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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 (*t*Z), isopentenyladenine (IP), *cis*-zeatin (*c*Z) 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

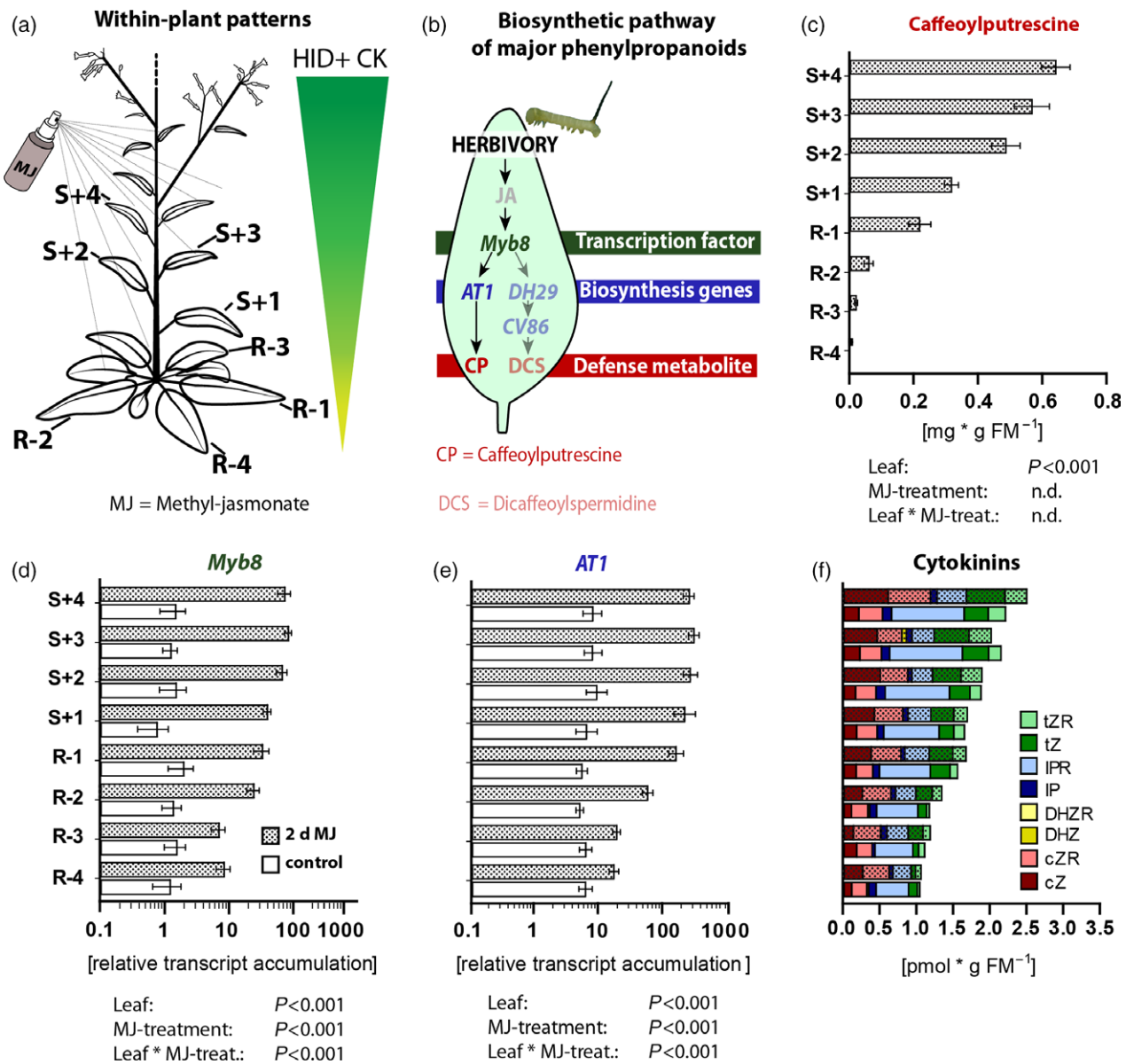

Figure 1. Herbivory-induced defense metabolites (HIDs) and cytokinins (CKs) follow the same within-plant distributions in *Nicotiana attenuata*.
(a) Experimental design and distribution of HID and CKs within a plant.
(b) Scheme of biosynthetic pathway of major phenolamides (PAs).
(c) Caffeoylputrescine (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.

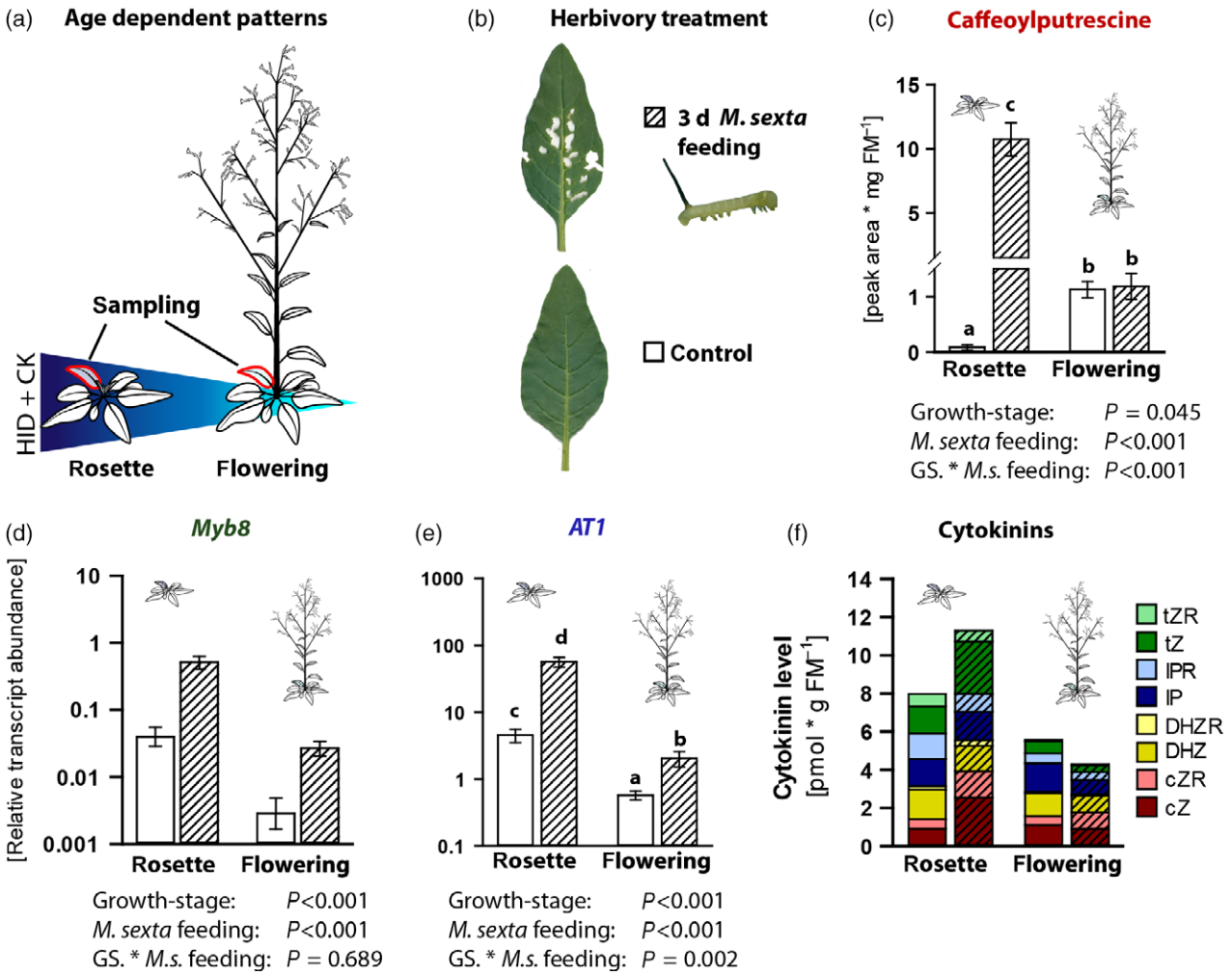

Figure 2. Herbivory-induced defense metabolites (HIDs) and cytokinins (CKs) follow similar developmental patterns in *Nicotiana attenuata*.
(a) Experimental design and the distribution of HIDs and CKs during plant development.
(b) Typical damage after 3 days *Manduca sexta* feeding and control leaf.
(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

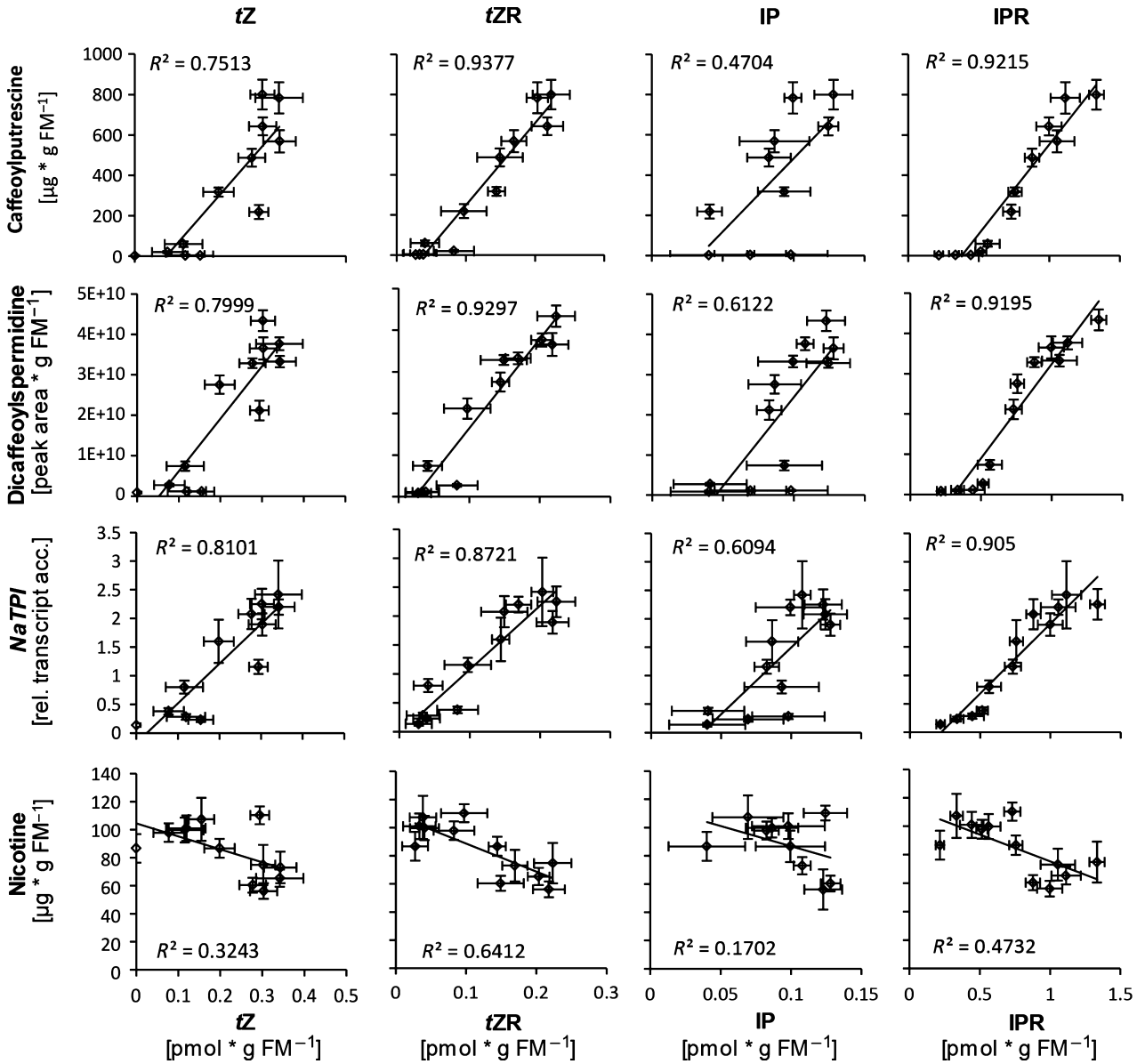

Figure 3. Correlations of cytokinin (CK) levels in untreated leaves with the accumulations of different defense compounds after methyl jasmonate (MJ)-induction in *Nicotiana attenuata*.

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).

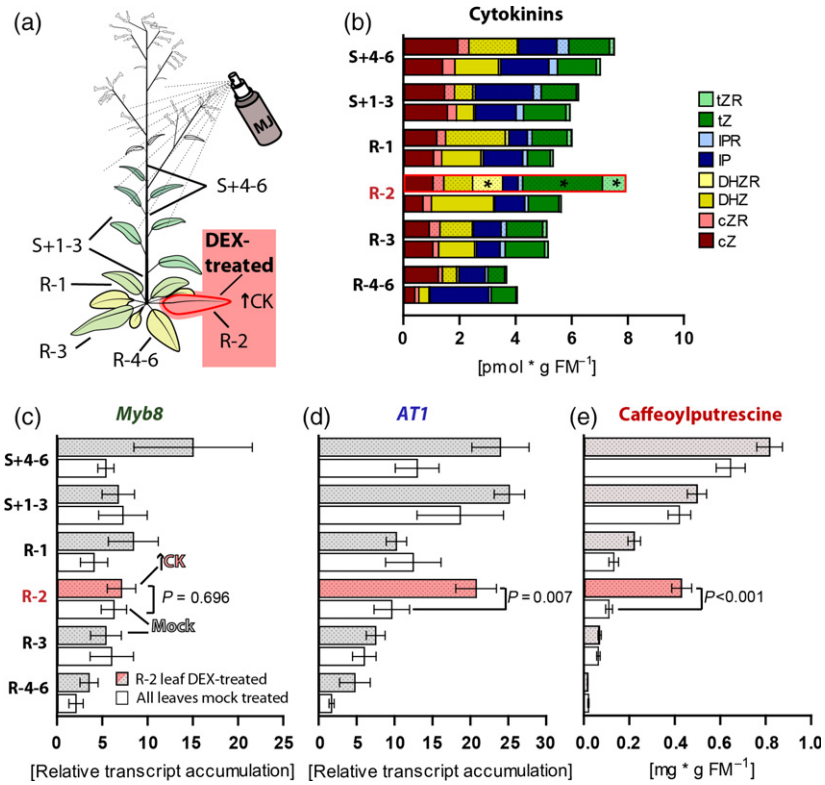

Figure 4. Manipulation of the within-plant cytokinin (CK) gradient alters the distribution of herbivory-inducible defenses in *Nicotiana attenuata*.
(a) Experimental design.
(b) CKs: *cis*-zeatin, *cZ*; *cis*-zeatin riboside, *c*ZR; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, IP; isopentenyladenosine, IPR; *trans*-zeatin, *tZ*; *trans*-zeatin riboside, *t*ZR; 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*, *t*ZR, 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

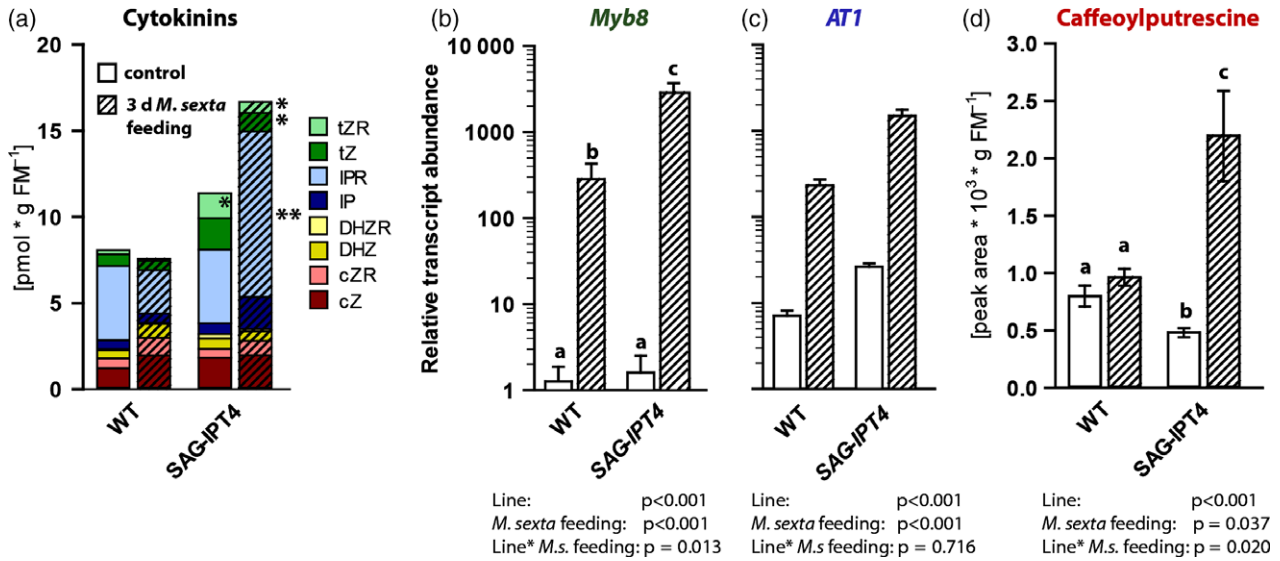

Figure 5. Restoring cytokinin (CK) levels to an earlier developmental stage increases defense gene expression and recovers inducibility of defenses in flowering *Nicotiana attenuata* plants.
Flowering wildtype (WT) and *SAG-IPT4* plants.
(a) CKs (*cis*-zeatin, *c*Z; *cis*-zeatin riboside, *c*ZR; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, IP; isopentenyladenosine, IPR; trans-zeatin, *t*Z; *trans*-zeatin riboside, *t*ZR; 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

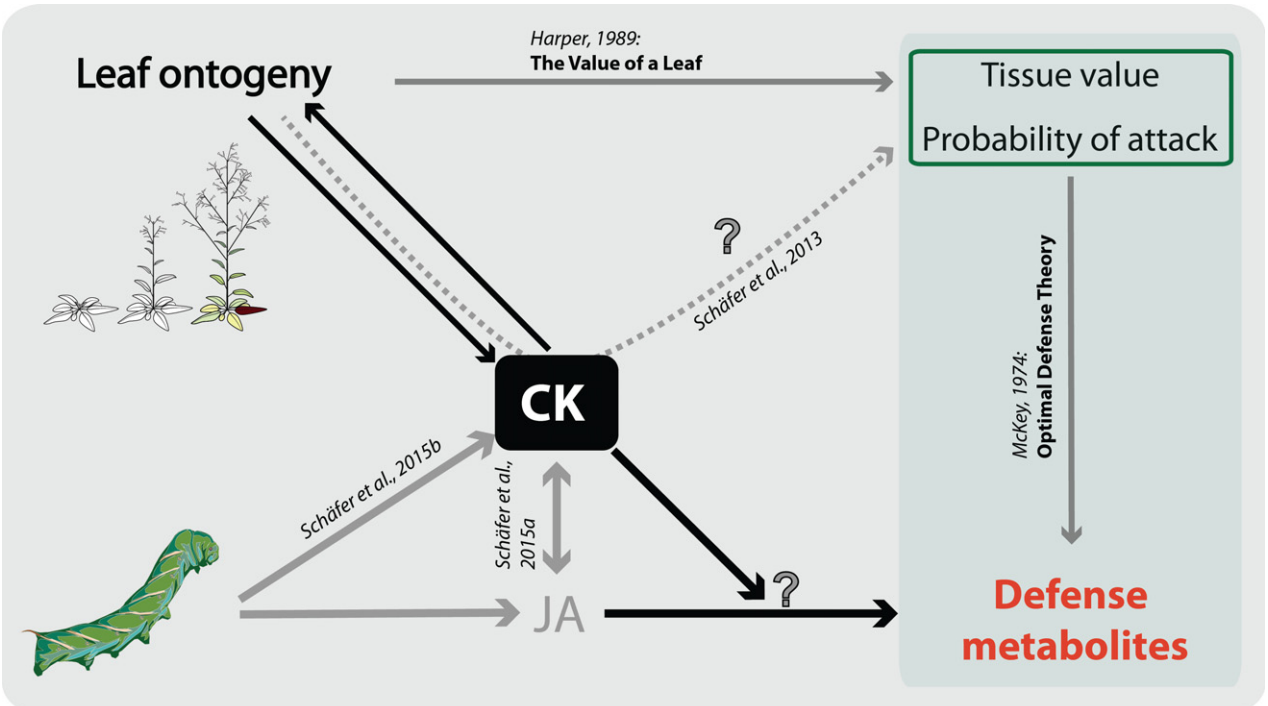

Figure 6. Cytokinins (CKs) influence the developmentally dependent distribution of defense metabolites in *Nicotiana attenuata*.
Black arrows: findings of this paper; gray arrows: previous publications as indicated next to the arrow. Leaf ontogeny/its developmental state change levels of CKs and vice 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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