also become a disadvantage.

Besides the higher susceptibility to herbivores, the seed production and fruit ripening will be likely delayed. With our *SAG-IPT4* plants, we observed a later onset of seed production. Although possibly at the very end of the seed production this deficit might be caught up or even overhauled by the transgenic plants, in nature a delayed seed production and ripening might account for some problems: First, the time span the plant is exposed to abiotic and biotic stresses is prolonged for plants with delayed senescence. This might increase the probability of factors like frost, heavy rain, drought or insect outbreaks to occur during their growth period. Second,

their delayed fruit ripening might be problematic in temperate regions, where possibly the growth period of a plant with inhibited senescence might exceed the favorable weather conditions and fruit ripening might be impaired by to dry, to cold or to humid weather conditions. This might be especially problematic in regions with short vegetation periods.

Regarding our findings that an inhibited senescence makes plants more susceptible to herbivores and possibly delays fruit and seed ripening, this breeding goal might be viewed critical. Nonetheless, under the right circumstances CK-dependent senescence inhibition in plants might be a useful tool for agriculture.

4.5 *Cytokinin manipulations as a potential opportunity to hijack the plants metabolism not only for endophytes*

The discussion so far has only been focused on the function of CKs from the plant side of the plant-herbivore interactions. I could demonstrate that increased levels of CKs do even benefit specialized insects on *N. attenuata*. Therefore, CKs are a possible point of attack for insects to improve the quality of their food. It is actually not surprising, that strategies to manipulate CK metabolism have evolved amongst insects. For leaf-miners and gall forming insects, it has been shown that an infestation with these insects causes an accumulation of nutrients and an increase in CKs in infested tissue (e.g. Matsubar.S and Nakahira 1967, Engelbrecht, et al. 1969, Hartley 1998, Mapes and Davies 2001, Giron, et al. 2007, Behr, et al. 2010, Body, et al. 2013, Zhang, et al. 2016). Leaf-miners and gall forming insects have been identified as a potential source of the CK increase, as their body and saliva contain CKs (e.g. Engelbrecht, et al. 1969, Matsui, et al. 1975, Mapes and Davies 2001, Body, et al. 2013, Tanaka, et al. 2013).

With the study presented in **manuscript VI**, I added two major new findings: 1) I showed that a non-endophytic, free living insect, *T. notatus* also contains CKs in its body and its saliva and is likely to produce IP itself or with the help of an endosymbiont. So far it was assumed that this behavior is exclusive to endophytes. 2) I used a ^{15}N labelling experiment to proof that IP originating from the insect is transferred to the plant. It was already proposed that the increase of CKs around the feeding sites of endophytes was caused by the transfer and not by reactions of the plant to the herbivore (Mapes and Davies 2001, Giron, et al. 2016), but so far this transfer itself has not been demonstrated to my knowledge.

Still it is unclear, what causes the high levels of CKs in *T. notatus*. Studies with the leaf miner *Phyllonorycter blancardella* have shown that CKs in the insects are produced by endosymbiotic bacteria (Kaiser, et al. 2010, Body, et al. 2013). If CKs in *T. notatus* are also produced by endosymbionts, remains to be found out in future studies. As I discussed in **manuscript VI**, CK production by endosymbionts seems to be the most likely explanation, as we found no evidence for an accumulation of plant derived CKs in the insect. We found transcripts of

bacteria, like *Wolbachia*, that are main suspects to produce CKs in insects (Kaiser, et al. 2010, Body, et al. 2013), in the transcriptome of *T. notatus* (Crava, et al. 2016). Also the increase in SA levels after *T. notatus* attack that I show in **manuscript VI**, provides another hint for an involvement of microbes in this interaction. From interaction with aphids it was already suggested that aphid induced defense responses are triggered by effectors like the chaperononin GroEL derived from the endosymbiont *Buchnera* (Bos *et al.* 2010, Chaudhary *et al.* 2014, Elzinga *et al.* 2014). Therefore, an exploration of the involvement of microbes in this interaction might be a worthwhile direction for future research. Nonetheless, I cannot exclude that CKs are produced by other organisms like fungi, or the insect itself, as I discussed in **manuscript VI**.

From a co-evolutionary perspective, another interesting question is, if the insect derived CKs are beneficial for the plant as well to tolerate the insect infestation. It is possible that those CKs are important to have an adequate response to this insect herbivore. We cannot answer this question yet, but at least the fact that plants with silenced CK receptors (ir*chk2/3*; **manuscript VI**) seem to suffer more from an attack by *T. notatus*, hints into that direction.

4.6 Do cytokinins function as direct effectors?

My results from **manuscript VI** show, that endophytic insects are not the only insects that contain CKs. The fact that I found it also in a free living sap feeding insect suggests that the CK mediated manipulation of plants by insects is a mechanism that is possibly much more widespread than previously thought. The fact that also endophytes that are known for their CK-dependent plant manipulation are phylogenetically not very closely related, hints that this mechanism is either evolutionary very old, or that this mechanism has evolved several times. In both cases it seems likely that many more species might be able to accumulate CKs. Further investigations would be very helpful to find out, how widespread this phenomenon is.

To go one step further, one could extend the question about the abundancy of CKs in insects to the question, if CK abundancy is limited to phytophagous insects and what additional roles CKs could play in insects. Especially IP has been found in many organisms, including animals like nematodes (Siddique *et al.* 2015). The tRNA derived synthesis of IP and IPR could be a potential source of IP in almost all organisms (Persson *et al.* 1994). But CKs have not only been found inside organisms but also in the environment. There are reports of CKs being found in litter and soil, as well as fresh and marine water (Stirk and van Staden 2010). These observations have even led to the hypothesis that CKs could function as some kind of a cross-kingdom signaling molecule, comparable to ethylene (Schultz 2002, Schultz and Appel 2004, Robischon 2015). The question is, if this almost ubiquitous abundancy of CKs is a by-product of degradation processes of tRNA (McLennan 1975) or if CKs are sequestered by organisms for signaling purposes remains unclear.

So far, I have discussed the role of CKs in plant-insect interactions only as being causative for metabolic changes like defenses or nutrient allocation in the plant, which influence the interaction between the plant and the insect. But it is also not inconceivable that CKs themselves have the potential to influence the insects physiology and development. This could be seen as the counterpart to the insect-triggered CK manipulations of plants (Robischon 2015). Indeed, CKs have also been shown to have a direct influence on insects and other animals. Most of the experiments about a direct influence of CKs have been made with kinetin. Kinetin in the diet of *Zaprionus* fruitflies for example had a direct influence on development and fecundity of the flies and turned out to have an "anti-aging" effect (Sharma *et al.* 1995, Sharma *et al.* 1997). Nevertheless, only little work on the direct influence of CKs on insects has been published since then. Some studies have even examined the function of CKs in humans. Kinetin and zeatin have been demonstrated to have a "gerontomodulatory and youth-preserving" effect on human fibroblasts (Rattan and Clark 1994, Rattan and Sodagam 2005, Yang 2013) or keratinocytes (Berge *et al.* 2006) which lead to their application in costly "anti-aging" creams (Cronin and Draelos 2010). Also their potential influence on cancer cells (Dudzik *et al.* 2011, Siveen *et al.* 2017) or thymus and immune function (Li *et al.* 2016) has been reported. While an application of CKs in medicine or cosmetics might only be an interesting potential, a direct influence of CKs on insects might have huge implications for their interaction with plants. CKs could potentially interfere with insect performance and population dynamics. Recently, colleagues published a shift in sex-ratio of *T. notatus* on ir*chk2/3* plants with silenced CK receptors (Adam, et al. 2017). Although this could also be an indirect effect of the impaired CK signaling, this gives at least a starting point for future research.

Generally, a test for a direct effect of CKs on phytophagous insects is tricky, as it is hard to distinguish between direct and indirect effects. In plants, indirect effects of CKs might mask the direct effects. Therefore, one could first try to use artificial diet experiments and perform empirical studies on different insect species. A follow up study on plants could then use potentially transgenic plants silenced in different metabolic pathways to unravel the indirect from the direct CK effects.

Still the hypothesis that there is a direct influence of CKs on insects and that CKs also evolved as a cross-kingdom signal is very speculative, but not completely unsubstantiated by previous observations. Examining CKs as cross-kingdom signals certainly would open a completely new field of research and increase the complexity and significance of CK research.

4.7 References

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5 SUMMARY

Phytohormones play a crucial role in the interaction between plants and insect. Besides jasmonates and jasmonate dependent signaling, which represent the core part of a plant's defense against insect herbivores, other phytohormones have been shown to influence this interaction. It has been known for decades that endpophytic insects, like gall-formers and leaf-miners can use cytokinins (CKs), a group of growth hormones, to manipulate the plants metabolism in their own favor. When I started my thesis, almost nothing was known about the relevance of CKs in the interaction of plants with free living phytophagous insects.

In my thesis I characterized the role of CKs in the interaction of the ecological model plant *Nicotiana attenuata* with two of its most abundant free living herbivores: the larvae of the Tobacco Hawkmoth *Manduca sexta* and the small mirid species *Tupiocoris notatus.*

We could show that real or simulated herbivory increased the levels of some CKs and changed the accumulation of CK-dependent transcripts in leaves of *N. attenuata*. We observed these changes after damage by *M. sexta*, as well as *T. notatus*, a sap-feeder with a totally different feeding behavior. As we also found comparable changes in CK levels in *Arabidopsis thaliana*, I hypothesize that reactions of the CK metabolisms could be a widespread reaction to herbivory.

To understand the function of a reaction of the CK metabolism to herbivory, we examined the influence of CKs on the defense reactions of the plant. I used several transgenic and non-transgenic approaches to change CK levels and perception to recognize and minimize side effects on growth and development that can be triggered by CK changes. Amongst others, we established transgenic plants silenced in the expression of two of three known CK receptors (ir*chk2/3*); plants with senescence activated biosynthesis of CKs (*SAG-IPT4*); as well as plants with a construct which allows for dexamethasone inducible CK biosynthesis (i-ov*ipt*), which we also established as a tool for field experiments.

CK increases always led to increases in herbivory induced defense metabolites (HID) independently from the method we used, whereas an impaired CK perception led to a decrease in defense metabolites.

During our research we recognized that CKs and HID follow the same distribution pattern: We found high levels of CKs and HID in young leaves and low levels in old leaves. This pattern has been predicted by the "optimal defense theory": Young leaves provide a larger fitness-value for the plant and are therefore better defended than old leaves. This developmentally regulated distribution of defense metabolites has been reported several times but the underlying mechanisms remained elusive so far. Based on our observations I hypothesized that CKs are playing a role in the developmental regulation of defenses in the plant. Indeed we found that increasing CK levels in old leaves, using *SAG-IPT4* and i-ov*ipt* plants was sufficient to increase

levels of HID to the levels found in young leaves, whilst HID levels in comparable old leaves in WT plants were barely detectable.

The transcription factor Myb8 has been identified as a potential link of CKs to the developmentally dependent regulation of defenses, as its transcript levels were influenced by CKs. So far Myb8 was thought to be a specific regulator of phenolamide biosynthesis. We could show that further HID that are developmentally regulated, such as trypsin proteinase inhibitors and a threonine deaminase, were also influenced by Myb8 expression.

Although CK increases enhanced the plants defenses, we found leaves with increased CK levels (i-ov*ipt*) more attractive to *T. notatus*, which might be due to simultaneously increased levels of nutrients. This led me to the hypothesis, that a manipulation of CK-metabolism could not only be beneficial for endophytes but also for free living insects. Therefore I examined if a CK-dependent manipulation of the host plant by a free living insect could also be possible. We found high levels of the CK N^6-isopentenyladenine (IP) in the *T. notatus* insects as well their oral secretions. Using ^{15}N labeled plants, we could show that IP is being transferred in big amounts from the insects to the host plant. Potentially this is a way to stabilize and improve nutrient content in infested tissue.

In my thesis, I could show that CKs significantly influence the regulation of plant defense against insects and could be used by free living insects to manipulate the host plant. This suggests that the role of CKs goes far beyond the known cases of endophytes and that CKs have rather a key role in the interaction of plants and insects.

6 ZUSAMMENFASSUNG

In der Interaktion von Pflanzen mit Insekten, spielen Phytohormone eine entscheidende Rolle. Neben Jasmonaten und dem Jasmonatweg, die das Herzstück in der pflanzlichen Verteidigung gegen pflanzenfressende Insekten darstellen, können auch andere Phytohormone diese Interaktionen beeinflussen. Von endophytischen Insekten, wie Gallbildnern oder Blattminierern ist seit Jahrzehnten bekannt, dass sie Cytokinine (CK), eine Gruppe von Wachstumshormonen nutzen um den Pflanzlichen Stoffwechsel zu ihrem Vorteil zu manipulieren. Zu Beginn meiner Arbeit war jedoch kaum etwas über die Relevanz von CK in der Interaktion von Pflanzen mit freilebenden pflanzenfressenden Insekten bekannt.

In meiner Doktorarbeit habe ich die Rolle von CK in der Interaktion der ökologischen Modellpflanze *Nicotiana attenuata* und zwei ihrer häufigsten freilebenden Fraßfeinde, den Larven des Tabakschwärmers *Manduca sexta* sowie der kleinen Wanzenart *Tupiocoris notatus* charakterisiert.

Wir konnten feststellen, dass tatsächlicher oder simulierter Insektenfraß die Konzentrationen einiger CK in den Blättern von *N. attenuata* erhöhte und die Akkumulation mehrerer CK abhängiger Transkripte veränderte. Diese Veränderungen konnten sowohl bei Schaden durch *M. sexta,* als auch *T. notatus*, einem Pflanzensaftsauger mit völlig anderem Fraßverhalten, beobachtet werden. Da ähnliche Veränderungen der CK Konzentrationen auch in *Arabidopsis thaliana* bestätigt werden konnten, stelle ich die These auf, dass Reaktionen des CK-Metabolismus eine weit verbreitete Reaktion auf Insektenfraß darstellen.

Um die Funktion einer Reaktion des CK Metabolismus auf Herbivorie zu verstehen untersuchten wir den Einfluss von CK auf die Verteidigungsreaktionen der Pflanze. Um Wachstums- und Entwicklungsstörungen, die CK-Veränderungen verursachen können, zu erkennen und zu minimieren, nutzte ich mehrere transgene und nicht-transgene Ansätze um CK-Konzentrationen und CK-Wahrnehmung der Pflanzen zu verändern. Unter anderem etablierten wir transgene Pflanzen, mit stark reduzierter Expression von zwei von drei bekannten CK Rezeptoren (ir*chk2/3*); Pflanzen, mit Seneszenz-aktivierter Biosynthese von CK (*SAG-IPT4*); sowie Pflanzen, deren CK Biosynthese mittels eines Dexamethason-induzierbaren Promotors aktiviert werden kann (i-ov*ipt*) und die wir auch als Werkzeug für Feldversuche etablieren konnten.

Erhöhungen von CK führte unabhängig von der Art der Manipulation zu einer Erhöhung von Verteidigungsmetaboliten, die durch Herbivorie induziert wurden (Herbivore induced defenses: HID), wohingegen eine eingeschränkte Perzeption von CKs niedrigere Konzentrationen von Verteidigungsmetaboliten hervorrief.

Bei unseren Forschungsarbeiten erkannten wir, dass CK und und HID einem ähnlichen Verteilungsmuster folgen: Wir fanden hohe Werte von CK und HID in jungen Blättern und niedrige Werte in alten Blättern. Diese Verteilung von Verteidigungsmetaboliten wird durch die „Optimale Verteidiguns-Theorie“ vorhergesagt: Junge Blätter sind für den pflanzliche Reproduktionserfolg wichtiger und sind daher besser Verteidigt als alte. Diese entwicklungsabhängige Verteilung von Verteidigungsmetaboliten wurde oft beschrieben, jedoch waren die zugrundeliegenden Mechanismen bisher unbekannt. Aus unserer Beobachtung erwuchs die Hypothese, dass CKs eine Rolle in der entwicklungsgesteuerten Regulation der Verteidigung der Pflanze spielen. Tatsächlich konnten wir feststellen, dass in alten Blättern CK-Erhöhungen mittels *SAG-IPT4* oder i-ov*ipt* Pflanzen ausreichend waren um eine Erhöhung von HID auf den Wert von jungen Blättern zu bewirken, wohingegen alte Blätter in WT pflanzen kaum noch messbare HID-Konzentrationen zeigten.

Als ein möglicher Angriffspunkt für CKs in der entwicklungsgesteuerten Regulation der Verteidigung stellte sich der Transkriptionsfaktor Myb8 heraus, dessen Transkriptakkumulation durch CK-Konzentrationen beeinflusst wurde. Von Myb8 war bislang angenommen worden, dass er spezifisch die Biosynthese von Phenolamiden reguliert. Wir konnten zeigen, dass weitere HID, die einer entwicklungsabhängigen Verteilung folgen, nämlich Trypsin Proteinase Inhibitoren und eine Threonindeaminase, von Myb8 beeinflusst werden.

Obwohl CK-Erhöhungen die Verteidigung der Pflanze verstärkte, waren Blätter mit höheren CK Konzentrationen (i-ov*ipt*) dennoch attraktiver für *T. notatus*, was möglicherweise auf einen gleichzeitig erhöhten Nährstoffgehalt zurückzuführen ist.

Dies veranlasste mich zu der Hypothese, dass eine Manipulation des CK-Metabolismus nicht nur für endophytische, sondern auch für freilebende Insekten vom Vorteil sein könnte. Daher untersuchte ich, ob eine CK-abhängige Manipulation der Wirtspflanze auch durch ein freilebendes Insekt möglich ist. Wir fanden in *T. notatus* Insekten, sowie in deren während des Fraßes abgegebenen Sekreten hohe Konzentrationen des CKs N^6-Isopentenyladenin (IP). Mittels ^{15}N markierten Pflanzen zeigten wir, dass IP von *T. notatus* in großen Mengen auf die Pflanze übertragen wird. Möglicherweise dient dies der Stabilisierung und Verbesserung des Nährstoffgehalts der befallenen Blätter.

Meine Arbeit zeigt, dass CKs sowohl die Regulation der Verteidigung gegen Insekten maßgeblich beeinflussen, als auch von freilebenden Insekten zur Manipulation der Wirtspflanze genutzt werden könnten. Dies legt nahe, dass die Rolle der CK weit über die bisherigen bekannten Fälle von endophytischen Insekten hinausgeht und CK vielmehr von zentraler Bedeutung in der Interaktion zwischen Pflanze und Insekt sind.

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Schäfer, M., Brütting, C., Meza-Canales, I.D., Großkinsky, D.K., Vankova, R., Baldwin, I.T. and Meldau, S. (2015) The role of *cis*-zeatin-type cytokinins in plant growth regulation and mediating responses to environmental interactions. *J. Exp. Bot.*

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9 CURRICULUM VITAE

Name, first name:	**Brütting, Christoph**
Date of birth	**28.07.1986**
Place of birth	**Neuendettelsau, Germany**

EDUCATION

2012-2017	**PhD student** at Max-Planck-Institute for Chemical Ecology, Jena Department for Molecular Ecology (Prof. I. T. Baldwin) Associated to Friedrich-Schiller-University Jena, Germany PhD Thesis: "Cytokinins shape plant herbivore interactions in *Nicotiana attenuata"*
2009-2012	**Master Molecular Ecology** at University of Bayreuth, Germany Master-thesis: „The role of cytokinins in plant responses to insect attack*"* Supervision*:* Prof. Dr. S. Clemens (Dep. Plant Physiology, University Bayreuth), Prof. I. T. Baldwin und Dr. Stefan Meldau (MPICE)
2006-2009	**Bachelor Biology** University of Bayreuth, Germany Bachelor-thesis: „Influence of adipokinetic hormone on the fat body of female larvae of the Mediterranean field cricket (*Gryllus bimaculatus*)" Supervisor: PD Dr. M. Lorenz (Dep. Animal Physiology)
1997-2006	Laurentius Gymnasium Neuendettelsau, Germany
1993-1997	Grundschule Dietenhofen, Germany

PROFESSIONAL POSITIONS

2012 - 2017	**Research Assistant (PhD),** Dep. Molecular Ecology, MPI for Chemical Ecology, Jena, Germany

2011 – 2012	**Research Assistant (HiWi),** Dep. Molecular Ecology, MPI for Chemical Ecology, Jena, Germany
2011	**Research Assistant (HiWi)**, Dep. Evolutionary Neuroethology, MPI for Chemical Ecology, Jena, Germany
2010	**Teaching Assistant (HiWi)**, Dep. Plant Ecology, University Bayreuth, Germany
2007 – 2010	**Teaching Assistant (HiWi),** Dep. Plant Physiology, University Bayreuth, Germany
2009 – 2010	**Teaching Assistant (HiWi),** Dep. Animal Physiology, University Bayreuth, Germany
2009	**Teaching Assistant (HiWi)**, Dep. Plant Ecology, University Bayreuth, Germany
2007 – 2009	**Research Assistant (HiWi)**, Dep. Animal Ecology I, University Bayreuth, Germany
2006	**Dance Instructor Assistance**, Tanzschule Springer, Ansbach, Germany
2004 – 2005	**Nursing Care Assistant,** Seniorenresidenz Dietenhofen (geriatric nursing home), Dietenhofen, Germany.

TEACHING AND STUDENT SUPERVISIONS:

2012 - 2015	Supervision of seven research projects of Bachelor and Master students.
2007 – 2011	Supervision and teaching in basic courses of the Biology bachelor's programme at University Bayreuth:

- Anatomy and morphology of plants
- Knowledge and identification of the domestic fauna
- Practical courses in animal physiology
- Practical courses in developmental biology of insects

PUBLIC RELATIONS:

2013 – 2015	Supervision of experiments for students (12-17 years old) for the „Forsche Schüler Tag“
2014	Contribution to the planning of show experiments a spart of the „Langen Nacht der Wissenschaften“
2012	Writing and editing of Wikipedia articles

LANGUAGES

German:	Mother tongue
English:	fluent, proficient level
Spanish:	Basic level

ADDITIONAL QUALIFICATIONS

- Training according to §15 GenTSV as certified project leader and responsible for biological safety
- Training with the portable photosynthesis system LICOR Li6400XT
- Several trainings and advanced knowledge in R
- Proficient knowledge in Adobe Photoshop und Adobe Illustrator.
- Sophisticated knowledge with Linux-based operating systems
- Training and advanced knowledge in LaTex

AWARDS

2014	Prize for outstanding oral presentation, 7th European Workshop on Plant Senescence, Sandbjerg Estate, Denmark
2013	DAAD grant within the framework of the RISE Southern Europe project for an intern for my research project for two months.

Jena, 03. April 2018

(Christoph Brütting)

PUBLICATIONS

1. **Brütting, C., Schäfer, M., Gase, K., Baldwin, I.T., Meldau, S.** (2017) Herbivory induced senescence processes increase resistance of *Nicotiana attenuata* against the specialist herbivore *Manduca sexta*. (in preparation)
2. **Brütting, C., Crava, C. M., Schäfer, M., Meldau, S., Schuman, M.C., Baldwin, I. T.** (2018) Cytokinin transfer by the free living insect *Tupiocoris notatus* to its host-plant *Nicotiana attenuata* recapitulates a strategy of endophytic insects. (submitted to ELIFE)
3. **Joo, Y, Schuman, M.C., Goldberg, J.K., Kim, S.-G., Yon, F., Brütting, C., Baldwin, I.T.** (2018) Herbivore-induced volatile blends with both "fast" and "slow" components provide robust indirect defense in nature. FUNCTIONAL ECOLOGY 32, 136-149
4. **Schäfer, M. Brütting, C., Xu, S., Ling, X., Steppuhn, A., Baldwin, I.T.,Schuman, M.C.** (2017) *NaMYB8* regulates distinct, optimally distributed herbivore defense traits. JIPB 59 (12), 844–850
5. **Brütting, C., Schäfer, M., Vankova R., Gase, K., Baldwin, I.T., Meldaus, S.** (2017) Changes in cytokinins are sufficient to alter developmental patterns of defense metabolites in *Nicotiana attenuata*. THE PLANT JOURNAL, 89, 15-30
6. **Crava, C. M., Brütting, C., Baldwin, I. T.** (2016). Transcriptome analysis of the tobacco-specialist mirid Tupiocoris notatus: de novo assembly, expression profiling and identification of candidate "adaptation" genes. BMC GENOMICS, 17, 15
7. **Schäfer, M., Brütting, C., Baldwin, I.T., Kallenbach, M.** (2016): High-throughput quantification of more than 100 primary- and secondary-metabolites, and phytohormones by a single solid-phase extraction based sample preparation with analysis by UHPLC-HESI-MS/MS. PLANT METHODS, 12, 1-18.
8. **Schäfer, M., Brütting, C., Meza-Canales, I, Großkinski, D. K., Vankova, R., Baldwin, I. T., Meldau, S.**(2015). The role of *cis*-zeatin-type cytokinins in plant growth regulation and mediating responses to environmental interactions. JOURNAL OF EXPERIMENTAL BOTANY, 66, 4873-84.
9. **Schäfer, M., Meza-Canales, I., Brütting, C., Baldwin, I. T., Meldau, S.** (2015).Cytokinin levels and NaCHK2- and NaCHK3-mediated perception modulate herbivory-induced defense-signaling and defenses in Nicotiana attenuata. NEW PHYTOLOGIST, 207, 645-658.
10. **Schäfer, M., Meza Canales, I. D., Navarro-Quezada, A., Brütting, C., Radomira, V., Baldwin, I. T., Meldau, S.** (2015). Cytokinin levels and signaling respond to wounding and

the perception of herbivore elicitors in *Nicotiana attenuata*. JOURNAL OF INTEGRATIVE PLANT BIOLOGY, 57, 198-212**.**

11. **Schäfer, M., Brütting, C., Gase, K., Reichelt, M., Baldwin, I. T., Meldau, S.** (2013). "Real time" genetic manipulation: a new tool for ecological field studies. THE PLANT JOURNAL, 76, 506-518.

SELECTED TALKS AND POSTERS

1. SAB MEETING 2016, MAX PLANCK INSTITUTE FOR CHEMICAL ECOLOGY, Jena, DE, 2016
   Poster: From small molecules to multispecies interactions – different layers of plant-herbivore interactions
   Brütting C., Schäfer M., Li J., Heiling S., Zhou W., Adam N., Meza Canales I.D., Pradhan M., Baldwin I.T
2. ICE SYMPOSIUM 2016, MAX PLANCK INSTITUTE FOR CHEMICAL ECOLOGY, Jena, DE, 2016
   Poster: From small molecules to multispecies interactions – different layers of plant-herbivore interactions
   Brütting C., Schäfer M., Li J., Heiling S., Zhou W., Adam N., Meza Canales I.D., Pradhan M., Baldwin I.T
3. 12TH ANNUAL CONFERENCE OF THE METABOLOMICS SOCIETY, Dublin, IE, 2016
   Poster: Targeted analysis of primary- and secondary-metabolites, and phytohormones from a single plant extract – a method accounting for complexity in plant metabolomics
   Schäfer M., Brütting C., Kallenbach M., van't Slot G., Kutyniok M., Baldwin I.T
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10 EIGENSTÄNDIGKEITSERKLÄRUNG

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Jena, 03. April 2018

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Christoph Brütting

11 APPENDIX

11.1 Method for figure 5

One fully expanded rosette leaf of rosette stage WT plants was wounded using a pattern-wheel and oral secretions of *M. sexta* were applied as described in **manuscript I and IV**. Additionally whole plants were sprayed two times a day (morning and evening) with 5 µM (*t*ZR spray) or 0 µM *t*ZR (mock spray) solution. *t*ZR stock solution in 70 % EtOH is dissolved in water with 0.02% Tween 20 to a final concentration of 0.07 % EtOH in water. Mock-solution contains only Tween 20 and EtOH.

After 48 h, leaves were harvested and flash frozen in liquid nitrogen. RNA was purified, transcribed to cDNA and a qPCR analysis of *NaTD* transcript levels was performed as described in **manuscript IV**. Effect of *t*ZR spraying was tested using a t-test.

11.2 Method for figure 6

Manduca sexta performance assay

We placed five freshly hatched *M. sexta* caterpillars on each plant. After 3 days we reduced the number of caterpillars to two on each plant to correct for caterpillars that died within the first 3 days. We then determined weight of every individual caterpillar (replicate) at 6, 8, 10, 12 and 14 days after hatching. We performed individual Bonferroni corrected t-tests for each time point between WT and each of the two transgenic *SAG-IPT4* lines.

Soluble protein:

Soluble proteins were determined in the youngest fully expanded rosette leaf of a flowering *N. attenuata* plant with a Bradford assay as described in **manuscript VI**.