Running title: *Phenolamide biosynthesis in tobacco*
MYB8 controls inducible phenolamide levels by activating three novel hydroxycinnamoyl-CoA:polyamine transferases in Nicotiana attenuata

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Abstract

A large number of plants accumulate N-acylated polyamines (phenolamides; PAs) in response to biotic and/or abiotic stress conditions. In the native tobacco (Nicotiana attenuata), the accumulation of two major PAs, caffeoylputrescine (CP) and dicafeoylspermidine (DCS) after herbivore attack is known to be controlled by a key transcription factor, MYB8. Using a broadly-targeted metabolomics approach, we show that a much larger spectrum of PAs composed of hydroxycinnamic acids and two polyamines, putrescine and spermidine, is regulated by this transcription factor. We clone several novel MYB8-regulated genes, annotated as putative acyltransferases, and analyze their function. One of the novel acyltransferases (AT1) is shown to encode a hydroxycinnamoyl-CoA: putrescine acyltransferase responsible for CP biosynthesis in tobacco. Another gene (DH29), specific for spermidine conjugation, mediates the initial acylation step in DCS formation. Although this enzyme was not able to perform the second acylation towards DCS biosynthesis, another acyltransferase CV86 gene proposed to act on mono-acylated spermidines was isolated and partially characterized. The activation of MYB8 in response to herbivore attack and associated signals required the activity of LIPOXYGENASE 3, a gene involved in JA biosynthesis in N. attenuata. These new results allow us to reconstruct a complete branch in JA signaling that defends N. attenuata plants against herbivores: JA via MYB8’s transcriptional control of AT1 and DH29 genes controls the entire branch of PA biosynthesis, which allows N. attenuata to mount a chemically diverse (and likely efficient) defense shield against herbivores.

237 words
**Introduction**

Phenolamides (PAs) are a diverse group of plant secondary metabolites found in many dicotyledonous plants as well as in monocots including wheat, barley, rice and maize (Martin-Tanguy et al., 1978; Martin-Tanguy 1985; Facchini et al., 2002; Edreva et al., 2007; Bassard et al., 2010). Although chemical structures of PAs are well studied, the regulation of their biosynthetic pathways, patterns of accumulation and the roles of PAs in plants remain largely unknown. PAs often accumulate in reproductive organs and developing tissues, suggesting their role in plant growth and development (Martin-Tanguy, 1985; Facchini et al., 2002; Edreva et al., 2007; Grienenberger et al., 2009; Luo et al., 2009). In addition, the involvement of PAs in plant defense against abiotic (mineral deficiency, drought and salt stress, and UV-irradiation) and biotic (pathogen infections and tissue chewing herbivores) stresses have been proposed (Stoessl and Unwin, 1970; Martin-Tanguy, 1985; Back et al., 2001; Tanaka et al., 2003; Camacho-Cristobal et al., 2005; Izaguirre et al., 2007; Kaur et al., 2010).

Jasmonates are important plant hormones that mediate plant responses to attack from herbivores and necrotrophic pathogens (Howe and Jander, 2008; Bari and Jones, 2009). Rapid accumulation of jasmonic acid (JA) after herbivore attack (Halitschke and Baldwin, 2003) results in altered secondary metabolite profiles in tobacco plants, as exemplified by the accumulation of alkaloids and phenylpropanoids (Baldwin, 1996; Shoji et al., 2000; Keinänen et al., 2001; Bernards and Bästrup-Spohr, 2008; Gaquerel et al., 2010). Goossens et al. (2003) demonstrated that the application of methyl jasmonate (MeJA) strongly induces the expression of genes involved in the alkaloid and phenylpropanoid biosynthetic pathways in tobacco cell cultures. Because JA-mediated responses are controlled by the activation of transcription factors (TFs) and synchronized expression of defense-related genes (Eulgem et al., 2000; Sugimoto et al., 2000; Singh et al., 2002; vom Endt et al., 2002; Zheng et al., 2007; Woldemariam et al., 2011), discovery of regulatory TFs greatly facilitates the identification of novel biosynthetic enzymes, and can potentially serve as important tools for the efficient regulation of metabolic pathways through the manipulation of a single gene in the plant genome.

In PA biosynthesis, the expression of MYB-TF (NtMYBJS1) was rapidly elicited by MeJA in BY-2 cell suspension cultures, and the over-expression of this gene resulted
in a strong ectopic accumulation of CP in tobacco cells (Galis et al., 2006). When Kaur et al. (2010) targeted a homologue of the NtMYBJS1 gene in *N. attenuata* (NaMYB8) to down-regulate the levels of CP and DCS, both metabolites appeared to be essential for *N. attenuata*’s defense against chewing herbivores. While it highlighted the role of PAs in plant defense, the identity of the specific CP and DCS biosynthetic genes controlled by NaMYB8-TF remained unknown.

It has been shown that the N-coupling reaction of polyamines to phenolic acids (such as cinnamic-, *p*-coumaric-, caffeic-, ferulic- and sinapic acids) in plants is catalyzed by a specific class of acyltransferase enzymes. A significant number of these proteins have been shown to belong to previously characterized BAHD protein superfamily (reviewed by D’Auria, 2006). However, the conventional use of sequence homology to identify substrates and enzyme activities of BAHD proteins often fails because many BAHD enzymes with similar substrate specificities have evolved independently through a process of convergent evolution (Pichersky and Lewinsohn, 2011). This may include the case of CP and DCS secondary metabolites: although being very abundant in plants, their biosynthetic genes remained unresolved. In addition, proteins from other groups can be also involved in the biosynthesis of phenolamides in plants, such as structurally divergent tyramine hydroxycinnamoyltransferases (Bassard et al., 2010) that contain a GCN5-related *N*-acetyltransferase (GNAT)-like domain in their structure.

Due to difficulties associated with identification of PA biosynthetic enzymes, development of novel approaches such as correlation analysis of metabolite accumulation and gene expression used in this work are essential to find and characterize novel enzymes (Luo et al., 2007). In the initial analysis, using a metabolomics approach, we show that NaMYB8 (referred to as MYB8 in this paper) TF controls a surprisingly wide variety of PAs in *N. attenuata* plants. Next, we used data mining and microarray analysis to identify several novel PA biosynthetic candidate genes controlled by the activity of the *MYB8* gene. Finally, silencing the expression of three most promising candidate genes by virus-induced gene silencing (VIGS), combined with metabolomics analysis revealed that MYB8-controlled *AT1, DH29* and *CV86* genes are directly responsible for PA biosynthesis in *N. attenuata* plants.
Results

**MYB8 controls a large spectrum of PAs in *N. attenuata* plants**

In our previous study, the silencing of the MYB8 transcription factor (TF) resulted in a strong suppression of CP and DCS in *N. attenuata* (Kaur et al., 2010). To examine whether MYB8 also controls other less abundant PAs that occur in tobacco plants (Bassard et al., 2010), we used a broadly targeted metabolomics approach to compare the herbivory-elicited metabolic profiles of wild type (WT) and MYB8-silenced (irMYB8) leaves. The young rosette leaves of WT and irMYB8 plants were elicited by treating puncture wounds (W) with diluted oral secretions (OS) from *Manduca sexta* larvae (W+OS), which strongly elicits plant defenses and the accumulation of CP and DCS in *N. attenuata*. The extracts from the leaves collected 24 h after elicitation were subjected to the ultra-performance liquid chromatography (UPLC) coupled to an electrospray ionization-time-of-flight mass spectrometer (Gaquerel et al., 2010). Peak matrices obtained from the analysis of WT and irMYB8 leaf extracts (see Material and Methods for data processing) were first subjected to a principal component analysis (PCA) to examine the variance in our dataset without referring to the class labels.

Metabolic profiles from both irMYB8 constitutive and induced leaf samples strongly separated from those of WT leaves in the PCA projection plots (Supplemental Fig. S1 online), suggesting the existence of major metabolic differences between the two genotypes (the cumulative variance of PC1 and PC2 accounted for 53.3 % of the total variance between WT and irMYB8 samples). *m/z* features (parameters defined as discrete *m/z* values at defined retention times) that were most affected by OS-elicitation exerted strong loading values on PC1, as observed by comparing the rankings of the loadings on two first PCs and their relative fold-changes (WT/W+OS vs. WT/control in Supplemental Fig. S2 online). The *m/z* patterns controlled by MYB8 (revealed from the comparison irMYB8/W+OS vs. WT/W+OS in Supplemental Fig. S2 online) affected the orientation of both PCs, indicating that other signals which are less affected by the W+OS, and likely developmentally controlled, could also be regulated through MYB8 signaling.

When we annotated the pseudo-spectra corresponding to *m/z* signals exhibiting the strongest signals on these two components, ions characteristic of caffeic acid-containing metabolites showed the highest contributions to the PCA separations.
Annotations of novel MYB8-dependent metabolites

We next performed a direct pairwise statistical analysis of irMYB8 vs. WT metabolic profiles (Supplemental Table S2 online). In total, 387 m/z signals (5.6 % of the normalized m/z profile) were up-regulated (2-fold change; unpaired t-test, P < 0.05) and 707 (10.3 %) down-regulated in irMYB8 compared to WT leaves at 24 h after W+OS elicitation. The m/z signal behavior was again strongly influenced by OS-elicitation treatment (Supplemental Fig. S3 online). To directly visualize the phenolic constituents controlled by MYB8 TF, we computed extracted ion traces corresponding to the typical signatures of coumaroyl- ([M+H]+ m/z 147.04 ± 0.05), caffeoyl- ([M+H]+ m/z 163.04 ± 0.05) and feruloyl- ([M+H]+ m/z 177.04 ± 0.05) moieties after in-source fragmentation of molecular ions and cleavage of the core molecules (e.g. sugars, polyamines and small acids). The overlay and visual inspection of extracted ion traces in Fig. 1 revealed that the MYB8 TF silencing affects a number of coumaric acid-, caffeic acid- and ferulic acid-containing metabolites with distinct retention times. In contrast, ion traces of cinnamic and sinapic acid moieties showed only minor differences between WT and irMYB8 leaf samples (data not shown). When the predicted parent ions of the most abundant phenolamides composed of coumaric acid-, caffeic acid- and ferulic acid coupled to putrescine or spermidine were extracted, even more striking differences in the accumulation of these metabolites were observed, further supporting a central role of MYB8 in PA biosynthesis (Supplemental Fig. S4 online). From this analysis, MYB8 TF can regulate at least 29 different (includes isomeric forms) hydroxyccinnamoylputrescine/spermidine conjugates (Table 1; Supplemental Fig. S4 online) in N. attenuata. Given the complexity of the observed regulated pool of metabolites, we predicted the existence of multiple MYB8 TF-controlled hydroxycinnamoyl-CoA: putrescine/spermidine transferases and pursued this prediction in the following text.
Identification of novel MYB8-regulated acyltransferases

In the previous report, a custom oligo-DNA microarray was used to identify differentially regulated genes in irMYB plants after W+OS elicitation (Kaur et al., 2010). Although the microarray contained a relatively small number of oligoprobes (1,421), two cDNA fragments from *N. attenuata* encoding potential acyltransferase genes controlled by MYB8-TF were found. The DH29 and CV86.1 (referred to as CV86 in this manuscript) gene fragments, originally cloned as herbivory-regulated ESTs (DH29, Hui et al., 2003; CV86.1, Voelckel and Baldwin, 2003), showed 3.8- and 3.7-fold down-regulation in irMYB8 compared to WT leaves at 90 min after W+OS elicitation, respectively (Kaur et al., 2010). First, we tried to extend the partial cDNA fragments of DH29 (CA591847.1) and CV86 (BU494535.1) genes by repeatedly blasting the fragments against public ESTs libraries from *N. tabacum* available in NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). When the putative full length size of *N. tabacum* DH29 and CV86 genes was reached (i.e., no additional overlapping ESTs were found), primers outside the longest ORF were designed (Supplemental Table S3 online) and full length CDS of *N. attenuata* DH29 and CV86 were cloned, sequenced and deposited in database under accession numbers JN390824 (*N. attenuata* DH29) and JN390825 (*N. attenuata* CV86).

We then used the *N. attenuata* DH29 and CV86 sequences as protein queries to search for additional acyltransferase-like enzymes in tobacco, exploring the *N. tabacum* EST libraries in NCBI (see Material and Methods for details). In total, we obtained 32 potential acyltransferase-like sequences that could be examined for their expression using our currently established herbivory-regulated microarray dataset. In this experiment, time kinetics of labeled cRNA probes from W+OS, W+ water (W+W) and control leaves treated for 1, 5, 9, 13, 17 and 21h were hybridized to a *N. attenuata*-specific Agilent microarray platform GPL13527. Raw and normalized data from the triplicated experiment showing gene expression in locally treated leaves, systemic leaves and roots were already deposited in public GEO microarray repository under sample numbers GSM750588 - GSM750721. Searching the expression patterns of our 32 putative acyltransferases, we found 15 novel herbivory-regulated transcripts, using a strong
elicitation by W and/or W+OS, and systemic induction in untreated leaves as main criteria for herbivory-regulated genes (Supplemental Table S4 online, worksheet C).

Next, we performed a visual inspection of all expression patterns, searching for those that matched the expression of *MYB8*, *DH29* and *CV86* genes, assuming that the use of common TF will result in significant co-regulation of downstream genes. The main criteria were (1) strong W+OS elicitation in local and (2) systemic leaves and (3) low expression of genes in the roots (Supplemental Table S4 online, worksheet C; Supplemental Fig. S5 online). After screening, the *AT1* gene represented by CUST_51472_PI422650789 oligoprobe on the microarray was selected; this acyltransferase-like gene was strongly induced and closely co-regulated with *DH29* and *CV86* genes. After cloning the entire *N. attenuata* *AT1* gene (deposited in database as JN390826), the *AT1* expression in W+OS-elicited WT and irMYB8 leaves was examined by RT-qPCR. *AT1* gene was strongly down-regulated in irMYB8 OS-elicited leaves compared to WT plants, suggesting that it is indeed located downstream of the MYB8 control element (Fig. 2). At the same time, MYB8-dependent expression of DH29 and CV86 in OS-locally induced and systemic unwounded leaves was confirmed by RT-qPCR (Fig. 2). In summary, our results indicated that MYB8 TF acts upstream of AT1, DH29 and CV86 putative acyltransferases, and that MYB 8 controls the expression of these genes in *N. attenuata* both locally and systemically during herbivory.

**Silencing of acyltransferases impairs PA accumulation**

When we compared our novel acyltransferases against the set of known BAHD proteins (Luo et al., 2009), adding several other characterized hydroxycinnamoyl-CoA:polyamine transferases (Bassard et al., 2010; Supplemental Fig. S6 online), the AT1 protein clustered with *Hordeum vulgare ACT* gene known to utilize a short chain polyamine agmatine as its acyl acceptor (Burhenne et al., 2003). In contrast, DH29 and CV86 clustered with proteins that contained the acetyl-CoA:benzylalcohol acetyltransferase (BEAT) protein from *Clarkia breweri* (Dudareva et al., 1998). Neither of the proteins closely grouped with the Arabidopsis spermidine utilizing SCT, SDT or SHT N-hydroxycinnamoyltransferases (Grienenberger et al., 2009; Luo et al., 2009) as
would be expected from our metabolic profiles shown in Fig. 1. We therefore proceeded
to direct functional characterization of AT1, DH29 and CV86 proteins.

When the expression of *AT1*, *DH29* and *CV86* was separately silenced by virus
induced gene silencing (VIGS), all three genes showed reduced transcript levels in
respective VIGS plants (Supplemental Fig. S7 online). Next, the phenolamides in an
empty vector (EV) control, AT1-, DH29- and CV86-VIGS plants that had their leaves fed
on by *M. sexta* neonates for 4 d were examined. Because of the large number of plants
involved in our functional screens, we performed our experiments in two groups, each
time using a new set of control EV-inoculated plants (Group 1: AT1-EV1; Group 2:
DH29-CV86-EV2). Herbivore-fed and control leaves were extracted and subjected to
high performance liquid chromatography coupled to photodiode array detection (HPLC-
PDA). An abundant UV-absorbing peak corresponding to CP was strongly suppressed in
AT1-VIGS plants (Fig. 3A), while the peak corresponding to pooled DCS increased. In
contrast, pooled DCS content was strongly reduced in DH29 leaves (Fig. 3B). Only small
amounts of mono-caffeoylspermidine (MCS) were typically detected in the samples,
suggesting a rapid conversion of mono-acylated spermidines into PAs like DCS; however,
CV86-VIGS plants accumulated abnormally high levels of MCS during herbivory (Fig.
3C).

Because HPLC-PDA only detects the most dominant metabolites in the otherwise
complex plant extracts, we utilized the more sensitive LC-ESI-TOFMS to examine the
metabolic changes in our herbivore-fed leaves from VIGS plants. As expected from
HPLC-PDA profiles, CP isomeric peaks at m/z 251.13 ± 0.05 were strongly reduced in
AT1-VIGS (Fig. 4; Supplemental Table S5 online). In addition, a putative p-
coumaroylputrescine peak (CoP; m/z 235.14±0.05) was reduced in AT1-VIGS but
surprisingly, putative feruloylputrescine (FP; m/z 265.15±0.05) content did not change
after silencing of AT1 enzyme, suggesting that FP might be produced by another yet
unknown MYB8-dependent enzyme. The isomeric peaks belonging to DCS (m/z 470.23
± 0.05) were strongly and partially reduced in DH29- and CV86-VIGS leaf extracts,
respectively. Silencing the expression of DH29 affected the entire spectrum of acylated
spermidines (Fig. 4) suggesting a broad donor substrate specificity of this enzyme. The
over-accumulation of MCS dominated the LC-ESI-TOFMS traces in CV86-VIGS leaves,
but this accumulation pattern now extended to include putative mono-\textit{p}-coumaroylspermidine and mono-feruloylspermidine metabolites (Fig. 4).

Even after a single W+OS elicitation treatment, similar metabolic patterns could be observed in the leaves from VIGS plants (Supplemental Fig. S8 online; Supplemental Table S6 online), however they were much less pronounced compared to herbivore-fed leaves. When W+OS-elicited samples were subjected to PCA analysis, the metabolic profiles of AT1-, DH29- and CV86-VIGS-silenced plants strongly separated from their respective EV-VIGS controls (Fig. 5A). Interestingly, while the majority of metabolites in irMYB8 leaves was down-regulated (Table 1; Supplemental Fig. S3 online), the regulation of the same set of metabolites in the individual \textit{AT1}-, \textit{DH29}- and \textit{CV86} gene-silenced plants was more complex, showing the highly interconnected character of PA biosynthesis (Fig. 5B). Typically, silencing of one acyltransferase enzyme impaired the accumulation of several compounds while increasing another set of metabolites – most likely a result of silencing-mediated “metabolic tension” in the PA biosynthetic pathway. This was particularly discernible in the case of \textit{AT1} and \textit{DH29} regulated putrescine- and spermidine-containing metabolites, respectively (Fig. 5B; follow the contrasting red/green colors in the heatmaps).

\textit{In vitro} activity of recombinant acyltransferase enzymes

Our \textit{in vivo} metabolic profiles of VIGS plants suggested that \textit{AT1} encodes a hydroxycinnamoyl-CoA: putrescine transferase, \textit{DH29} showed properties predicted for a hydroxycinnamoyl-CoA: spermidine transferase, and the role of \textit{CV86} was suggestive of hydroxycinnamoyl-CoA: hydroxycinnamoylspermidine conjugating activity. \textit{AT1}, \textit{DH29} and \textit{CV86} were therefore expressed and purified (using 6xHis tag sequence attached to C-terminus of each of the proteins; Supplemental Fig. S9A online) to examine their biochemical function \textit{in vitro}. While the proteins accumulated in \textit{E. coli} cells, native \textit{CV86} protein was recovered exclusively as inactive protein in pellets during extraction. We therefore purified soluble \textit{CV86} protein under denaturing conditions and subsequently renatured the protein by dialysis. Despite numerous attempts under numerous conditions, the \textit{CV86} protein showed no activity with substrates predicted from our previous metabolic studies.
We therefore focused on the AT1 (50.8 kDa) and DH29 (52 kDa) proteins and successfully determined their enzymatic activity. Notably, recombinant DH29 protein showed significant tendency for precipitation, especially when frozen in the elution buffer with imidazole and it was unstable during storage at -80°C. The protein precipitation could be efficiently avoided by directly desalting the protein after elution from the Ni-column, and frozen protein aliquots were used immediately after defrosting on ice to minimize the unavoidable loss of enzyme activity. Compared to DH29, the AT1 recombinant enzyme was fully soluble and more resistant to temperature changes during handling.

Enzyme activity assays with recombinant AT1 and DH29 proteins, informed by close examination of our VIGS metabolomics data, were performed with enzymatically prepared \( p \)-coumaroyl-, caffeoyl- and feruloyl-CoA esters as acyl donors, and polyamines putrescine, spermidine and spermine as acyl acceptors (Supplemental Fig. S9B, C online). When using caffeoyl-CoA as acyl donor, AT1 was only active with putrescine (Fig. 6A; Table 2), while DH29 required spermidine for its activity (Fig. 6B; Table 2). Neither of the proteins was active with spermine. These data were fully consistent with metabolic changes observed in our VIGS-silenced plants (Fig. 3-5). When using fixed concentrations of caffeoyl-CoA as an acyl donor and variable spermidine levels, a low apparent Km value (27.2 ± 5.3 μM) of DH29 for spermidine was observed. In contrast, an apparent Km value of AT1 for putrescine was significantly higher (664 ± 56 μM) in a similar experiment with putrescine (variable) and caffeoyl-CoA (fixed). In a relative comparison, caffeoyl-CoA was the best substrate for both AT1 (Km = 24.0 ± 4.9 μM) and DH29 (Km = 325 ± 12 μM) when their obligatory polyamines were used (Table 2). DH29 also metabolized feruloyl-CoA (70% relative activity compared to caffeoyl-CoA) and showed minor activity with \( p \)-coumaroyl-CoA (8% relative activity of caffeoyl-CoA). AT1 showed low but detectable activity with feruloyl-CoA (10% relative activity of caffeoyl-CoA), while it was almost inactive in vitro with \( p \)-coumaroyl-CoA as a substrate (Table 2). AT1 and DH29 were found to be most active at pH 8 and pH 7.5, respectively (Supplemental Fig. S10 online). Finally, the main reaction product of AT1, determined by UPLC-ESI-TOF was matching the structure of synthetic CP standard (Fig. 6C, left), while the product of DH29 showed the expected spectral properties of MCS (Fig. 6C,
right). In our experiments, we did not detect any reaction products corresponding to higher-acylated (di-, tri-) forms of polyamines.

In conclusion, we annotated AT1 as an authentic CP synthase that correlates with the accumulation of CP but not dicaffeoylputrescine in *N. attenuata*. The function of DH29 was tentatively assigned to the biosynthesis of MCS (as well as coumaroylspermidine and feruloylspermidine). However, DCS in *N. attenuata* occurs in at least 4 isomeric forms (Supplemental Fig. S4 online). Currently, the silencing of CV86 gene did not provide sufficiently conclusive information as only some DCS isomers were significantly reduced in CV86-VIGS leaves (Supplemental Fig. S8 online; Supplemental Table S6 online). Therefore, the identification of an enzyme(s) involved in the conversion of MCS to DCS, which partially overlapped with the experimentally determined function of CV86 enzyme, still remains to be resolved.

**Accumulation of DCS during ontogeny**

While the pattern of CP accumulation during ontogeny was published earlier by our group (Kaur et al., 2010), a comparably detailed accumulation profile of DCS was not reported. Returning to the original dataset used by Kaur et al. (2010) for CP quantification, we now determined the pooled DCS content in the W+OS-elicited *N. attenuata* plants at 5 distinct developmental stages (analyzed by HPLC-PDA in all available tissues). The highest concentration of DCS was found in the young rosette leaves, which was further enhanced by W+OS elicitation of the young leaves. Basal stems of early elongated plants also contained very high induced levels of DCS (Fig. 7). The overall accumulation patterns of DCS were similar to that of CP (Kaur et al., 2010), where younger tissues contained significantly more DCS (or CP) compared to the older leaves and roots. The levels of DCS in the vegetative parts of the flowering and mature plants were low and less inducible by W+OS treatment (Fig. 7). However, DCS was still detected in the capsules of mature *N. attenuata* plants (Fig. 7). When the accumulation of two other phenolic metabolites, rutin and chlorogenic acid (CGA), was determined, these metabolites lacked the herbivory-inducible character (Supplemental Fig. 11 online). Indeed, the accumulation of rutin and CGA does not depend on MYB8 TF that
specifically regulates the accumulation of direct defenses in tobacco plants against herbivores.

**MYB8 connects JA to metabolic genes and PAs**

Previously, low levels of CP and DCS were reported in both JA-biosynthesis and JA-perception deficient tobacco plants (Paschold et al., 2007; Demkura et al., 2010). To determine if MYB8 directly links JA to downstream metabolic genes, we examined the accumulation patterns of MYB8, AT1, DH29 and CV86 transcripts (Fig. 8A) in plants silenced in the expression of the LOX3 gene (asLOX3; Fig. 8B), and therefore having very low accumulated levels of JA (Halitschke and Baldwin, 2003; Onkokesung et al., 2010). Relative transcript levels of all studied genes were coordinately reduced in asLOX3 compared to WT plants before and 1, 6, 20 and 60h after W+OS elicitation (Fig. 8A). The levels of CP and DCS determined in the same samples confirmed the previously demonstrated reduction of CP and DCS in the asLOX3 genetic background (Fig. 8C), establishing the important role of JA, MYB8 and MYB8-controlled acyltransferase gene network in the PA accumulation process in response to herbivory in *N. attenuata* (Fig. 9).

**Discussion**

Although the biochemical activity leading to CP and DCS biosynthesis in tobacco was demonstrated nearly 20 years ago (Negrel et al., 1989, 1991 and 1992), cloning and characterization of the respective acyltransferase genes has not been reported. Currently, the combination of gene and metabolite profiling methods in *N. attenuata* provided a new powerful molecular tool for the functional genetics in this established ecological plant model. Taking the advantage of this novel platform, we show that JA-regulated *AT1*, *DH29* and *CV86* genes encode three essential acyltransferases in PA biosynthesis, including the two most abundant PAs - CP and DCS - in tobacco plants.

**Distribution and role of PAs in *N. attenuata***

PAs, such as CP, di-*p*-coumaroylspermidine and *p*-coumaroyltyramine are abundant floral constituents in several *Solanaceae* plant species. It was therefore
proposed that PAs should play important role(s) in plant development, and the occurrence of PAs in plants was intensively examined (Cabanne at al., 1981; Martin-Tanguy, 1997 and 1985). In *N. attenuata*, CP and DCS can be readily determined by HPLC in all aboveground tissues (CP, Kaur et al., 2010; DCS, Fig. 7). In addition, both CP and DCS are more abundant in the young vegetative tissues and reproductive organs, and their accumulation is strongly amplified by the attack of chewing herbivores in *N. attenuata* plants (CP, Kaur et al., 2010; DCS, Fig. 7 in this report). These accumulation patterns therefore strongly support a defense-related function of PAs against herbivores (Kaur et al., 2010). Notably, the plants that were completely deprived of CP and DCS by MYB8 silencing showed no developmental abnormalities and produced normal flowers and seeds (Kaur et al., 2010). Consistent with stable-silenced irMYB8 plants, VIGS plants silenced separately in CP (AT1-VIGS) and DCS (DH29-VIGS) biosynthesis showed completely normal morphology, providing another argument against proposed developmental roles of PAs.

While the regulatory role of MYB8 in Kaur et al. (2010) was limited to CP and DCS, Gaquerel et al. (2010) demonstrated that a much broader range of PAs can be elicited by simulated herbivore treatment (W+OS) in *N. attenuata* leaves. Indeed, we found that the majority (if not all) putrescine- and spermidine-containing PAs in *N. attenuata* accumulate in MYB8-dependent manner (Fig. 1; Supplemental Figs. S1, S2, S3, S4 online, Table 1). Although a direct application of CP to tobacco leaves retarded growth of *M. sexta* larvae (Kaur et al. 2010), the exact mode of toxicity of PAs in insects remains unknown. A broad spectrum of PAs regulated during herbivory shown in this paper suggests that a mixture of various PAs may be required to exert maximal efficiency of PAs against insects. In addition, as the application of synthetic PAs to insect artificial diet usually did not affect the growth of insects (Bassard et al., 2010), the presence of plant factor(s), such as low molecular compounds or enzymes, may be required for transformation of PAs to toxic metabolites, most likely during digestion of plant material in insect guts.

**Limited functionality of DH29 suggests a two-step biosynthesis of DCS**
Previously published data in Arabidopsis suggested that acyltransferases are most likely multi-functional enzymes. For example, Luo et al. (2009) reported two novel spermidine specific acyltransferases - namely spermidine dicoumaroyltransferase (SCT) and spermidine disinapoyltransferase (SDT) - in Arabidopsis, which mediated diacylation of spermidine in vitro. The Arabidopsis spermidine-hydroxycinnamoyltransferase (SHT) recombinant enzyme was even able to produce tri-feruloylspermidine in vitro (Grienenberger et al., 2009). In contrast, the DH29 enzyme reported here, which readily transferred one acyl group to spermidine, failed to produce DCS or tri-caffeoylspermidine from the caffeoyl-CoA and spermidine in vitro. While this could be an artifact of our in vitro reaction conditions and/or recombinant origin of the protein, we obtained a potential explanation of our data by partially solving the function of CV86 protein. This enzyme, while being structurally very similar to the DH29 protein was not involved in the first acylation of spermidine (shown in vivo by silencing of CV86). Instead, the inhibition of CV86 activity by VIGS resulted in a strong accumulation of mono-acylated spermidines (products of DH29) in herbivore attacked N. attenuata leaves, strongly suggesting that the CV86 protein might be utilizing monoacylated spermidines as its acyl acceptor substrates.

According to our high resolution mass spectrometry data and MS² experiments, at least four isomeric peaks of DCS exist in N. attenuata (Supplemental Fig. S4 online; Table 1). Interestingly, even when CV86 gene was strongly silenced by VIGS (Supplemental Fig. S7 online), the accumulation of most of the DCS isomers was only partially affected (Figs. 3B, 4; Supplemental Fig. S8 online; Supplemental Tables S5, S6 online). This suggests that CV86 might not be the only acyltransferase involved in DCS biosynthesis, and the existence of additional stereospecific acyltransferases is therefore predicted by our data. Perhaps, as already proposed in Bassard et al. (2010), the ability to separately control the accumulation of the different DCS isomers became important if the different isomers acquired specific functions in plant defense and/or development. Functional analysis of CV86 enzyme and/or identification of the additional CV86-like acyltransferases in tobacco will allow this hypothesis to be directly falsified.

Evidence for metabolic exchange in the PA pathway

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Previously, it was shown that silencing MYB8 TF results in the complete shutdown of PA biosynthesis without substantially affecting fluxes into other potentially interconnected metabolic pathways (nicotine, CGA and rutin; Kaur et al. 2010). However, working with the singly-silenced acyltransferase plants allowed us to appreciate the interconnections and substantial plasticity of the PA biosynthetic pathway. For example, silencing of AT1 (and therefore CP biosynthesis) increased the accumulation of DCS in the herbivore-attacked leaves (Fig. 3A, B; Supplemental Table S5 online). In contrast, plants silenced in DH29 expression accumulated significantly more FP (Fig. 4; Supplemental Table S5 online). It shows that in PA biosynthesis, hydroxycinnamoyl-CoA substrates can be diverted into the production of other PAs pending the enzymatic activity of AT1 or DH29 was not sufficient. In addition to limiting acyltransferase activity, similar substrate switch scenarios, although not demonstrated here, can be predicted if one or more of the polyamine substrates become limiting in the biosynthesis.

Interestingly, AT1 gene product was required for CoP and CP biosynthesis but not for FP formation \textit{in vivo} (Fig. 4; Supplemental Table S5 online). Previously, it has been shown that O-methyltransferase enzymes may contribute to the formation of feruloyl-containing PAs in Arabidopsis pollens (Grienenberger et al., 2009), suggesting an alternative pathway, and indirect production of FP from CP by O-methylation in \textit{N. attenuata} plants. In our \textit{in vitro} recombinant assays, we were still able to detect a limited activity of the AT1 enzyme with feruloyl-CoA as a substrate (Table 2), suggesting that production of FP in \textit{N. attenuata} plants may be facilitated by two or more functionally overlapping biosynthetic pathways. Notably, FP production was strongly suppressed in irMYB8 plants, predicting an existence of MYB8-regulated O-methyltransferase(s) genes in \textit{N. attenuata}.

\textbf{Functional diversification of PAs}

As shown above, tobacco plants are able to accumulate many PAs, and this number currently increases with the analytical ability to resolve isomeric and hydrated forms of PAs. Such broad spectrum of PAs may reflect the natural variety of stresses, biotic and abiotic, giving rise to existence of highly diversified chemical defenses in plants. For example, the accumulation of putrescine/spermidine PAs in \textit{N. attenuata} was...
strongly induced by herbivory that is efficiently coordinated by MYB8 TF (Kaur et al., 2010; this report); however we did not find substantial amounts of other polyamine conjugate forms featuring tyramine, agmatine, or serotonin in the *N. attenuata* plants challenged with herbivores and/or under regulation of herbivory-related MYB8 protein (Fig. 1; Table 1). Interestingly, the content of feruloyltymamine, coumaroyltymamine, feruloylagmatine and coumaroylagmatine is known to strongly increase in response to pathogen infections in plants (Stoessl and Unwin, 1970; Fattorusso et al., 1999; Newman et al., 2001; Matsuda et al., 2009; Muroi et al., 2009; Bassard et al., 2010). This observation suggests that the biological activity of PAs may be linked to the polyamine structure present in the particular defense metabolite. To test this hypothesis, the role of MYB8 in defense against pathogens should be examined in order to complement its already demonstrated role in defense against herbivores.

**Future perspectives of MYB8 research**

While we can now fully appreciate the role of MYB8-regulated PAs in defense against herbivores in *N. attenuata*, the role of PAs in defense against pathogens and/or UV-stress requires more attention (Izaguirre et al., 2007, Demkura et al., 2010). The previously found induction of MYB8 by UV irradiation (Pandey and Baldwin, 2008) thus provides another independent chapter in the functional repertoires of MYB8 TF and PAs in plants exposed to multitude of stress conditions in nature. In our future experiments, by studying plants in their natural environment, we hope to uncover the full crosstalk of signaling pathways and understand the complete role of MYB8 and PAs in stress resistance of plants.

**Acknowledgments**

We thank Aura Navarro Quezada for advice and help with the phylogenetic analysis of acyltransferase genes; Klaus Gase and Gustavo Bonaventure for help with the preparation of materials for microarray construction, Wibke Kröber for hybridization of Agilent microarrays; San Gyu Kim for providing plant materials for a time course microarray...
experiment; Antje Wissgott for help with VIGS vector constructs; John D’Auria for donation of cloned 4CL enzyme in an expression vector; Andreas Weber and Andreas Schünzel for growing the plants in the glasshouse; Aleš Svatoš, Nicolas Heinzel and Mathias Schöttner for help with elucidation and consultation of unknown phenolamide structures.

Author Contributions

Nawaporn Onkokesung: performed research (DH29, CV86 isolation and activity, expression analysis, metabolomics analysis); designed the research; wrote the paper. Emmanuel Gaquerel: performed research; contributed new analytic/computational tools (LC-TOF data analysis, PCA analysis); wrote the paper. Hemlata Kotkar: performed research (AT1 isolation and activity). Harleen Kaur: performed research (accumulation of CP, DCS, rutin and CGA in N. attenuata plants). Ian T. Baldwin: designed the research; wrote the paper; head of the department; provided financial support. Ivan Galis: designed the research (supervised N. Onkokesung, H. Kaur and H. Kotkar); performed research (enzyme activity in vitro); wrote the paper.

Material and methods

Plant material and growth conditions

Nicotiana attenuata Torr. Ex S. Watson (31st inbred generation) seeds, originally collected from a native population at the field site located in Utah, USA, were used in all experiments. The transformants used were inverted repeat (ir) MYB8 plants previously described in Kaur et al. (2010) and antisense (as) LOX3 plants as described in Halitschke and Baldwin (2003). Seeds were germinated on Gamborg’s B5 media (Duchefa) as previously described in Krügel et al. (2002). Seedlings were maintained at 26°C /16 h, 155 µmol m⁻² s⁻¹ light; 24°C/8 h dark cycle (Percival, Perry, IA, USA) for 10 d and then transplanted to soil in Teku plastic pots. After 10 d, early rosette plants were transferred to 1 L pots and grown in the glasshouse with day/night cycle of 16 (26-28°C)/8 (22-24°C) h under supplemental light from Master Sun-T PIA Agro 400 or Master Sun-T PIA Plus 600W high-pressure sodium lamps (Philips Sun-T Agro);
Plants for VIGS experiments were grown in the climate chambers with day/night cycle of 16 (20°C)/8 (20°C) h under supplemental light from Master Sun-T PIA Agro 400 or 600.

**Plant elicitations**

*Elicitation by direct feeding*: one freshly hatched *M. sexta* neonate was placed on stem leaves of AT1-, DH29-, CV86-, and EV-VIGS plants and neonates were allowed to feed on the plants for 4 d. After removing the larvae, the remaining leaf tissues were collected for metabolite and transcript analyses. The untreated leaf tissues from a similarly positioned EV leaves were collected at the same time and used as a control. Leaf tissues were frozen in liquid nitrogen and kept at -80°C until analysis.

*Simulated herbivory treatment*: transition rosette leaves (stable lines) or stem leaves (VIGS plants) were wounded with a fabric pattern wheel and immediately treated with 20 µL of 1:5 (v/v) water-diluted oral secretions (OS) from *M. sexta*. Control plants remained untreated. Treated and untreated leaf tissues were collected at designated time points, frozen in liquid nitrogen and kept at -80°C until analysis. *M. sexta* larval OS were collected after larvae were reared on *N. attenuata* WT plants until the 3rd to 5th instar; OS were collected after regurgitation through a Teflon tube connected to vacuum and stored under argon at -20°C until use.

**Virus induced gene silencing (VIGS)**

Vector construction, plant growth and inoculation condition were performed as described in Saedler and Baldwin (2004). Briefly, 200-300 bp fragments of *N. attenuata* AT1, DH29 and CV86 were amplified by PCR using specific primer pairs as listed in Supplemental Table S3 online. Amplified fragments were cloned into pTV00 vector and plasmids were transformed by electroporation into *Agrobacterium tumefaciens* strain GV3101. A pTV00 plasmid without insert (empty vector; EV) was used as a negative control in all experiments. Three leaves of 24-25 d-old *N. attenuata* plants were infiltrated with a 1:1 mixture of *A. tumefaciens* transformed with pBINTRA, and one of the pTVAT1, pTVDH29, pTVCV86 or pTV00 constructs. *Phytoene desaturase* (pTVPDS) causing bleaching of tobacco leaves due to the depletion of carotenoids was
used as a positive control to monitor the progression of VIGS in a separate set of inoculated plants. AT1-, DH29-, CV86- and EV-VIGS silenced plants were used for treatments after PDS-VIGS leaves developed strong bleaching phenotype. Silencing efficiency was verified by RT-qPCR of target gene transcripts after RNA extraction and cDNA synthesis.

**RT-qPCR analysis**

Total RNA was extracted by adding Trizol reagent (Invitrogen; http://www.invitrogen.com) to approximately 150 mg of powdered leaf material ground in liquid nitrogen following manufacturer’s protocol. Crude RNA samples were treated with RQ1 DNase (Promega; http://www.promega.com), followed by phenol/chloroform/isoamylalcohol (25:24:1) extraction and ethanol precipitation. 500 ng of DNA-free RNA samples were reverse-transcribed using oligo (dT) 18 primers and Superscript II enzyme (Invitrogen) following the manufacturer’s recommendations. All RT-qPCR assays were performed with Stratagene MX3005P instrument (Stratagene; http://www.stratagene.com) as recommended by the manufacturer. For normalization of transcript levels, primers specific for the elongation factor-1α gene from tobacco (EF1-α; Acc. D63396) were used. Specific primers in 5’-3’ direction used for SYBR Green-based analyses are listed in the Supplemental Table S3 online. Typically, data from 3-5 biological replicates were used for statistical analysis.

**Identification of novel acyltransferase gene AT1**

The NCBI "Non-human, non-mouse ESTs (est_others)" database limited to "N. tabacum (taxid:4097)" and tblastn program with default settings were used to identify a set of 500 tblastn hits with corresponding e-values < 0.059 and < 0.020 for DH29 and CV86, respectively. All FASTA-formatted EST sequences similar to DH29 and CV86 were uploaded from the database (Supplemental Table S4 online, worksheet A) and included in the assembly of ESTs using publically available CAP3 program with default parameters (http://deepc2.psi.iastate.edu/cgi-bin/cap_pm.pl). Each assembled contig (Supplemental Text S2 online) was blasted using our local blastn engine against the *N. attenuata*-specific microarray 60-mer Agilent oligoprobe database [all 43,533 oligo
sequences can be downloaded from Gene Expression Omnibus (GEO) microarray repository as part of the *N. attenuata* Agilent platform GPL13527]. All contigs that did not provide a significant hit against microarray oligo database were re-blasted against *N. tabacum* ESTs using NCBI *blastn* program, and where possible the 5’ and 3’ ends of the contigs were extended (Supplemental Text S2 online). Extended contigs (e.g. Contig5-1 and Contig5-2 represent the extensions of Contig5) were re-blasted against Agilent 60-mer oligos to obtain a set of 53 contigs that showed positive >84% match to 60-mer Agilent oligo sequence database, representing 32 unique genes from *N. attenuata* (Supplemental Table S4 online, worksheet B).

**Microarray analysis**

*N. attenuata* seedlings were cultivated in 1L pots with sand to facilitate sampling of the roots. Plants were watered by flood irrigation system with 200 g CaNO₃·4H₂O and 200 g Flory B1 fertilizer in 400 L water, and grown in the glasshouse under standard conditions. Three rosette leaves at positions 0, +1 and +2 of early elongated stage plants were elicited with simulated herbivory (W+OS), wounding (W+W) or remained untreated. Local treated leaves, systemic unwounded leaves (two younger leaves above the treated leaves) and roots were collected at 1, 5, 9, 13, 17 and 21 h after treatment, tissues were frozen in liquid nitrogen and kept at -80°C until analysis. Microarrays were performed exactly as described in Gilardoni et al. (in press). Raw and normalized data for each triplicated treatment (totally 134 samples) were deposited in Gene Expression Omnibus (GEO) repository under sample numbers GSM750588 - GSM750721 (Agilent platform identification number was GPL13527). In this report, raw in Agilent “gProcessedSignal” column were normalized by dividing each value by 75 percentile value of each experiment. Expression data for individual genes shown in Supplemental Fig. S5 online were manually extracted from array matrix and plotted in XY-plots using an Excel program from Microsoft Office 2003.

**Expression and purification of recombinant proteins**

Full-length cDNA of *AT1, DH29* and *CV86* without natural stop codons were amplified using specific primers as listed in Supplemental Table S3 online. Sequence
verified PCR products were cloned into pET23a (Novagen; http://www.merck-chemicals.de) as NdeI-XhoI fragment (AT1, DH29) or NdeI-NotI (CV86) to produce histidine tagged fusion proteins (C-terminal 6X His-tag) in the expression vector (pET23a-AT1, pET23a-DH29 and pET23a-CV86). Expression vectors were transformed into BL21 (DE3) pLysS E. coli host cells (Novagen) by standard electroporation. For each protein, expression conditions were first optimized in 10 mL bacterial cultures. For large scale protein extractions, 250 mL cultures were allowed to grow at optimal temperature (28°C) until culture reached 1 OD₆₀₀; isopropyl β-D-1-thiogalactopyranoside (IPTG; Roth; http://www.carlroth.com) was added to a final concentration of 1 mM to induce recombinant gene and cultivation was continued for 24 h. Bacterial cultures were centrifuged at 2,500 g at 4°C for 20 minutes and pellets were frozen in liquid nitrogen after completely removing the supernatants and stored at -80°C. Cell pellets were re-suspended in lysis buffer (50 mM Na₂HPO₄/300 mM NaCl, 1 mg/mL lysozyme, 10 mM imidazole) and incubated on ice for 30 min. All buffers were supplemented with 6 M urea in case of CV86 protein extraction under denaturing conditions. Homogenates were disrupted by 3 min sonication in a sonicator Bandelin UW2070 (Sonoplus, Berlin, Germany; power at 60%, 4X cycle) and centrifuged at 10,000g, 4°C for 30 min. Supernatants were loaded onto 2 mL Hi-Trap charged nickel chloride (Jena Biosciences, Jena, Germany) columns in 5 ml plastic reservoirs. Proteins were eluted from the columns with 50 mM Na₂HPO₄ buffer containing 300 mM NaCl and a gradient of 0 to 0.5 M imidazole. 20 μL of eluted protein fractions were subjected to SDS-PAGE to identify recombinant protein-containing fractions. The positive fractions were pooled and desalted with Zeba Spin Desalting Columns (7K MWCO, 10 mL, Thermo Scientific; http://www.piercenet.com). 100 μL aliquots of purified AT1 and DH29 proteins were snap-frozen in liquid nitrogen and stored at -80°C. CV86 protein extracted under denaturing conditions was renaturated by dialysis of the protein fraction against 50 mM Tris/HCl buffer pH 7.5 containing gradually decreasing concentrations of urea at 4°C in the cold room. SDS-PAGE was performed using 8-10% acrylamide gels with Tris-glycine SDS running buffer. Protein bands were visualized using bio-safe Coomassie blue stain (Bio-Rad).
**In vitro enzyme assays**

Hydroxycinnamoyl-CoAs of *p*-coumaric, caffeic and ferulic acids were synthesized using tobacco recombinant 4CL enzyme as described in Beuerle and Pichersky (2002). However, this enzyme was found completely inactive with cinnamic and sinapic acids under our conditions. All enzymatic assays were performed in total volume of 100 μL containing available acyl donors (caffeoyl-, feruloyl-, and *p*-coumaroyl-CoA) and polyamines (putrescine, spermidine and spermine) as acyl acceptors in 100 mM Tris/HCl-buffer pH 7.5 containing 5 mM EDTA (pH 8). For testing enzyme activity, the reactions were initiated by addition of purified enzymes, incubated at 30°C for 15 minutes and stopped by adding 1 μL 12N HCl and 15 μL acetonitrile. Undiluted samples were injected to HPLC-PDA and analyzed for reaction products based on RT and UV-absorbance spectra. Identity of reaction products was confirmed by UPLC/ESI-TOFMS analysis. The kinetic constants of AT1 and DH29 were determined using 0-750 μM caffeoyl-CoA as acyl donor and 0-2 mM polyamine as acyl acceptor. Reactions were monitored in a spectrophotometer Infinite M200 Tecan (Tecan; [http://www.tecan.com](http://www.tecan.com)) at 1 min intervals by following decrease of specific UV absorbance at 365 nm from caffeoyl-CoA substrate.

**Phylogenetic analysis**

BAHD protein sequences described in Luo et al. (2009) and other several functionally characterized hydroxycinnamoyl-CoA:amine *N*-hydroxycinnamoyl transferases (Bassard et al., 2010) were supplemented with AT1, DH29 and CV86 sequences from *N. attenuata* and used to generate a multiple alignment by Muscle program ([http://www.ebi.ac.uk/Tools/msa/muscle/](http://www.ebi.ac.uk/Tools/msa/muscle/)) using default parameters (Supplemental Dataset 1 online). The protein alignment was trimmed in order to remove highly variable regions of the aligned proteins that contained frequent insertions and deletions.Trimming of sequence alignment shown in Supplemental Dataset 1 online was performed using Gblocks ([http://molevol.cmima.csic.es/castresana/Gblocks.html](http://molevol.cmima.csic.es/castresana/Gblocks.html)) with the maximum number of contiguous nonconserved positions set to 100 and the minimum length of a block set to 2. Gblocks trimming has defined 16 blocks showing sequence conservation which gave 133 amino acid-long trimmed sequences as shown in
Supplemental Dataset 2 online. A tree was built using the Neighbor-Joining clustering method with MEGA 4 program. One thousand iterations were used to calculate the bootstrap values assessing the confidence of each tree clade.

**Secondary metabolite quantification by HPLC-PDA**

PAs in VIGS experiments were quantified by HPLC coupled with photodiode array (PDA) detector. Approximately 100 mg of liquid nitrogen-ground leaf powders were extracted by adding 1 mL of acidified 40% MeOH prepared with 0.5% acetic acid water to each sample in 2 mL microcentrifuge tubes with metal balls. The samples were homogenized in the ball mill (Genogrinder 2000, SPEX CertiPrep, Metuchen, New Jersey, USA) for 45 sec at 1x rate and 250 strokes per min. Homogenized samples were centrifuged at 16,000 g, 4°C for 30 minutes, supernatants were transferred into 1.5 mL microcentrifuge tubes and re-centrifuged as before. 400 µL of supernatants were transferred to 2 mL glass vials before analyzing on Agilent-HPLC 1100 series (http://www.chem.agilent.com): 1 µL of the sample was injected in a Chromolith FastGradient RP 18-e column (50 x 2 mm; monolithic silica with bimodal pore structure, macropores with 1.6 µm diameter, Merck, Darmstadt, Germany) attached to a pre-column (Gemini NX RP18, 2 x 4.6 mm, 3 µm). The mobile phases (0.1% formic acid + 0.1% ammonium water, pH 3.5) as solvent (A) and methanol as solvent (B) were used in a gradient mode with the following conditions: time/concentration (min/%) for B: 0.0/0; 0.5/0; 6.5/80; 9.5/80; and reconditioning for 5 min to 0% B. The flow rate was 0.8 mL/min and column oven temperature was set to 40ºC. DCS, CGA and rutin determination in *N. attenuata* development was performed as described previously in Kaur et al. (2010). Peaks from individual DCS isoforms were pooled and reported as total DCS in Figs. 3, 7 and 8. CP and DCS were quantified based on the calibration curves constructed for CGA and expressed as CGA equivalents.

**UPLC/ESI-TOFMS measurements**

Two microliters of the leaf extracts prepared as above were separated using a Dionex RSLC system (Dionex, Sunnyvale, USA) equipped with Dionex Acclaim 2.2 µm 120A 2.1x150 mm column, applying either a short separation binary gradient (flow rate
300 µl min⁻¹) with the following parameters, 0 to 0.5 min isocratic 80 % A (deionized water, 0.1 % [v/v] acetonitrile [Baker, HPLC grade] and 0.05 % formic acid), 20 % B (acetonitrile, 0.05 % formic acid); 0.5 to 2 min linear gradient to 40 % B; 2 to 6 isocratic 40 % B, 6 to 10 min linear gradient to 80 % B or a long separation gradient optimized for phenolamides (flow rate 300 µl min⁻¹) 0 to 5 min isocratic 95 % A, 5 % B; 5 to 20 min linear gradient to 32 % B; 20 to 22 min linear gradient to 80 % B; isocratic for 6 min. Eluted compounds were detected by a MicroToF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization source in positive ionization mode. Typical instrument settings were as follows: capillary voltage 4500 V, capillary exit 130 V, dry gas temperature 200 °C and dry gas flow of 8 L min⁻¹. Ions were detected from m/z 200 to 1400 at a repetition rate of 1Hz. Mass calibration was performed using sodium formate clusters (10 mM solution of NaOH in 50/50% v/v isopropanol/water containing 0.2 % formic acid). The level of identification of the metabolites reported in this study is denoted in Table 1 using the four levels of the metabolite annotation nomenclature proposed by the Metabolome Standard Initiative (Sumner et al., 2007) employed as described in Matsuda et al. (2010).

**Processing of UPLC/ESI-TOFMS data**

Raw data files were converted to netCDF format using the export function of the Data Analysis version 4.0 software (Bruker Daltonics, Bremen, Germany) and processed using the XCMS package (http://metlin.scripps.edu/download/). Peak detection was performed using the ‘centWave’ method and parameter settings ppm = 20, snthresh = 10, peakwidth= c(5,18). Retention time correction was achieved using the parameter settings minfrac = 1, bw = 10 s, mzwid = 0.1 D, span = 1, missing = extra = 0. After peak grouping and filling in of missing features using the fillPeaks routine of the XCMS package, the obtained data matrix was imported into Microsoft Excel for statistical analysis. Ion traces were deconvoluted and putative in-source pseudo-spectra reconstructed with the R package CAMERA (http://www.bioconductor.org/packages/release/bioc/html/CAMERA.html) with defaults parameters. Isotopic peaks and multi-charged m/z signals detected by CAMERA were excluded to reduce the redundancy within the data matrix. Consistent mass features,
which were at least present -- for a single factorial group -- in four out of the five biological replicates with Rt > 1 min were considered for further analysis. Zero values remaining after applying the ‘filling in’ function in XCMS were replaced by half of the minimum positive value of the row in the original data.

**Statistical analysis**

For principal component analysis (PCA) of UPLC/ESI-TOFMS profiling data, we first filtered the data in order to identify and remove m/z signals that are unlikely to be of use when modeling the data. No phenotype information was used during the filtering process, so the result can be used with any downstream analysis. 40% of the original m/z features were considered as near-constant throughout the experiment conditions based on their coefficient of variation (mean divided by standard deviation) and removed. Normalization was used to make each variable comparable to each other within the same sample. We used the Pareto scaling -- mean-centered and divided by the square root of the standard deviation of each variable -- method for data normalization as recommended in van den Berg et al. (2006). The PCA analysis was performed using the ‘prcomp’ package for R via the MetaboAnalyst interface. The calculation is based on singular value decomposition. The HPLC data and RT-qPCR data were analyzed with PASW statistic 18 (SPSS) software.

**Sequence accession numbers:**

DH29 (fragment: CA591847; full length CDS: JN390824); CV86 (fragment: BU494534; full length CDS: JN390825); AT1 (full length CDS: JN390826); EF1-α (D63396).
### Table 1

Major coumaroyl-, caffeoyl- and feruloyl-containing metabolites measured by UPLC-TOF-MS in methanol-water leaf extracts of *N. attenuata* WT and irMYB8 plants.

<table>
<thead>
<tr>
<th>Label in Fig. 1</th>
<th>F-value, unpaired t-test</th>
<th>Fold-change</th>
<th>Rt (s)</th>
<th>Precursor m/z</th>
<th>Ion type</th>
<th>Elem. Formula</th>
<th>Error (ppm)</th>
<th>Ab.</th>
<th>Annotation</th>
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<td>0.00</td>
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<td>-</td>
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<tr>
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<td>1.71</td>
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<td>646</td>
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Table 2
Summary of determined biochemical properties of the recombinant proteins AT1 and DH29 in vitro.

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<td>DH29$_{spd}$</td>
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<table>
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<th>DH29 CafCoA</th>
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ND: Not determined
References:


Figure and Table legends

Tables in main text:
(2 Tables)

Table 1
Major coumaroyl-, caffeoyl- and feruloyl-containing metabolites measured by UPLC-TOFMS in methanol-water leaf extracts of *N. attenuata* WT and irMYB8 plants. Rosette leaves of WT and irMYB8 plants were wounded (W) with a fabric pattern wheel, immediately treated with *M. sexta* oral secretions (OS) and harvested 24 h later. Elemental formulas (Elem. Formula) and relative mass errors (in ppm) were calculated using Smart Formula from the UPLC-TOFMS operating software (cf. Method section). Candidate formulas were ranked according to both mass deviation and isotope pattern accuracy reflected in the sigma value. MS/MS+ spectra for some of the reported parent ion have been published by our group in Gaquerel et al. (2010) and the strategy used for compound annotation are summarized into Supplemental Text S1. Asterisks indicate significant differences (unpaired *t*-test, performed on log2-transformed data) in the relative intensity of hydroxycinnamic acid-containing metabolites between W+OS elicited WT leaves and untreated WT controls (24 h; WT W+OS vs. WT Control), and between irMYB8 leaves induced by W+OS compared to identically treated WT leaves (24 h; irMYB8 W+OS vs WT W+OS): * *P* < 0.05, ** *P* < 0.001, *** *P* < 0.0001.

Shading of cells indicates either significant increases (dark grey) or decreases (light grey) due to W+OS elicitation or MYB8 silencing. Numbers in the compound name column refer to the different annotation levels using the four levels of the metabolite annotation nomenclature proposed by the Metabolome Standard Initiative. Indexes after nitrogen atoms indicate that structural rearrangements during in-source or CID-MS/MS fragmentation did not allow the unequivocal assignment of the phenylpropanoid residues to either of the N1, N5, or N10 positions of spermidine. Ab.: Abbreviated name. Elem. Formula: Elemental formula. N.V.: not visible in Fig. 1 but presented in Supplemental Fig. S4 online. Rt: Retention time.
Table 2

Summary of determined biochemical properties of the recombinant proteins AT1 and DH29 in vitro. Recombinant HisTag-containing AT1 and DH29 enzymes were examined for substrate specificity based on the observed spectrum of naturally occurring metabolites found in N. attenuata. Km values were determined with caffeoyl-CoA (0-750 μM) as the best substrate and 0-2 mM polyamines putrescine (AT1) and spermidine (DH29).

Figures in main text:

(9 Figures)

Figure 1

MYB8-silencing induces specific alterations in the accumulation of phenylpropanoid-containing metabolites. (A)-(C) show representative ion chromatograms (n = 5) calculated for (A) coumaroyl- (m/z 147.05, peaks Co1-7), (B) caffeoyl- (m/z 163.04, peaks C1-15) and (C) feruloyl- (m/z 177.05, peaks F1-8) ion moieties generated upon in-source fragmentation during the analysis by UPLC-TOFMS in positive mode of methanol-water extracts of the 24 h W+OS-elicited leaves from untransformed WT and MYB8-silenced (irMYB8) plants; raw data were deconvoluted with the R package XCMS prior statistical analysis. Up and down arrows indicate respectively significant (unpaired t-test, $P < 0.05$) increases and decreases in the accumulation of coumaroyl-, caffeoyl- and feruloyl-containing metabolites in irMYB8 compared to WT leaves. C, Co and F numbers refer to the major phenylpropanoid derivatives present in N. attenuata leaves as summarized in Table 1.

Figure 2

AT1, DH29 and CV86 transcript levels are dependent on MYB8 transcriptional activity in local and systemic tissues of W+OS elicited N. attenuata plants. RT-qPCR analysis was performed with cDNA samples prepared from local (wounded) and systemic young (unwounded) leaves before, 1.5, 5 and 24 h after W+OS elicitation. Values are means of three replicate measurements ±SE.
Figure 3

HPLC-PDA determination of differentially accumulated metabolites in herbivory-elicited leaves of AT1-, DH29- and CV86-VIGS plants. Caffeoylputrescine (A), dicaffeoylspermidine (B) and mono-caffeoylspermidine (C) contents expressed as chlorogenic acid (CGA) equivalents were determined in control (un-elicited) and 4 d continuously *M. sexta* neonate-fed leaves (elicited). VIGS plants treated with an empty vector construct (EV) were used as control in all experiments; EV1 and EV2 are showing respective controls in two VIGS experiments conducted with AT1 (EV1) and DH29+CV86 (EV2). Asterisks or different letters indicate significantly down- and up-regulated metabolites in response to silencing of the respective acyltransferase gene activity; AT1-EV1, Student’s *t* test *P < 0.05* (*), **P < 0.05** (**; *n* = 5); DH29-CV86-EV2, one-way ANOVA, *n* = 5. FM, fresh mass.

Figure 4

Representative (*n* = 4) extracted ion chromatograms (EIC) for mono-acylated putrescines and mono- and di-acylated spermidines in the 4 d herbivory-elicited leaves of EV and AT1-, DH29- and CV86-silenced plants. Ion types selected for the calculation of EIC traces correspond to the protonated [M+H]+ *m/z* signals of the different isomers of (1st Row, left to right): N-coumaroylputrescine; N-cafeoylputrescine; N-feruloylputrescine; (2nd Row): N'-coumaroylspermidine; N-caffeoylspermidine; N-feruloylspermidine; (3rd Row): N', N''-coumaroyl, cafeoylputrescine; N', N''-di-caffeoylspermidine; N', N''-di-feruloylspermidine. Indexes after nitrogen atoms indicate that structural rearrangements during in-source or CID-MS/MS fragmentation did not allow the unequivocal assignment of the phenylpropanoid residues to either the N1, N5, or N10 positions of spermidine. Metabolites showing at least 1.5-fold reduced and increased content are highlighted using light grey and dark grey background, respectively (data available in Supplemental Table S5 online). EV1 and EV2 show respective control treatments with an empty vector infiltration in two independent VIGS experiments conducted separately for AT1 (EV1) and DH29+CV86 (EV2).
Figure 5
AT1, DH29 and CV86 contribute to distinct, but inter-connected MYB8-controlled hydroxycinnamoyl-putrescine and -spermidine pools. (A) Principal component analysis (PCA) showing a separation of herbivory-elicited large-scale metabolic profiles in AT1-, DH29- and CV86-VIGS leaves compared to an empty vector (EV)-treated leaf profile. PCA analysis was performed after pre-processing using the R-package XCMS and log2-transformation of the raw UPLC-TOFMS files obtained from the measurement of the leaf tissues harvested 24 h after W+OS treatment. (B) Hierarchical clustering analysis – using Pearson correlation as distance metric – and the heatmap of MYB8-dependent m/z signals from UPLC-TOFMS analysis (log2-scaled intensities shown in Supplemental Table S2 online) that were differentially regulated in AT1-, DH29- and CV86 -VIGS plants (fold change vs. EV < 0.5 or > 2, unpaired t-test on log2-transformed data * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$) compared to EV plants. The hierarchical clustering analysis separated AT1-dependent putrescine-based and DH29 and putatively CV86-dependent spermidine-based PA metabolic clusters.

Figure 6
Biochemical characterization of AT1 and DH29 using recombinant proteins and in vitro enzymatic assays. HPLC chromatograms (UV320 nm) show reaction products without and after addition of AT1 (A) and DH29 (B) recombinant enzymes to reaction mixtures containing caffeoyl-CoA and polyamines putrescine (A) or spermidine (B). Lower chromatograms show formation of CP at retention time 5.1 min (A) and MCS at retention time 4.3 min (B) in samples supplied with recombinant AT1 and DH29 enzymes, respectively. Putrescine and spermidine that do not absorb UV are not visible in the chromatograms. (C) MS spectra of the reactions products of AT1 (CP) and DH29 (MCS) recombinant enzymes determined with high resolution UPLC-TOFMS.

Figure 7
Time- and tissue-scale resolved accumulation of DCS in N. attenuata plants. Wild-type (WT) plants were grown in sand; at each developmental stage, a single fully expanded rosette leaf at (+1) position was OS-elicited, and 3 d later the samples from
representative plant parts were collected and analyzed by HPLC-PDA. The dotted arrow shows position of the locally W+OS-elicited rosette leaf at different stages of development. DH29 transcripts, shown in the gray inset, were analyzed by RT-qPCR from tissues at the elongated stage of development. R, Root; SB, stem basal; SU, stem upper; SeL, senescent leaf; OL, old leaf; LL, locally W+OS-induced rosette leaf; SL, systemic rosette leaf; ISL, first stem leaf; YL, young stem leaf; B, flower buds; EB, elongating flower buds; F, open flowers; GC, green capsules with seeds; RC, ripe capsules with seeds; FM, fresh mass; nd, metabolite not detected in the sample. Only samples with indicated x-label in each graph and developmental stage were used for HPLC-PDA. Distribution and accumulation of two herbivory non-inducible phenylpropanoid metabolites, CGA and rutin, from identical same set of samples are shown for comparison in Supplemental Fig. S11 online.

Figure 8
Silencing of jasmonate biosynthesis in N. attenuata impairs CP and DCS accumulation that is mediated by the MYB8 regulatory network. (A) The transcript abundance of MYB8 and MYB8-dependent genes AT1, DH29 and CV86 was determined by RT-qPCR in the W+OS-elicited leaves from WT and jasmonic acid biosynthesis-deficient (asLOX3) plants; (B) shows silencing of LOX3 gene in asLOX3 plants; (C) strongly reduced content of CP and DCS in the leaves of asLOX3 plants; FM, fresh mass. Values are means of three replicate measurements with SE indicated.

Figure 9
Schematic summary of AT1, DH29 and CV86 action in herbivore attacked N. attenuata plants. Metabolic function of AT1, DH29 and putative function of CV86 is placed in the context of phenylpropanoid metabolism regulated by MYB8 transcriptional activity after herbivore attack. AT1 is directly involved in the biosynthesis of p-coumaroylpurescine (CoP), caffeoylputrescine (CP), while feruloylputrescine (FP) biosynthesis involves another unknown acyltransferase enzyme. In another branch of PA metabolism, DH29 mediates biosynthesis of monoacylated spermidines such as mono-coumaroylspermidine (MCoS), mono-caffeoylspermidine (MCS) and mono-
feruloylspermidine (MFS). CV86 is proposed to encode the second acylation of monoacylated spermidines to di-caffeoylspermidine (DCS) and related metabolites in *N. attenuata*. PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase, C3H, coumarate-3-hydroxylase; COMT, caffeate O-methyltransferase; 4CL, 4-coumarate:CoA ligase; CoCS, *p*-coumaroylcaffeoylspermidine; di-feruloylspermidine (DFS); Putr, putrescine; Sperm, spermidine.
Supplemental material legends on line:
(11 figures, 2 Supplemental texts, 2 supplemental datasets, 6 tables)

Supplemental Figures 1-11

Supplemental Figure S1
Principal component analysis (PCA) separates herbivory-induced large-scale metabolic profiles in irMYB8 leaves from those of WT leaves. The PCA analysis was performed after pre-processing of the raw UPLC-TOFMS files obtained from the measurement of uninduced control (C) and 24 h-elicited (W+OS) leaf tissues using the R package XCMS and Pareto scaling methods. Ellipses delimit the 95% statistical confidence areas for each biological group in the score plot. As revealed by the examination of the loading plots obtained for the 4129 m/z variables, m/z values corresponding to the pseudo-molecular and/or in-source ion fragments from phenolamides -- red color labeled with * -- explained most of the group separation on the projection plot visualized for PC1 and PC2; m/z values corresponding to 25 variables exerting the strongest influence on the projection plot are highlighted on the loading plot. Elemental formulas and annotation of most of the reported phenylpropanoid-containing metabolites are summarized in Table 1 and in Supplemental Table S1 online.

Supplemental Figure S2
PC ranking analysis, W+OS-inducibility and irMYB8-silencing effects on leaf metabolomics profiles. Rankings of loadings exerted on PC1 and PC2 (data in Supplemental Table S1 online) by 4128 m/z signals obtained after pre-processing with R package XCMS were compared with the log2-transformed fold changes calculated for the following comparisons: 24 h elicited WT leaves W+OS compared to WT controls ($, ‘W+OS inducibility in WT’) and irMYB8 leaves induced by W+OS compared to OS-induced WT leaves (#, ‘MYB8-silencing effect’). The regression analysis on the rankings and fold-changes demonstrate that PC1 is strongly influenced by W+OS-inducible and repressible m/z signals while the consequences of MYB8-silencing affect both PCs. This
highlights that although MYB8 controls a significant proportion of W+OS inducible compounds, other signals less affected by the W+OS and likely developmentally controlled are also regulated through MYB8 signaling.

**Supplemental Figure S3**

**MYB8-silencing results in large-scale alterations in *N. attenuata* leaf secondary metabolism.** (A) Volcano plots summarizing the intensity and degree of significance of the changes occurring in molecular fragment relative intensity in irMYB8 compared to WT plants (data available in Supplemental Table S2 online). Un-induced (Control) and induced leaves by W+OS were harvested 24 h after elicitation. UPLC-TOFMS raw data files from the analysis of methanol-water extracts were pre-processed with the XCMS package from R. Up- or down-regulation was assigned to m/z features increasing or decreasing in irMYB8 compared to in WT plants with a fold change (FC) above 2 and *P* value below 0.05 (unpaired *t*-test on log2-transformed data). (B) Venn diagrams showing the number of overlapping and non-overlapping m/z features differentially regulated (up-regulated: up arrow, down-regulated: down arrow) in Control and W+OS-induced irMYB8 leaves compared to WT. (C) Hierarchical clustering analysis (z-score transformed data, Pearson correlation as distance metric) showing that W+OS-induced irMYB8 leaf samples cluster independently compared to their WT counterparts.

**Supplemental Figure S4**

**Representative (*n* = 4) extracted ion chromatograms (EIC) for mono-acylated putrescines and mono- and di-acylated spermidines in the 24 h W+OS-elicited leaves of WT and irMYB8 plants.** The mass accuracy and quality of the isotope pattern fit derived from the elemental formula of these phenylpropanoid polyamine conjugates is summarized in Table 1. Ion types selected for the calculation of EIC traces corresponded to the protonated [M+H]^+ m/z signals of the different isomers of (1): R1= H, N-coumaroylputrescine; R1 = OH, N-caffeoylputrescine; R1 = OCH3, N-feruloylputrescine; (2): R1= H, N- coumaroylspermidine; R1 = OH, N- caffeoylspermidine; R1 = OCH3, N-feruloylspermidine; (3): R1= H and R2 = OH, N’, N”- coumaroyl, caffeoyl-putrescine; R1 and R2 = OH, N’, N”-di-caffeoylspermidine; R1 and R2 = OCH3, N’, N”-di-
feruloylspermidine. Indexes after nitrogen atoms indicate that structural rearrangements during in-source or CID-MS/MS fragmentation did not allow the unequivocal assignment of the phenylpropanoid residues to either the N₁, N₅, or N₁₀ positions of spermidine.

**Supplemental Figure S5**

**Expression profiles of MYB8, AT1, DH29 and CV86 determined by microarray analysis in the Control (untreated), W+water (W) and W+OS elicited tobacco tissues.** Data showing **MYB8, AT1, DH29 and CV86** gene expression were extracted from the microarray dataset using rosette stage WT plants. Control plants remained untreated; “W+W” plants had (+1) rosette leaf elicited with wounding and immediate application of 20 μL water; “W+OS” plants had (+1) rosette leaf elicited with wounding and immediate application of 20 μL oral secretions from *M. sexta* diluted in water (1:5). Graphs show gene expression in the local treated leaf or equivalent control leaf (left column), systemic young unwounded leaves in (0; -1) positions (middle column) and in roots of the same plant (right column). Inducible expression of MYB8, AT1, DH29 and CV86 in local and systemic leaves and low accumulation of respective transcripts in roots should be particularly noted. Microarrays used the Agilent Custom Gene Expression Microarray 4x44K system produced by 60-mer SurePrint technology ([http://www.genomics.agilent.com](http://www.genomics.agilent.com)). Arrays were hybridized with labeled samples using one color labeling system as described in Methods. Values are means of three biological replicate measurements with SE indicated.

**Supplemental Figure S6**

**Phylogenetic tree of the BAHD enzymes from Arabidopsis and other PA biosynthetic enzymes identified from other plant species.** Protein sequences of BAHD genes listed in Luo et al. (2009) and functionally characterized hydroxycinnamoyl-CoA:amine N-hydroxycinnamoyltransferases (Bassard et al., 2010), together with AT1, DH29 and CV86 proteins from *N. attenuata* were aligned with Muscle and the alignment was trimmed with Gblocks to obtain 133 positions in 16 blocks that were used to calculate the phylogenetic tree using MEGA 4 and Neighbor-Joining (NJ) clustering method with 1000 iterations to calculate bootstrap values. The sequences used were
CbBEAT (AAC18062), FvAAT (CAC09062), FxAAT (AAG13130), RhAAT (AAW31948), CmAAT4 (AAW51126), CcAT1 (AAN85435), PsSalAT(AAK73661), CaAT3 (AAV66308), Pun1 (AAV66311), CrDAT (AAC99311), CrMaT (AAO13736), RsVS (CAD89104), Ss5MaT2 (AAR26385), AsHHT1 (BAC78633), AsHHT2 (BAC78634), AtHCT (NP_199704), NtHCT (CAD47830), LeHQT (CAE46933), NtHQT (CAE46932), DcHCBT (CAB06430), HvACT (AAO73071), BanAAT (CAC09063), TcTAT (AAF34254), TcDBAT (AAF27621), TcTBT (AAG38049), TcBAPT (AAL92459), TcDBNTBT (AAM75818), AtCHAT (AAN09797), NtBEBT (AAN09798), PhBPBT (AAU06226), CmAAT3 (AAW51125), CbBEBT (AAN09796), LaHMT (BAD89275), VIAMAT (AAW22989), MpAAT1 (AAU14879), CmAAT1 (CAA94432), CmAAT2 (AAL77060), CcAT2 (AAN85436), Cm3MaT1 (AAQ63615), Cm3MaT2 (AAQ63616), Sc3MaT (AAO38058), Dv3MaT (AAO12206), Lp3MaT1 (AAS77404), Vh3MaT1 (AAS77402), NtMaT1 (BAD93691), Pf3AT (BAA93475), Ss3AT (AAR28757), Pf5MaT (AAL50565), SsAT201 (AAR26386), Ss5MaT1 (AAL50566), Gt5AT (BAA74428), AtCER2 (AAM64817), At4g29250 (CAB79683), At5g02890 (AAQ56813), At2g39980 (AAB95283), At5g01210 (BAE99201), At5g07860 (AAN15449), At5g07870 (BAF01091), At5g07850 (NP_196402), At5g42830 (NP_199097), At3g50280 (NP_190597), At3g50300 (AEE78650), At5g38130 (AAU95437), At3g50270 (AEE78647), At5g67150 (AAM51419), At5g67160 (NP_201517), At5g23940 (AAN31909), At1g32910 (NP_174567), At1g78990 (NP_178020), At5g16410 (AAU95445), At1g31490 (NP_174434), At3g26040 (NP_189323), At1g24430 (NP_173852), At3g30280 (NP_189647), At4g15390 (AAM20603), At5g47950 (AAO63428), At1g24420 (AEE30528), At4g15400 (AAY56447), At5g47980 (AAO64765), At5g23970 (ABH04621), At2g19070; AtSHT (AAO22784), At5g57840 (NP_200592), At1g65450 (AEE34376), At1g27620 (AAT71925), At2g40230 (NP_181552), At1g03990 (ABF59176), At3g48720 (AAQ62868), At5g63560 (AAN13119), At5g41040 (AAK59460), At2g23510; AtSCT (AAC23766), At2g25150; AtSDT (AAP81804), At2g47170 (NP_190301), At5g07080 (AAL07032), At1g28680 (BAE98427), At3g62160 (AAW30017), At5g17540 (NP_197256), At4g31910 (CAB79909), At3AT2 (NP_171849), At3AT1 (NP_171890), At5MaT (NP_189600), At3g29635 (NP_189605),
At3g29670 (AAP04017), At3g29680 (NP_189610), At5g39050 (NP_568561), At5g39080 (AAP49522), At5g39090 (AAP49516), At5g61160 (NP_200924), SITHT7-8 (AAL99191), SITHT7-1 (AAL99190), SITHT1-3 (AAL99189), NtTHT (P80969), CaCASHT (AAK15312), StTHT (BAC23029), CaTHT (AAX15704), NaAT1 (JN390826), NaDH29 (JN390824), NaCV86 (JN390825).

Supplemental Figure S7

Silencing efficiency of AT1, DH29 and CV86 genes in the VIGS plants. Transcript abundances were determined in VIGS plants using gene specific primers and RT-qPCR after cDNA synthesis. Normalized gene expression was determined in untreated and 24 h W+OS-leaves. MYB8 expression was examined to show the normal presence of the transcripts encoding upstream transcription factors while the expression of enzyme is specifically down-regulated by VIGS. Asterisks or different letters indicate significantly down- and up-regulated transcript levels in response to silencing of the respective acyltransferase gene; AT1-EV, Student’s t-test *P < 0.05 (*; n = 3); DH29-CV86-EV2, one-way ANOVA, n = 3.

Supplemental Figure S8

Representative (n = 3) extracted ion chromatograms (EIC) for mono-acylated putrescines and mono- and di-acylated spermidines in the 24h W+OS-elicited leaves of EV and AT1-, DH29- and CV86-silenced plants. Ion types selected for the calculation of EIC traces corresponded to the protonated [M+H]+ m/z signals of the different isomers of (1st Row, left to right): N-coumaroylputrescine; N-cafeoylputrescine; N-feruloylputrescine; (2nd Row): N-coumaroylspermidine; N-cafeoylspermidine; N-feruloylspermidine; (3rd Row): N’, N’’-coumaroyl, cafeoylputrescine; N’, N’’-di-cafeoylspermidine; N’, N’’-di-feruloylspermidine. Indexes after nitrogen atoms indicate that structural rearrangements during in-source or CID-MS/MS fragmentation did not allow the unequivocal assignment of the phenylpropanoid residues to either the N1, N5, or N10 positions of spermidine. Metabolites showing reduced content are highlighted with light grey background (data available in Supplemental Table S6 online). EV1 and EV2
are respective control treatments with empty vector infiltration in two independent VIGS experiments conducted with AT1 (EV1), and DH29+CV86 (EV2).

**Supplemental Figure S9**

**Characterization of AT1 and DH29 recombinant enzymes in vitro.** (A) 10 μL of purified fractions containing recombinant proteins AT1 (50.8 kDa), 4CL (60.6 kDa), CV86 (51.3 kDa) and DH29 (52 kDa) were separated on 10% SDS-PAGE gels together with known amounts of bovine serum albumin (BSA, 66.5 kDa) protein. P, pooled fraction of AT1; 1, 500 mM imidazole fraction; 2, 250 mM imidazole fraction. Red arrows indicate positions of purified proteins with expected size. CV86 protein could not be purified. PL= PageRuler TM Plus Prestained Protein Ladder (Fermentas). (B) Determination of kinetic properties of purified AT1 and DH29 enzymes using fixed concentration of polyamine (1 mM) and variable amounts of caffeoyl-CoA as substrates. (C) Determination of kinetic properties of purified AT1 and DH29 enzymes using fixed concentration of caf-CoA (75 μM) and variable amounts of polyamines (AT1, putrescine; DH29, spermidine). In (B) and (C), one of 3-5 times replicated experiments are shown.

**Supplemental Figure S10**

**Determination of pH optimum of AT1 and DH29 recombinant enzymes in vitro.** The enzymes were subjected to reactions in 100 mM Tris/HCl buffers ranging from pH 6.5 to 9.0, using caffeoyl-CoA/putrescine (AT1) and caffeoyl-CoA/spermidine (DH29) as reaction substrates. AT1 showed pH optimum at pH 8, while DH29 was the most active at pH 7.5.

**Supplemental Figure S11**

**Time and tissue resolved accumulation of chlorogenic acid (CGA) and flavonoid rutin in N. attenuata plants.** Wild-type (WT) plants were germinated in sand and supplemented with nutrients dissolved in water to the roots. At each developmental stage, a single fully expanded rosette leaf at (+1) position was OS elicited, and 3 d later the samples from representative plant parts were collected and analyzed by HPLC-PDA. The dotted arrow shows position of the locally W+OS-elicited rosette leaf at different stages.
of development. R, Root; SB, stem basal; SU, stem upper; SeL, senescent leaf; OL, old leaf; LL, locally W+OS-induced rosette leaf; SL, systemic rosette leaf; ISL, first stem leaf; YL, young stem leaf; B, flower buds; EB, elongating flower buds; F, open flowers; GC, green capsules with seeds; RC, ripe capsules with seeds; FM, fresh mass; nd, metabolite not detected in the sample. Only samples with indicated x-values in each graph and stage were subjected to HPLC-PDA.

**Supplemental Text 1-2 (in attached file)**

**Supplemental Text S1**
Description of method and tandem MS measurements for the precursor ions of selected phenolamides.

**Supplemental Text S2**
CAP3-assembled contigs with significant similarity to DH29 and CV86 proteins represent a pool of novel acyltransferase enzymes in tobacco.

**Supplemental Datasets 1-2 (in attached file)**

**Supplemental Dataset S1**
BAHD protein alignment used for trimming by Gblocks program before generating phylogenetic tree by MEGA 4 displayed in Supplemental Fig. S6 online.

**Supplemental Dataset S2**
Gblocks-trimmed BAHD protein alignment used for generating phylogenetic tree by MEGA 4 displayed in Supplemental Fig. S6 online.
Supplemental Tables S1-S6 (in attached separate Excel files)

Supplemental Table S1
Summary of loadings exerted on PC1 and PC2 by 4128 m/z signals obtained after pre-processing with R package XCMS, and log2-transformed fold changes calculated for the following comparisons: 24 h elicited WT leaves after wounding with a fabric pattern wheel and treatment with *M. sexta* oral secretions (W+OS) compared to WT controls (‘W+OS inducibility in WT’) and irMYB8 leaves induced by W+OS compared to induced WT leaves (‘MYB8-silencing effect’). The regression analysis on the rankings and fold-changes are shown in Supplemental Figure S2 online.

Supplemental Table S2
Summary of molecular fragment relative intensities and fold changes in irMYB8 compared to WT leaves. Uninduced (Ctrl) and induced leaves by wounding with a fabric pattern wheel and treatment with *M. sexta* oral secretions (W+OS) were harvested 24 h after elicitation. UPLC-TOFMS raw data files from the analysis of methanol-water extracts were pre-processed with the XCMS package running under R software.

Supplemental Table S3
Primer sequences used for qRT-PCR and cloning of VIGS vectors into pTV00 or full length cDNA sequences into pET23a for protein expression analysis.

Supplemental Table S4
Identification of the *AT1* gene as novel hervivory-regulated acyltransferase in *N. attenuata*. DH29 and CV86 protein sequences were used to search for additional MYB8-regulated acyltransferases among *N. tabacum* ESTs available in public NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using strategy described in Worksheets A-D. The expression of identified herbivory-regulated acyltransferase-like genes was examined in *MYB8* gene-silenced plants (irMYB8) using semi-quantitative RT-PCR and the expression of positive clones (down-regulated in the absence of MYB8 TF) was further
confirmed by RT-qPCR; *AT1* gene was selected as main candidate for CP biosynthetic enzyme.

**Supplemental Table S5**
Fold changes and related statistical analysis (*n*=4) of metabolites depicted in Fig. 4. Tissues were harvested from *M. sexta* 4 d-fed leaves transiently silenced by VIGS for *CV86, DH29* and *AT1* expression, and up- and down-regulated m/z features and predicted metabolites in the leaves were compared to a respective set of empty-vector (EV)-infiltrated plants. UPLC-TOFMS raw data files from the analysis of methanol-water extracts were pre-processed with the XCMS package and deconvoluted by the CAMERA from R software package.

**Supplemental Table S6**
Summary of up- and down-regulated m/z features and predicted metabolites in the leaves of plants transiently silenced by VIGS for *CV86, DH29* and *AT1* expression compared to an empty-vector (EV) infiltrated plants. Leaves were harvested 24 h after wounding with a fabric pattern wheel and treatment with *M. sexta* oral secretions (W+OS). UPLC-TOFMS raw data files from the analysis of methanol-water extracts were pre-processed with the XCMS package and deconvoluted by the CAMERA from R software package.
Figure 1
Figure 3
Figure 4
Figure 5
Figure 8