Running title: *Phenylpropanoid-polyamine conjugates in plant defense*

Corresponding author:
Ivan Gális
Department of Molecular Ecology
Max Planck Institute for Chemical Ecology
Hans Knöll Str. 8
D-07745 Jena
Germany
Phone: 49-(0)3641-571129
Fax: 49-(0)3641-571102
Email: igalis@ice.mpg.de

Category: Plants Interacting with Other Organisms
R2R3-NaMYB8 regulates the accumulation of phenylpropanoid-polyamine conjugates which are essential for local and systemic defense against insect herbivores in *Nicotiana attenuata*

Harleen Kaur¹, Nicolas Heinzel¹,², Mathias Schöttner¹, Ian T. Baldwin¹ and Ivan Gális¹*

¹Department of Molecular Ecology, Max-Planck-Institute for Chemical Ecology, Beutenberg Campus, Hans-Knöll Strasse 8, D-07745 Jena - Germany
²Present address: Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Correns Strasse 3, D-06466 Gatersleben - Germany

*Corresponding author email:
igalis@ice.mpg.de; fax +49-3641-571102

The author responsible for distribution of materials integral to the findings presented in this article is: Ian T. Baldwin (baldwin@ice.mpg.de)
ABSTRACT

Although phenylpropanoid-polyamine conjugates (PPCs) occur ubiquitously in plants, their biological roles remain largely unexplored. The two major PPCs of *Nicotiana attenuata* plants, caffeoylputrescine (CP) and dicaffeoylspermidine (DCS), increase dramatically in local and systemic tissues after herbivore attack and simulations thereof. We identified *NaMYB8*, a homolog of *NtMYBJS1*, which in BY2 cells regulates PPCs biosynthesis, and silenced its expression by RNAi in *N. attenuata* (ir-MYB8), to understand the ecological role(s) of PPCs. The regulatory role of *NaMYB8* in PPCs biosynthesis was validated by a microarray analysis, which revealed that transcripts of several key biosynthetic genes in shikimate and polyamine metabolism accumulated in a *NaMYB8*-dependent manner. Wild-type (WT) *N. attenuata* plants typically contain high levels of PPCs in their reproductive tissues; however, *NaMYB8*-silenced plants that completely lacked CP and DCS showed no changes in reproductive parameters of the plants. In contrast a defensive role for PPCs was clear; both specialist (*Manduca sexta*) and generalist (*Spodoptera littoralis*) caterpillars feeding on systemically pre-induced young stem leaves performed significantly better on ir-MYB8 plants lacking PPCs compared to WT plants expressing high levels of PPCs. Moreover, the growth of *M. sexta* caterpillars was significantly reduced when neonates were fed ir-MYB8 leaves sprayed with synthetic CP, corroborating the role of PPCs as direct plant defense. The spatial-temporal accumulation and function of PPCs in *N. attenuata* are consistent with the predictions of the Optimal Defense Theory: plants preferentially protect their most fitness enhancing and vulnerable parts, young tissues and reproductive organs, to maximize their fitness.

(247 words/ 250 word limit)

Keywords: caffeoylputrescine, dicaffeoylspermidine, MYB transcription factor, herbivores, *Nicotiana attenuata*, phenylpropanoid-polyamine conjugates
INTRODUCTION

In nature, plants are frequently exposed to abiotic and biotic stress factors, including drought, extreme temperatures, high winds, UV-exposure, pathogens and herbivores. These selection pressures enabled plants to refine their constitutive and inducible defenses (Purrington, 2000; Zangerl, 2003; Howe and Jander, 2008; Walling, 2009). A shift from constitutive to inducible defense strategies can be considered a potential cost-saving mechanism whereby plants timely tune the production and accumulation of defenses with the need for the defenses, and thereby forgo the production and opportunity costs they incur when they are not needed, for example ultraviolet (UV) light induces secondary metabolite-flavonoid accumulation to intensify their UV-protective screen (Li et al., 1993; Zhao et al., 2007; Jenkins, 2009; Wang et al., 2009).

Once recognized via specific receptors, stress factors activate phytohormone signaling networks that trigger downstream defense responses in plants. Jasmonic acid (JA) is known to mediate wound and herbivore stress signals in plants that activate local and systemic defenses, and lead to the accumulation of antifeedants and/or ovipositioning deterrents against herbivores. These toxins largely impair insect growth and reduce their survivorship rates, helping plants to diminish further damage (Steppuhn and Baldwin, 2007; Chen, 2008). In a similar manner, salicylic acid (SA) coordinates the elicitation of defenses against invading pathogens, resulting in the accumulation of phytoalexins that limit the spread of pathogens in the plant tissues, but an increasing amount of evidence points to antagonism between SA and JA signaling (Loake and Grant, 2007; Diezel et al., 2009). Therefore, defense-related hormones in plants are engaged in a complex cross-talk that still needs to be fully examined (Koornneef and Pieterse, 2008).

After herbivore attack, the wounds in plants often come in direct contact with herbivores’ oral secretions (OS), a potential carrier of herbivore-specific elicitors, which can be recognized by plant cells. Upon perception of these elicitors -- e.g. fatty acid-amino acid conjugates (FACs), inceptins or caeliferins -- plants trigger herbivore-specific defense responses, which involve large-scale transcriptional, translational and post-translational changes in plants at both local and systemic levels (Halitschke et al., 2001; Gatehouse, 2002; Howe and Jander, 2008). In N. attenuata plants, FACs are known to rapidly activate mitogen-activated protein kinase (MAPK) cascade, followed by JA accumulation and conjugation of JA to isoleucine (Ile), yielding the active signal molecule, JA-Ile (Wu et al., 2007; Meldau et al., 2009).
Ile is required for the interaction of JAZ (jasmonate-ZIM domain) repressor proteins with SCF<sup>coi1</sup> (Skp1-Cullin-F-box) E3-ubiquitin ligase complex that results in degradation of JAZ repressors and activation of MYC2 transcription factor (Santner and Estelle, 2007; Chico et al., 2008; Eckardt, 2008; Kazan and Manners, 2008; Browse, 2009; Chini et al., 2009a; Chini et al., 2009b; Chung et al., 2009; Fonseca et al., 2009; Santner and Estelle, 2009; Yan et al., 2009). MYC2 and a putative suite of MYC2-controlled secondary transcription factors like ANAC019 and ANAC055 in <i>Arabidopsis thaliana</i> (Bu et al., 2008) then activate transcription of genes encoding defense-related enzymes responsible for biosynthesis of defense-related metabolites in plants (Kazan and Manners, 2008; Chini et al., 2009a). Notably, with exception of two already mentioned NAC proteins, the identity of other putative MYC2-regulated secondary transcription factors involved in JA signaling cascade is unknown (Bu et al., 2008; Chini et al., 2009a).

Secondary metabolites constitute an important part of both inducible and constitutive plant defenses that target insects, plant pathogens and other competitors, including plants (Sudha and Ravishankar, 2002; Zhao et al., 2005). One of the widespread secondary metabolic pathways in plants activated by stress is the biosynthesis of phenylpropanoid-polyamine conjugates (PPCs), which has been frequently found to be positively correlated with increased plant resistance to pathogens, viruses and fungi (review by Edreva et al., 2007); however, the exact role and mode of action of these metabolites in plants remain unclear (Edreva et al., 2007; Cowley and Walters, 2005). In addition, the enzymes responsible for conjugation of acetyl CoA-activated phenylpropanoid moieties to polyamines have only started to be identified (Grienenberger et al., 2009; Luo et al., 2009).

<i>NtMYBJS1</i> gene, previously reported from tobacco, is a JA-dependent R2R3-MYB transcription factor that regulates the accumulation of PPCs in BY-2 tobacco cell cultures (Gális et al., 2006); however, the physiological and ecological functions of this gene in intact plants are not known. We therefore cloned a functional homolog of NtMYBJS1 from an established ecological model <i>N. attenuata</i> (designated NaMYB8 in this study) and explored the potential role of this transcription factor in plant defense. The transcripts of NaMYB8 accumulated rapidly and transiently upon mechanical wounding of local <i>N. attenuata</i> leaves; however, a simultaneous application of <i>Manduca</i> sexta’s OS to the wounds amplified the wound-induced increase in NaMYB8 transcripts at 1-2 days after OS-elicitation, suggesting that
NaMYB8 could play an important role in plant-herbivore interactions, possibly by regulating PPC levels in response to herbivory.

Following the initial characterization of NaMYB8 expression and the accumulation of CP in *N. attenuata*, we used NaMYB8 transcription factor as a genetic tool to examine the ecological relevance of OS-induced PPCs accumulation, and significance of PPCs presence in *N. attenuata*. The plants specifically silenced in expression of NaMYB8 gene were generated through RNAi technology. As expected, the inverted repeat NaMYB8-silenced (referred to as ir-MYB8) plants were unable to accumulate two major PPCs (CP and DCS) in their tissues. A microarray study conducted with ir-MYB8 and WT plants revealed that NaMYB8 protein is required for transcriptional activation of genes involved in phenylpropanoid and polyamine biosynthesis, in addition to several other genes with unknown functions. As both specialist (*M. sexta*) and generalist (*Spodoptera littoralis*) caterpillars performed better on ir-MYB8 plants compared to the WT plants, we propose that PPCs and their regulation by NaMYB8 are vital part of direct defense mechanisms used by plants against attacking herbivores.

RESULTS

*Isolation and expression of NtMYBJS1 homolog in N. attenuata*

The transcripts of NtMYBJS1 gene accumulated 3h after treatment of tobacco BY-2 cell cultures with methyl jasmonate (MJ; Gális et al., 2006). We therefore used a 1h W+OS-elicited cDNA pool -- a time point known to be associated with accumulation of many JA-responsive genes -- to clone a homolog of NtMYBJS1 from *N. attenuata*. The cloned sequence, which was designated NaMYB8 in this study (Supplemental Fig. S1A; GenBank Acc. GU451752), occurs as a single copy gene in *N. attenuata*’s genome (Supplemental Fig. S2C).

We then examined transcriptional response of NaMYB8 gene to wounding and herbivore cues: a fully-expanded rosette leaf was wounded with a pattern wheel and either water (W+W) -- mimicking mechanical wounding -- or *M. sexta*’s OS (W+OS) -- representing simulated herbivory -- were applied to the wounds. Biotic stresses are known to trigger transcriptional responses that typically follow one of the expression profiles: (i) rapid and transient up-regulation of transcripts encoding primary regulators, and (ii) steadily increasing but long-lasting transcriptional responses of genes encoding defensive metabolites [e.g. trypsin proteinase inhibitors (TPIs)] or enzymes involved in their biosynthesis (Zhao et al., 2005). Interestingly, NaMYB8
gene showed a mixed pattern of its transcript regulation in the local leaves, including rapid transient increase of transcripts 45 min after W+W- and W+OS-elicitations (Fig. 1A, inset), followed by stably elevated levels of NaMYB8 transcripts in the W+OS-elicited leaves for additional 1-2 days (Fig. 1A). Because NaMYB8 transcripts responded differentially to the presence of M. sexta’s OS, we assumed that it could play an important role in the inducible plant defenses employed by plants against attacking herbivores. The NaMYB8 transcript levels in unwounded control leaves did not change significantly over 2 days (0-50h; Fig. 1A), excluding the possibility that NaMYB8 transcript accumulation is controlled by circadian rhythm.

**Transient and stable silencing of NaMYB8 expression in planta**

In order to confirm the role of NaMYB8 transcription factor in plant-insect interactions, we used post-transcriptional gene silencing approaches to generate transiently silenced NaMYB8-VIGS [virus-induced gene silencing (VIGS); (Saedler and Baldwin, 2004)] as well as stably transformed ir-MYB8 plants. In the VIGS experiment, Agrobacterium cultures carrying vectors pTV-MYB8 (containing a fragment of NaMYB8 sequence) and pTV-00 (empty vector control; EV) were used to inoculate WT plants, generating NaMYB8-VIGS and EV-VIGS plants, respectively. The endogenous levels of NaMYB8 transcripts in NaMYB8-VIGS plants were reduced by ~70% relative to EV-VIGS plants (Supplemental Fig. S3A); however, no visible changes in plant morphology or growth were observed in these plants compared to the EV-VIGS plants (data not shown). This largely ruled out the possibility that NaMYB8 is involved in vegetative or reproductive development of N. attenuata, allowing preparation of the stably-silenced transgenic plants.

Stably silenced ir-MYB lines were produced by A. tumefaciens-mediated transformation method (Krügel et al., 2002), using pSOL8 binary vector containing ir fragment of NaMYB8 sequence (Supplemental Fig. S2A). After screening for single T-DNA insertion lines (Supplemental Fig. S2B), all further experiments were performed with two independently transformed homozygous diploid lines, 810 and 818 (referred to as ir-MYB8-818 and ir-MYB8-810). The lines were analyzed for their endogenous NaMYB8 transcript levels before and 1 h after W+OS-elicitation, showing significantly reduced levels of NaMYB8 transcripts (ir-MYB8-818: ~90 % and ir-MYB8-810: ~96 % silenced; ANOVA, Tukey post-hoc test; F2,6 = 128.512; P_{ir-MYB8-818} < 0.001; P_{ir-MYB8-810} < 0.001 ) relative to similarly elicited WT plants (Fig. 1B).
In order to determine the position of NaMYB8 upstream or downstream of hormonal signals in plant defense against herbivores, we quantified JA, JA-Ile accumulation and ethylene emission levels in ir-MYB8 and WT plants, before elicitation and 60, 90 and 180 min after W+W- and W+OS-elicitations. No significant differences in maximal JA (Supplemental Fig. S4A; 60 min, ANOVA, Tukey post-hoc test; \( F_{2, 12} = 2.547; P_{\text{ir-MYB8-818}} = 0.124; P_{\text{ir-MYB8-810}} = 0.904 \)) and JA-Ile accumulation (Supplemental Fig. S4B; 60 min, ANOVA, Tukey post-hoc test; \( F_{2, 12} = 1.148; P_{\text{ir-MYB8-818}} = 0.487; P_{\text{ir-MYB8-810}} = 0.65 \)) or ethylene emission (Supplemental Fig. S4C) were observed between the ir-MYB8 and WT plants, which was consistent with the previous phytohormone analyses conducted with NaMYB8-VIGS and EV-VIGS plants (Supplemental Fig. S4D-F). We therefore concluded that NaMYB8 should function downstream of JA and ethylene signals in *N. attenuata*, most probably below or in parallel with the putative transcriptional activator MYC2.

**NaMYB8 regulates the accumulation of PPCs in *N. attenuata* leaves**

To obtain a comprehensive information about the changes in herbivory-elicited secondary metabolite profiles in ir-MYB8 plants, we examined the content of secondary metabolites in (+1) rosette leaves of transgenic and WT plants that were fed by *M. sexta* caterpillars for 4 days. A simple comparison of the chromatograms, obtained from acidic methanolic plant extracts separated by HPLC, showed clear differences between ir-MYB8 and WT plants (Fig. 2A). In particular, CP (Fig. 2B) and DCS (Fig. 2C) could not be detected in ir-MYB8-818 and -810 leaves on which caterpillars fed, confirming previously observed trends observed in NaMYB8-VIGS leaves (Supplemental Fig. S3B, C). Moreover, CP and DCS were not detected in unelicited leaves of ir-MYB8 plants, suggesting that both constitutive and inducible levels of CP and DCS in *N. attenuata* are dependent on activity of the NaMYB8 transcription factor (Fig. 2B, C). However, an expected over-accumulation of parental compounds - putrescine, spermidine and caffeic acid (Fig. 7) due to NaMYB8 silencing had no impact on accumulation of several compounds derived from interconnected metabolic pathways (Fig. 7), including nicotine (Fig. 2F, control, ANOVA, Tukey post-hoc test; \( F_{2, 12} = 1.488; P_{\text{ir-MYB8-818}} = 0.939; P_{\text{ir-MYB8-810}} = 0.424 \); herbivory, ANOVA, Tukey post-hoc test; \( F_{2, 12} = 2.019; P_{\text{ir-MYB8-818}} = 0.987; P_{\text{ir-MYB8-810}} = 0.804 \)) and rutin (Fig. 2E; control, ANOVA, Tukey post-hoc test; \( F_{2, 12} = 3.746; P_{\text{ir-MYB8-818}} = 0.071; P_{\text{ir-MYB8-810}} = 0.101 \); herbivory, ANOVA, Tukey post-hoc test; \( F_{2, 12} = 2.989; P_{\text{ir-MYB8-818}} = 0.077; P_{\text{ir-MYB8-810}} = 0.326 \). In contrast, a significant
reduction in chlorogenic acid levels (CGA; Fig. 2D) was found in both ir-MYB8 lines (control, ANOVA, Tukey post-hoc test; $F_{2, 12} = 22.1; P_{ir-MYB8-818} < 0.001; P_{ir-MYB8-810} < 0.001$; herbivory, ANOVA, Tukey post-hoc test; $F_{2, 12} = 23.792; P_{ir-MYB8-818} < 0.001; P_{ir-MYB8-810} < 0.001$). Finally, no changes in the accumulation of diterpene glycosides (DTGs) -- unrelated but very abundant defensive metabolites (Jassbi et al., 2008) -- were observed in ir-MYB8 plants compared to the WT plants (Fig. 2G, control, ANOVA, Tukey post-hoc test; $F_{2, 12} = 0.654; P_{ir-MYB8-818} = 0.569; P_{ir-MYB8-810} = 0.994$; herbivory, ANOVA, Tukey post-hoc test; $F_{2, 12} = 2.411; P_{ir-MYB8-818} = 0.130; P_{ir-MYB8-810} = 0.864$). In summary, silencing NaMYB8 abolished the accumulation of CP and DCS, reduced CGA levels and showed no statistically significant effects on other examined metabolites, namely nicotine, rutin and DTGs in stably silenced ir-MYB8 lines.

**Transcriptional targets of NaMYB8**

In order to correlate the observed metabolic changes in NaMYB8-silenced lines with the NaMYB8 transcript accumulation in *N. attenuata*, we used a custom oligonucleotide microarray (Biochip ver. 4), spotted specifically with herbivory-activated genes from *N. attenuata* and other related species (1421 gene probes; Meldau et al., 2009). Referring to the maximal accumulation of NaMYB8 transcripts at 45 min after W+OS treatment (Fig. 1A), we used leaf samples collected at 45 and 90 min after treatment with W+OS from each ir-MYB8-818 and WT plants for the microarray analysis. Three pairs of labeled cDNA probes (WT-Cy5 / ir-MYB8-Cy3) were hybridized with the microarray chip to obtain differential expression data for each time point (45 min; 90 min).

Statistical analysis with three biological replications of the experiment, revealed transcript abundances of 8 and 44 genes to be significantly down-regulated in ir-MYB8 leaves after W+OS-elicitation at 45 and 90 min, respectively (2-fold threshold; $P$-value $\leq 0.05$; Supplemental Table S1). These genes predominantly included sequences from phenylpropanoid metabolism, such as phenylalanine ammonia lyase (PAL) and 4-coumaroyl-CoA ligase (4CL), and genes encoding enzymes involved in the synthesis of polyamines (Supplemental Table S1); we assume that these genes represent direct targets of NaMYB8 transcriptional activity in *N. attenuata*. The transcripts of the key biosynthetic gene involved in putrescine biosynthesis, ornithine decarboxylase (ODC), were also less abundant in ir-MYB8 leaves, but only at 1.8-fold-change level, and therefore below our arbitrarily-selected...
threshold (2-fold). In contrast, NaMYB8 silencing significantly influenced the accumulation of spermidine synthase transcripts (~3.9-fold reduction) which are required for DCS biosynthesis from spermidine.

Only few genes were actually up-regulated in NaMYB8-silenced plants compared to the WT plants (Supplemental Table S1), demonstrating that NaMYB8 generally functions as positive transcriptional regulator in *N. attenuata*.

**Young systemic leaves accumulate high levels of CP after simulated herbivory**

Resource allocation and spatial-temporal accumulation of defense-related metabolites varies within plant tissues and often reflects the degree and frequency of stresses that plants have to face during their development (Boege and Marquis, 2005; Boege et al., 2007). We therefore analyzed NaMYB8-dependent CP accumulation in plant ontogeny, namely at rosette, early elongated, elongated, flowering and mature stages of the development, using 3 day W+OS-elicited (stressed) and unelicited (control) WT plants. Even though CP accumulation followed a complex developmental pattern (Fig. 3), several general trends could be recognized. For example, the high constitutive levels of CP in the vegetative tissues at rosette and early elongated stages clearly shifted towards reproductive tissues after flowering and capsule development. In the mature plants, almost no CP could be detected in the leaves. Interestingly, while CP levels always increased in the local leaves following W+OS elicitation, CP accumulated even more in the systemically induced young stem leaves of these plants, even at flowering stage when it could be barely detected in the vegetative plant parts (Fig. 3).

We examined the transcript profile of *NaMYB8* in WT plants at an elongated stage of development to address whether the systemic accumulation of CP could be due to metabolite mobilization to the systemic leaves or the transmission of systemic signal from the locally W+OS-induced leaves to systemic ones, which would require NaMYB8 to mediate the up-regulation of downstream biosynthetic genes. The accumulation of CP coarsely correlated with the *NaMYB8* transcript abundances in both control and OS-elicited leaves (Fig. 3, inset), suggesting that CP accumulation in the distal leaves is most likely subject to systemic activation of *NaMYB8* gene expression and hence, transcriptional activity of the NaMYB8 protein.

As both local and systemic accumulation of CP and DCS was completely abolished in ir-MYB8 plants (Fig. 5A), we propose that *NaMYB8* gene serves as an universal master regulator for CP and DCS biosynthesis in *N. attenuata* plants.
However, in contrast to a strong inducible character of CP accumulation in the leaves (Fig. 5A), no significant increase in DCS accumulation was observed in the systemically induced young stem leaves of WT plants (Fig. 5A), while locally W+OS-induced leaves still showed a small but significant increase in DCS levels (Fig. 5A). In addition, the constitutive levels of DCS were usually higher compared to the constitutive levels of CP. This suggests that other cis-acting regulatory elements most probably contribute to the regulation of CP and DCS-synthase genes, which additionally modifies the rate-limiting NaMYB8 transcriptional activity. Alternatively, a variation in substrate availability of putrescine and spermidine could be contributing to the differential spatial-temporal accumulation of CP and DCS in N. attenuata plants (Paschalidis and Roubelakis-Angelakis, 2005).

Examination of putative role of CP and DCS in plant development

The preferential accumulation of CP in the young leaves and reproductive tissues of N. attenuata suggested that CP might play multiple defensive and/or developmental roles in N. attenuata. Several previous reports have associated high CP levels with flower initiation and bud tissue development in tobacco (Martin-Tanguy, 1985; Martin-Tanguy, 1997; Balint et al., 1987), indirectly proposing a role of CP in flower development. Moreover, the accumulation of phenylpropanoids, polyamines and their conjugates in the reproductive tissues has been previously associated with pollen fertility and floral initiation in plants (Wada et al., 1994; Imai et al., 2004; Kasukabe et al., 2004; Paschalidis and Roubelakis-Angelakis, 2005; Fellenberg et al., 2008, 2009; Grienenberger et al., 2009; Matsuno et al., 2009).

A prediction of the above hypothesis would be that CP (and DCS) deficiency should strongly influence flower development and seed set of ir-MYB8 N. attenuata plants. At first, the content of CP and DCS was examined in reproductive tissues of ir-MYB8-810 transgenic line; the content of CP (Fig. 4A) and DCS (Fig 4B) was heavily reduced in mature MYB8-silenced plants compared to corresponding WT tissues. We then closely examined the reproductive fitness associated traits of ir-MYB8 and WT plants, and found that ir-MYB8 plants grew better during their vegetative developmental phase, having slightly but significantly taller stalks compared to the WT plants (Fig. 4C, Repeated-measures ANOVA; F2, 33 = 55.609; P_{ir-MYB8-818} < 0.001; P_{ir-MYB8-810} < 0.001). In addition, their lifetime seed capsule production -- which is considered as an important fitness measure -- was comparable in WT and ir-MYB8 plants (Fig. 4D, ANOVA, Tukey post-hoc test; F2, 33 = 0.864;
We further quantified the seed mass in two seed capsules located on uppermost lateral branch, one located nearest (T0) and one farthest (T1) from the branching point (see Fig. 4E for details); the seed mass was not statistically different between ir-MYB8 and WT plants (Fig. 4F; T0: ANOVA, Tukey post-hoc test; $F_{2, 33} = 0.793$; $P_{\text{ir-MYB8-818}} = 0.469$; $P_{\text{ir-MYB8-810}} = 0.609$; T1: ANOVA, Tukey post-hoc test; $F_{2, 33} = 0.188$; $P_{\text{ir-MYB8-818}} = 0.986$; $P_{\text{ir-MYB8-810}} = 0.902$). The lack of any observable statistically significant differences between ir-MYB8 and WT plants in terms of reduced growth and/or reproductive fitness supports the idea that flower and seed development in *N. attenuata*, despite being positively correlated with the occurrence of high levels of PPCs, are independent of CP and DCS accumulation. We next focused on an alternative hypothesis that CP and DCS could be involved in direct defenses targeted against attacking herbivores, accounting for the high levels of PPC accumulation in reproductive plant parts.

**Lack of CP and DCS accumulation makes plants susceptible to herbivores**

To test whether CP accumulation is part of plant defense activated against attacking herbivores, we assessed the performance of *M. sexta* (*N. attenuata* specialist) and *S. littoralis* (*N. attenuata* generalist) caterpillars on WT and NaMYB8-silenced plants. It is known that the elicitors present in herbivore’s OS -- for instance, the FAC *N*-linolenoyl-L-glutamic acid (C18:3-Glu) -- are responsible for activation of defense mechanisms targeted against herbivores in *N. attenuata* (Halitschke et al., 2003; Giri et al., 2006); C18:3-Glu elicitor was found predominantly in OS of both *N. attenuata* generalist and specialist herbivores (Diezel et al., 2009). We therefore analyzed the CP and DCS accumulation in both W+C18:3-Glu-induced local rosette and systemically induced young stem leaves of *N. attenuata*. This treatment enhanced both local and systemic accumulation of CP in the plants, while the levels of DCS did not changed significantly (Fig. 5B). Because the overall pattern of CP accumulation in W+FAC-treated leaves was comparable to W+OS-elicited leaves, we therefore used the standardized FAC treatment to maximize the accumulation of CP in the systemically induced young leaves in following herbivory bioassays (see Fig. 3 and related paragraph).

To precondition the plants, we treated rosette leaves of early elongated WT and ir-MYB8-810 *N. attenuata*, one at the time, with W+C18:3-Glu, 1–2 days before placing the caterpillars onto the young, systemically pre-induced leaves. This experimental setup was specifically designed to mimic the initial feeding of the
Rosette leaves by neonates just hatched from oviposited eggs, before they increased size and mobility of first instar larvae to other plant parts (Stork et al., 2009). Both generalist and specialist herbivores were allowed to feed only on systemically induced young stem leaves (see schematic in Fig. 5C) to highlight the effect of systemic accumulation of CP, in combination with the constitutive levels of DCS (Fig. 5A, B); in the course of the experiment, additional rosette leaves were elicited with W+C18:3-Glu every 4<sup>th</sup> day to maintain the elicitation effect.

To conduct the actual bioassays, freshly hatched <i>M. sexta</i> (specialist herbivore) neonates were placed directly on to the FAC pre-induced stem leaves. Due to high sensitivity of <i>S. littoralis</i> (generalist herbivore) to <i>N. attenuata</i>’s defenses, hatched neonates had to be first pre-reared on artificial diet for 6 days. To eliminate the effect of artificial diet present in caterpillar’s gut, a short feeding on WT or ir-MYB8 leaves was then carried out with the larvae and subsequently, pre-weighed caterpillars were transferred to WT and ir-MYB8 young stem leaves, and their body mass gain was recorded every day. Both <i>M. sexta</i> (unpaired <i>t</i> test, <i>P</i> = 0.004) and <i>S. littoralis</i> (unpaired <i>t</i> test, <i>P</i> < 0.001) caterpillars performed better on ir-MYB8-810 plants compared to the WT plants (Fig. 5C). A similar trend in caterpillar performance was previously observed in <i>M. sexta</i> neonates that were allowed to feed directly on NaMYB8-VIGS and EV-VIGS plants (Supplemental Fig. S5, Repeated-measures ANOVA; <i>F</i><sub>1, 28</sub> = 6.265; <i>P</i> = 0.018). These results provided strong evidence that PPCs function as indispensable defensive metabolites targeted against leaf-chewing herbivores in <i>N. attenuata</i> plants.

**Exogenous application of synthetic CP impairs growth of <i>M. sexta</i> caterpillars**

We further tested the role of CP as plant specific-defensive metabolite against herbivores by spraying physiologically relevant concentration of synthetic CP on ir-MYB8 leaves, known to be deficient in accumulation of CP and DCS. We first examined the turnover and stability of the exogenously applied CP on the leaf surface, and found that CP sprayed on ir-MYB8-810 leaves remained stable for at least two days after application. This shows that CP was neither degraded nor it was mobilized to other plant parts. After the spray, we recovered about 130 μg CP per g fresh mass (FM), which was just below the CP concentrations accumulated in the leaves after W+OS-elicitation (~170 μg CP per g FM; Fig. 6A). In the control treatment, no CP was found in the water-sprayed leaves of ir-MYB8-810 plants (Fig. 6C).
6A). We then clip-caged two neonates on CP- or water-sprayed ir-MYB8-810 leaves and examined the growth of the caterpillars. The caterpillars fed for 4 days on CP-sprayed leaves showed less mass gain compared to caterpillars fed on water-sprayed (control) ir-MYB8-810 leaves (Fig. 6B; unpaired t test, P = 0.011), further highlighting the role of CP as direct defense metabolite in response to herbivory in *N. attenuata* plants.

**DISCUSSION**

Phenylpropanoid-polyamine conjugates (PPCs) belong to a group of ubiquitously occurring secondary metabolites in plants (Edreva et al., 2007). While their chemistry and distribution in plants is relatively well documented, much less is known about their biological functions. PPCs predominantly accumulate after pathogen and viral infections, during tuberization, and/or in response to fungal elicitors (Martin-Tanguy, 1985; Rabiti et al., 1998; Martin-Tanguy, 2001; Facchini et al., 2002; Walters, 2003; Cowley and Walters, 2005; Rodríguez-Kessler et al., 2008; Muroi et al., 2009), suggesting that PPCs might be involved in plant defense. Previously, the accumulation of several PPCs in *N. attenuata* leaves in response to herbivory has been reported (Kessler and Baldwin, 2004; Paschold et al., 2007) but no conclusive experimental evidence exists for the role of PPCs in plant-herbivore interactions. Here, we show that CP and DCS accumulation in *N. attenuata*, regulated by NaMYB8 transcription factor, plays an important role in plant defense against leaf-chewing herbivores.

**JA signaling is required for CP accumulation**

The accumulation of CP was previously shown to be strongly induced by jasmonic acid (JA) in *N. attenuata* and *Solanum lycopersicum* (tomato) plants (Keinanen et al., 2001; Chen et al., 2006), and CP accumulated in dose-dependent manner in JA-treated *Capsicum annuum* (sweet pepper) cotyledons (Tebayashi et al., 2007). The dependence of CP accumulation on JA was further highlighted by using *N. attenuata* transgenic plants silenced in their expression of *lipoxygenase 3* (antisense-LOX3), an enzyme essential for JA biosynthesis, which resulted in strongly reduced CP levels in *M. sexta* OS-elicited leaves (Paschoold et al., 2007). The accumulation of CP in antisense-LOX3 plants could be rescued by exogenously applied JA, suggesting that JA is one of the major limiting factors in CP accumulation. The role of JA and its active metabolite JA-Ile in CP biosynthesis was
further examined in ir-COI1 *N. attenuata* plants impaired in JA-Ile perception due to non-functional SCF<sup>coi1</sup> protein complex. ir-COI1 plants showed strongly reduced CP levels (Paschold et al., 2007), similar to tomato *jai-1* mutants defective in JA perception, which failed to accumulate CP in the flowers and in the MeJA-treated leaves (Chen et al., 2006). All together, these observations provide a strong link between JA signaling and CP accumulation; however, the actual regulatory mechanisms initiated after JA perception, particularly those downstream of putative MYC2 protein regulation in *N. attenuata*, leading to PPCs’ biosynthesis in plants, such as PAL (phenylpropanoid biosynthesis) or ODC/ADC (polyamines) remain elusive.

**Transcriptional regulation of phenylpropanoid pathway**

The phenylpropanoid biosynthesis in plants is often controlled by specific transcription factors, including the members of MYB gene family [*A. thaliana* – (Stracke et al., 2001); *Populus trichocarpa* (Wilkins et al., 2009)]. The most abundant sub-group of MYB transcriptional regulators, R2R3-MYBs, is frequently activated by biotic and abiotic stress factors -- UV-exposure, wounding, osmotic stress, anaerobic conditions, herbivory and pathogen infections, which further regulates the accumulation of secondary metabolites in stressed plants (Jin et al., 2000; Vailleau et al., 2002; Mengiste et al., 2003; De Vos et al., 2006; Gigolashvili et al., 2007; Sonderby et al., 2007; Tong Geon et al., 2007; Lippold et al., 2009; Mellway et al., 2009). In contrast, several MYB genes have been shown to regulate processes different from secondary metabolism, including cellular morphogenesis, cell cycle, meristem formation, fibre and trichome development, and lignification in plants (Baumann et al., 2007; Legay et al., 2007; Bomal et al., 2008; Petroni et al., 2008; Cominelli and Tonelli, 2009; Machado et al., 2009; Zhang et al., 2009).

The direct connection between MYB transcriptional activity and the regulation of PPC biosynthesis was first reported by Gális et al. (2006) and Shinya et al. (2007). Ectopically expressed R2R3-MYB transcription factors -- NtMYBJS1 (MeJA-responsive) and NtMYBGR1 (glucan elicitor-responsive) -- increased the accumulation of CP and feruloylputrescine (another PPC) in unelicited BY-2 tobacco cell cultures. Both transcription factors selectively responded to their own elicitors; however, both proteins converged in a regulation of similar group of genes that were involved in phenylpropanoid biosynthesis, and controlled the accumulation of PPCs in transformed plant cells (Gális et al., 2006; Shinya et al., 2007). Relatively large
spectrum of elicitors that could induce various MYB transcription factors, and enhance the accumulation of PPCs, suggests that these metabolites could be mediating a broad range of resistances to fungi, necrotrophic pathogens and herbivores.

**NaMYB8: OS-responsive regulator of CP and DCS accumulation**

*NaMYB8* transcripts accumulated rapidly in *N. attenuata* rosette leaves after wounding and returned to the pre-elicited levels within three hours after treatment (Fig. 1A, inset); however, the *M. sexta*’s OS-elicited rosette leaves showed delayed reinstatement of transcripts to their basal levels at late hours after induction, reflecting that *NaMYB8* expression discriminates between herbivory-associated damage and simple mechanical wounding. Similar trends in transcript accumulation are typically found in genes involved in defense triggered by *M. sexta*’s OS-elicitation, for instance, genes encoding TPIs (Halitschke et al., 2001). Previously, we showed that *NaMYB8* gene is also induced by UV-B in the glasshouse and by cumulative stress conditions in the natural environment of *N. attenuata*, both following an RdR2-dependent induction pattern (Pandey et al. 2008). It suggests that apart from regulating *N. attenuata*’s response to herbivores, the *NaMYB8* gene may also be regulated by various abiotic stresses, namely high levels of UV-B in the natural environment.

We examined the transcription regulatory activity of NaMYB8 by performing microarray analysis with OS-elicited ir-MYB8-818 leaves hybridized against identically elicited WT leaves. This analysis confirmed that *NaMYB8* gene specifically activates transcription of genes involved in phenylpropanoid and polyamine biosynthesis (Supplemental Table S1). Similar transcriptional regulatory pattern of genes involved in phenylpropanoid pathway was observed in BY-2 tobacco cell cultures ectopically expressing *NtMYBJIS1* gene, using an independent tobacco microarray system (Gális et al., 2006). Interestingly, several novel NaMYB8-controlled genes with a potential role in PPC biosynthesis have been identified in the current microarray experiment, and the functional characterization of these genes is currently in progress. Remarkably, one of the NaMYB8-regulated genes encodes a functional DCS synthase in *N. attenuata* (N. Onkokesung; manuscript in preparation).

The accumulation of CP and DCS in WT plants correlated well with the pattern of *NaMYB8* transcript abundance. The absence of both metabolites in NaMYB8-silenced lines, regardless of plant treatment, provided final conclusive
proof that NaMYB8 transcription factor is essential for CP and DCS biosynthesis and accumulation in *N. attenuata* plants (Fig. 7), similar to the role of *PAPI* gene, an AtMYB75 transcriptional regulator that governs anthocyanin biosynthesis in *A. thaliana* (Teng et al., 2005; Tohge et al., 2005).

**CP accumulates in the young vegetative and reproductive tissues**

The ecological role of metabolites can be often deduced from their spatial-temporal accumulation pattern. CP and DCS accumulated in the shoot apices, young leaves, and female reproductive organs during flower induction and development in tobacco (Cabanne et al., 1981; Martin-Tanguy, 1985; Edreva et al., 2007), and a similar pattern of PPC accumulation was also observed in some *Araceae* species (Ponchet et al., 1982). The accumulation of CP in reproductive organs is widely reported in the literature, which is generally linked to plant development. However, when the distribution of PPCs was examined across seven species belonging to *Solanaceae* family, CP accumulated predominantly in the pistils of tobacco flowers but this pattern was absent in several other species, indicating that PPCs may not be the universal regulators of flower initiation in Solanaceous plants (Leubner-Metzger and Amrhein, 1993). Moreover, the use of specific inhibitors that can reduce the accumulation of feruloylputrescine and CP in *in vitro* tobacco cultures did not affect the growth and floral bud formation in tobacco stem explants (Wyss-Benz et al. 1990), further supporting the alternative role of PPCs in plants.

In the search for ecologically meaningful interpretations of CP accumulation patterns, we decided to treat the plants with simulated herbivory (Halitschke et al., 2001) at five developmental stages and compared the CP accumulation with untreated plants. Interestingly, both constitutive and inducible levels of CP were higher in the young stem leaves compared to the mature rosette leaves (Fig. 3, 5A). In agreement with previous studies (Edreva et al., 2007), CP was most abundant in the buds at the early elongated stage but these levels progressively declined as plants aged (Fig. 3). At mature stage, plants retained high levels of CP almost exclusively in the reproductive organs, flowers and capsules. However, when ectopically silencing the ability of plants to accumulate PPCs, we found no aberrant morphological changes associated with this novel trait. In summary, the CP accumulation shifted primarily from photosynthetically active young leaves during vegetative growth to flower buds, flowers and seed capsules at maturity, as well as it was significantly stimulated by simulated herbivory treatments (Fig. 3).
**CP and DSC are indispensable for plant defense against herbivores**

In our follow up hypothesis, CP could be a direct defensive metabolite that accumulates upon herbivory and against herbivores. According to the Optimal Defense Theory, plants tend to allocate more defense-associated metabolites to the valuable plant parts during development -- photosynthetically active tissues, meristems and reproductive tissues (flowers and seeds) -- to protect these organs from stress factors, including herbivores (McKey, 1974; Ohnmeiss and Baldwin, 2000; Stamp, 2003). A shift in CP accumulation, consistent with the theory, was indeed observed in *N. attenuata* plants during development and in response to herbivore associated cues, suggesting that *N. attenuata* plants allocate PPCs to the young leaves in an attempt to protect them from attack by herbivores and/or pathogens. Defensive role of several PPCs in plant resistance to pathogens has already been demonstrated, including preferential accumulation of PPCs in systemically induced tissues: systemic leaves of *Hordeum vulgare* (barley) accumulated higher concentrations of PPCs compared to MJ-induced local leaves, and this higher accumulation of PPCs in systemic leaves was correlated with stronger resistance to powdery mildew infection (Walters et al., 2002).

Previously, CP was tested as an ovipositioning deterrent in cotyledons of sweet pepper (*Capsicum annuum*) by dipping leaves in CP solutions (Tebayashi et al., 2007). Treated leaves showed fewer oviposition-associated punctures from *Liriomyza trifolii* compared to non-treated leaves, demonstrating for the first time a defensive function of CP against herbivores in plants. PPCs, in particular *N*<sup>1</sup>-coumaroylspermine, have been shown to inhibit mammalian and crayfish neuroreceptors *in vitro*, being structurally similar to acylated polyamines found in spider and wasp toxins. However, when the shorter naturally occurring polyamine conjugates in plants (*N*<sup>1</sup>- and *N*<sup>8</sup>-coumaroylspermidine) were supplied in artificial diet to European corn borer, tobacco budworm and the oblique banded leaf roller, no toxic effects of these metabolites on insects have been found (Fixon-Owoo et al., 2003). It suggests that certain leaf-derived metabolites and/or enzymatic activities may be required for demonstrated toxicity of PPCs against herbivores in natural situations.

Here, we made a targeted attempt to investigate the role of CP and DCS in plant-insect interaction, using reverse genetic approach and plants silenced in the expression of the master transcriptional regulator responsible for the accumulation of CP and DCS in native tobacco plants. In accordance with the proposed defensive role
against herbivores, both generalist and specialist caterpillars gained more body mass when allowed to feed on the systemically induced leaves of ir-MYB8 plants compared to the WT plants. The last piece of supporting evidence resulted from experiments using direct application of synthetic CP at physiological concentrations to the ir-MYB8 leaves: ir-MYB8 leaves, when sprayed with synthetic CP, supported less *M. sexta* larval growth than did the water sprayed ir-MYB8 leaves. This experiment further showed that PPCs should be tested in natural context, together with plant leaves rather than artificial diet to show their toxic effects.

**Conclusion**

We used a well established ecological model plant, *N. attenuata*, combined with state-of-the-art molecular tools to construct transgenic plants deficient in expression of a master transcriptional activator required for CP and DCS biosynthesis, both at constitutive and elicited levels. The use of a master regulator (NaMYB8) allowed us to specifically down regulate the biosynthetic pathway leading to CP and DCS accumulation, without disturbing other defense-related mechanisms in plants. The functional analysis of ir-MYB8 plants then highlighted the important ecological role of CP and DCS in plant defense operating against herbivorous insects. Further studies with ir-MYB8 plants will enable us to understand the role of CP and DCS in defense against pathogens, as well as the results from our microarray analysis shall be used to identify novel genes responsible for CP and DCS biosynthesis.

**MATERIALS AND METHODS**

*Plant growth conditions in the glasshouse*

*Nicotiana attenuata* Torr. Ex S. Watson (22nd inbred generation) seeds, originally collected from a native population from a field site located in Utah, USA, were used for all described experiments, including transformation and generation of transgenic lines. The seeds were germinated on sterile Gamborg B5 medium (Sigma, http://www.sigmaaldrich.com) after 1h treatment with diluted smoke (House of Herbs) and 1μM GA3 (www.carl-roth.de). Ten days after germination, seedlings were transferred into Teku pots containing peat-based substrate and after additional 10-12 days, the plantlets were transplanted into individual 1L pots with the same substrate. In the glasshouse, plants were grown at 24-26°C, relative humidity ~ 55%,
and supplemented with light from 400- and 600-W sodium lamps (Philips Sun-T Agro; http://www.nam.lighting.philips.com) for 16h.

**Virus induced gene silencing (VIGS)**

A VIGS system based on the tobacco mosaic rattle virus was used as described in Saedler and Baldwin (2004). Three-week-old *N. attenuata* plants were inoculated with *Agrobacterium tumefaciens* and pTVMYB8 plasmid -- carrying a fragment of *NaMYB8* (*NaMYB8*-VIGS) or pTV00 -- carrying empty vector construct as control (EV-VIGS).

**Generation and characterization of ir-MYB8 transgenic lines**

Stably transformed ir-MYB8 lines were generated by introducing an ir construct containing a 309-bp *NaMYB8* gene fragment and a gene for hygromycin resistance (*hptII*) as a screening marker in a pSOL8 transformation vector (Supplemental Figure S2A) into *N. attenuata* plants as described by Bubner et al. (2006). *A. tumefaciens*-mediated plant transformation was followed as described by Krügel et al. (2002). Transformed lines, each containing a single insertion of the *hptII* marker gene as determined by Southern hybridization, were further screened by segregation analysis of T2 seedlings for their hygromycin resistance to obtain homozygous transformed lines. Quantitative real-time PCR (RT-qPCR) was used to quantify the transcript accumulation of *NaMYB8* gene and two independently transformed homozygous diploid lines with efficiently silenced expression of *NaMYB8* gene, ir-MYB8-810 and ir-MYB8-818, were selected for all subsequent experiments.

**Southern blot hybridizations**

A modified cetyltrimethylammonium bromide (CTAB) method as described by Paschold et al. (2007) was followed for genomic DNA extraction from the fully expanded rosette leaves of WT, ir-MYB8-810 and ir-MYB8-818 *N. attenuata* plants (Rogers and Bendich, 1985). For Southern blot hybridizations, 15 µg of the DNA was digested overnight with two different restriction enzymes (New England Biolabs, http://www.neb.com) at 37°C, size-fractionated on a 0.8% (w/v) agarose gel, and blotted onto a nylon membrane (GeneScreenPlus; PerkinElmer, http://www.perkinelmer.com). Fragments of *hptII* (forward primer: 5’-CGTCTGTCGAGAAGTTTCTG-3’; reverse primer: 3’-
CCGGATCGGACGATTGCG-5’) were PCR-amplified and used as probe for Southern hybridizations to confirm the single-insertion of hptII gene in ir-MYB8 transformed lines. The DNA probes were labeled with α-32P using Rediprime™ II DNA labeling system (Amersham Biosciences; http://www.amershambiosciences.com).

Expression analysis by RT-qPCR

To analyze NaMYB8 gene expression, total RNA was extracted from fully expanded control and W+OS-elicited rosette leaves of WT and ir-MYB8 plants, following the TRIZOL method as recommended by manufacturer (Invitrogen; http://www.invitrogen.com). A 30 µg of RNA was given DNase treatment by adding 10 units of RQ1 RNase-free DNase in 1x enzyme buffer following the manufacturer’s instructions (Promega; http://www.promega.com). Each sample was incubated at 65 ºC for 20 min to inactivate DNase enzyme, extracted in phenol/chloroform/isoamylalcohol (25:24:1) and precipitated in the presence of 0.1 volume 3M sodium acetate, pH 5.2 and 3 volumes of ice-cold pure ethanol at -20ºC. A 2 µg of total RNA was reverse-transcribed using oligo-dT (Fermentas; http://www.fermentas.com) as primer and Superscript II reverse transcriptase (Invitrogen). All RT-qPCR assays were performed with cDNA corresponding to 100 ng RNA before reverse transcription and gene-specific primers using qPCR core kit for SYBR Green I (Eurogentec GmbH; http://www.eurogentec.com), following the manufacturer’s instructions.

To determine the NaMYB8 genes transcript levels in the ir-MYB8 lines, gene-specific primers were designed outside the region used for making ir silencing construct. All gene-specific primers were designed with Primer3 online available software (http://frodo.wi.mit.edu/primer3). For all RT-qPCR analyses, unless stated differently, cDNA from five replicate biological samples was used and an assay was carried out on a Stratagene Mx3005P™ real-time PCR system (http://www.stratagene.com). Relative gene expression was calculated using a 10-fold dilution series of cDNA containing NaMYB8 as well as the elongation factor-1α house keeping gene from N. tabacum (EF1-α; Acc. D63396) as an endogenous reference.

To determine whether herbivore attack elicits NaMYB8 transcription, 5 fully expanded leaves at rosette-stage were either W+W or W+OS elicited, or left
untreated as control. All 5 leaves were pooled and cDNA was prepared from each
time point and treatment over a 48 h elicitation period. The transcript abundance of
*NaMYB8* gene was quantified with RT-qPCR in three technical replicates of the
pooled samples.

**Microarray analysis**

We used a custom oligonucleotide DNA microarray, selectively enriched with
herbivore-activated genes from *N. attenuata* and related species (Biochip ver. 4; 1421
gene probes; Meldau et al., 2009). The hybridizations were carried out with cDNA
synthesized from pooled leaf tissues representing 15 plants in three biological
replicates from each WT and ir-MYB8-818 OS-elicited rosette leaves, which were
harvested 45 and 90 min after elicitation. The cDNA samples from ir-MYB8-818 and
WT were labeled with Cy3- and Cy5-fluorescent dyes, respectively and three pairs of
labeled probes were hybridized with the microarray (WT-Cy5 / ir-MYB8-Cy3) to
obtain differential expression data for each time point. Hybridisation and analysis
were carried out essentially as described in Wang et al. (2008).

**Phytohormone analysis**

A (+1) ir-MYB8/WT or EV/NaMYB8-VIGS fully expanded *N. attenuata*
rosette leaves were either W+W or W+OS-elicited or left unelicited; the leaves were
then collected, snap-frozen in liquid nitrogen and stored at -80 °C until phytohormone
analysis. Phytohormones JA and JA-Ile were extracted following the procedure
described in Wu et al. (2007). In brief, 150 mg of leaf tissue was extracted with 1 mL
ethyl acetate spiked with 200 ng JA$^{13}$C$_2$, and JA-Ile$^{13}$C$_6$ as internal standards in
FastPrep tubes containing 0.9 g of FastPrep Matrix (Sili GmbH;
http://www.sigmund-lindner.com). The tissue was homogenized and samples were
centrifuged at 13,000 rpm for 15 min at 4 °C before the supernatant was transferred
to clean 2-mL Eppendorf tube. Each pellet was re-extracted with 1 mL of ethyl
acetate and centrifuged; supernatants were combined and then dried in a vacuum
concentrator to near dryness (Eppendorf; http://www.eppendorf.com). The residue in
the tubes was re-suspended in 0.5 mL of 70% methanol (v/v). A 10 μL extract was
subjected to reverse-phase HPLC coupled to tandem mass spectrometry and the peaks
were identified with a 1200LC MS/MS/MS system (Varian, Palo Alto, CA, USA;
http://www.varianinc.com) after negative ionization with parent-ion/daughter-ion
selections: 209/59 (JA), 211/61 (JA$^{13}$C$_2$), 322/130 (JA-Ile), 328/136 (JA-Ile$^{13}$C$_6$). JA
and JA-Ile were quantified with respect to each compound’s labeled internal standard (Wu et al. 2007).

**Secondary metabolite analysis**

The secondary metabolite analysis was carried out on (+1) leaves after *M. sexta* caterpillar feeding on WT/ir-MYB8 and EV/NaMYB8-VIGS plants for four days, using high-performance liquid chromatography (HPLC) as described by Keinänen et al. (2001). For analysis of the locally and systemically induced responses in WT/ir-MYB8-810/ir-MYB8-818, a (+1) leaf was either W+W, W+OS-elicited or left unelicited. The 3-day OS-elicited rosette leaf served as local and second stem leaf was used for systemic response analysis.

To examine the spatial-temporal distribution of CP, a study was conducted on *N. attenuata* WT plants at 5 different stages of development (rosette, early elongated, elongated, flowering and mature). At each stage, a (+1) leaf was either OS-elicited or left unelicited and three days after elicitation, representative tissue samples were collected and analyzed by HPLC. A 200 mg aliquot of tissue in FastPrep tubes containing 0.9 g of FastPrep matrix (Sili GmbH) was extracted with 1 mL of 40% MeOH prepared with 0.5% acetic acid water. The sample in FastPrep tubes was homogenized on a FastPrep homogenizer (Thermo Electron) for 45 sec and then centrifuged for 12 min at 13000 rpm. The supernatant was transferred into 1.5-mL Eppendorf tubes, centrifuged, and finally transferred to a glass vial where it was analyzed by an Agilent-HPLC 1100 series (http://www.chem.agilent.com). The ODS Inertsil C-18 column (3 µm, 150 x 4.6 mm i.d.) was attached to a Phenomenex Security Guard C18 pre-column (http://www.phenomenex.com). The solvents were (A) 0.25% H3PO4 in water and (B) acetonitrile. The elution system was as follows: 0-6 min, 0-12% of B; 6-10 min, 12-18% of B; 10-30 min, 18-58% of B. The flow rate was 1 mL/min, the injection volume was 10 µL, and the column oven was set at 24°C. The nicotine eluted at retention time 1.83 min was detected at 254nm; CP, CGA, DCS eluted at retention times 8.3 min, 12.3 min, 12.7 min respectively were detected at 320nm; rutin eluted at 16.4 min was detected at 360 nm and diterpene glycosides eluting at 24-26 min were detected at 210nm.

**Measuring ethylene accumulation**

At least three replicate measurements were used to quantify ethylene production in WT and NaMYB8 transgenic *N. attenuata* plants. Three leaves were
treated either with W+W or W+OS, in the case of EV/NaMYB8-VIGS; or W+OS in the case of WT/ir-MYB8-818/ir-MYB8-810; or plants were left untreated (controls). Leaves were cut from stems, immediately sealed in a three-neck 250-mL round bottom flasks and kept in the glasshouse under light conditions for 5 h. The headspace of the flasks was flushed into a photoacoustic laser spectrometer with hydrocarbon-free clean air, and the ethylene concentration was quantified by comparing ethylene peak areas with peak areas generated by a standard ethylene gas as previously described by Von Dahl et al. (2007).

**Herbivore performance**

The growth performance of *M. sexta* (*N. attenuata* specialist) and *Spodoptera littoralis* (*N. attenuata* generalist) caterpillars was examined using WT and ir-MYB8-silenced plants. Due to the high sensitivity of the generalist herbivore to *N. attenuata* defenses, *S. littoralis* neonates were first reared on artificial diet for 6 days and then placed on WT or ir-MYB8 leaves for one day to get rid of artificial diet present in the caterpillars’ guts, and subsequently, the pre-weighed *S. littoralis* caterpillars were placed on the plants. Freshly hatched *N. attenuata* specialist *M. sexta* neonates, were placed directly on WT/ir-MYB8-810 stem leaves. Both generalist and specialist herbivores were allowed to feed only on systemically pre-induced stem leaves of plants, which had their single rosette leaf elicited with FAC (18:3-Glu; 0.07 nmol/μL in 0.02% (v/v) Tween-20/water; 20 μL per leaf) every 4th day from the start of the experiment. *S. littoralis* caterpillar mass was recorded daily over 5 days, while the *M. sexta* caterpillar mass was recorded on 4, 7 and 11 day of feeding.

**CP synthesis**

CP was synthesized in the laboratory as described in (Hu and Hesse, 1996).

**STATISTICAL ANALYSIS**

All statistical analyses were performed with SPSS software (http://www.spss.com).

**ACKNOWLEDGMENTS**

We thank Thomas Hahn for sequencing; Klaus Gase and Antje Wissgott for vector construction; Susan Kutschbach and Wibke Kröber for generating the stably transformed plants; E. Rothe for technical assistance with HPLC; Tamara Krügel,
Andreas Weber, and Andreas Schüenzel for growing the plants in the glasshouse; Emily Wheeler for editorial assistance and the Max Planck Society for funding.
LITERATURE CITED

Balint R, Cooper G, Staebell M, Filner P (1987) N-caffeoyl-4-amino-n-butyric acid, a new flower-specific metabolite in cultured tobacco cells and tobacco plants. J Biol Chem 262: 11026-11031

Baumann K, Perez-Rodriguez M, Bradley D, Venail J, Bailey P, Jin H, Koes R, Roberts K, Martin C (2007) Control of cell and petal morphogenesis by R2R3 MYB transcription factors. Development 134: 1691-1701

Boege K, Dirzo R, Siemens D, Brown P (2005) Onset of cell and petal morphogenesis by R2R3 MYB transcription factors. Development 134: 1691-1701

Boege K, Marquis RJ (2005) Facing herbivory as you grow up: the ontogeny of resistance in plants. Trends Ecol Evol 20: 441-448

Bomal C, Bedon F, Caron S, Mansfield SD, Levasseur C, Cooke J, Blais S, Tremblay L, Morency M, Pavy N, Grima-Pettenati J, Seguin A, MacKay J, Pavy N, Grima-Pettenati J, Seguin A, MacKay J (2008) Involvement of Pinus taeda MYB1 and MYB8 in phenylpropanoid metabolism and secondary wall biogenesis: A comparative in planta analysis. J Exp Bot 59: 3925-3939

Browse J (2009) Jasmonate passes muster: A receptor and targets for the defense hormone. Ann Rev Plant Biol 60: 183-205

Bu Q, Jiang H, Li C-B, Zhai Q, Zhang J, Wu X, Sun J, Xie Q, Li C (2008) Role of the Arabidopsis thaliana NAC transcription factors ANAC019 and ANAC055 in regulating jasmonic acid-signaled defense responses. Cell Res 18: 756-767

Bubner B, Gase K, Berger B, Link D, Baldwin I (2006) Occurrence of tetraploidy in Nicotiana attenuata plants after Agrobacterium-mediated transformation is genotype specific but independent of polysomaty of explant tissue. Plant Cell Reports 25: 668-675

Cabanne F, Dalebroux MA, Martin-Tanguy J, Martin C (1981) Hydroxycinnamic acid amides and ripening to flower of Nicotiana tabacum var. xanthi n.c. Physiol Plant 53: 399-404

Chen H, Jones AD, Howe GA (2006) Constitutive activation of the jasmonate signaling pathway enhances the production of secondary metabolites in tomato. FEBS letters 580: 2540-2546

Chen M-S (2008) Inducible direct plant defense against insect herbivores: A review. Insect Sci 15: 101-114

Chico JM, Chini A, Fonseca S, Solano R (2008) JAZ repressors set the rhythm in jasmonate signaling. Curr Opin Plant Biol 11: 486-494

Chini A, Boter M, Solano R (2009a) Plant oxylipins: COI1JAZsMYC2 as the core jasmonic acid-signalling module. FEBS J 276: 4682-4692

Chini A, Fonseca S, Chico JM, Fernández-Calvo P, Solano R (2009b) The ZIM domain mediates homo- and heteromeric interactions between Arabidopsis JAZ proteins. Plant J 59: 77-87

Chung HS, Niu Y, Browse J, Howe GA (2009) Top hits in contemporary JAZ: An update on jasmonate signaling. Phytochem 70: 1547-1559

Cominelli E, Tonelli C (2009) A new role for plant R2R3-MYB transcription factors in cell cycle regulation. Cell Res 19: 1231-1232

Cowley T, Walters DR (2005) Local and systemic changes in arginine decarboxylase activity, putrescine levels and putrescine catabolism in wounded oilseed rape. New Phytol 165: 807-811

De Vos M, Denekamp M, Dicke M, Vuylsteke M, Van Loo L, Smeekens SCM, Pieterse CMJ (2006) The Arabidopsis thaliana transcription factor
AtMYB102 functions in defense against the insect herbivore *Pieris rapae*. Plant Signal Behav 1: 305-311

Diezel C, von Dahl CC, Gaquerel E, Baldwin IT (2009) Different lepidopteran elicitors account for cross-talk in herbivory-induced phytohormone signaling. Plant Physiol 150: 1576-1586

Eckardt NA (2008) Oxylipin signaling in plant stress responses. Plant Cell 20: 495-497

Edreva AM, Velikova VB, Tsonov TD (2007) Phenylamides in plants. Russian J Plant Physiol 54: 287-301

Facchini PJ, Hagel J, Zulak KG (2002) Hydroxycinnamic acid amide metabolism: physiology and biochemistry. Canadian J Botany-Revue Canadienne De Botanique 80: 577-589

Fellenberg C, Böttcherb C, Vogt T (2009) Phenylpropanoid polyamine conjugate biosynthesis in *Arabidopsis thaliana* flower buds. Phytochem 70: 1392-1400

Fellenberg C, Milkowski C, Hause B, Lange P-R, Böttcher C, Schmidt J, Vogt T (2008) Tapetum-specific location of a cation-dependent O-methyltransferase in *Arabidopsis thaliana*. Plant J 56: 132-145

Fixon-Owoo S, Levasseur F, Williams K, Sabado TN, Lowe M, Klose M, Mercier AJ, Fields P, Atkinson J (2003) Preparation and biological assessment of hydroxycinnamic acid amides of polyamines. Phytochemistry 63: 315-334

Fonseca S, Chico JM, Solano R (2009) The jasmonate pathway: the ligand, the receptor and the core signalling module. Curr Opin Plant Biol 12: 539-547

Gális I, Simek P, Narisawa T, Sasaki M, Horiguchi T, Fukuda H, Matsuoka K (2006) A novel R2R3 MYB transcription factor NtMYBJS1 is a methyl jasmonate-dependent regulator of phenylpropanoid-conjugate biosynthesis in tobacco. Plant J 46: 573-592

Gatehouse JA (2002) Plant resistance towards insect herbivores: a dynamic interaction. New Phytol 156: 145-169

Gigolashvili T, Yatusevich R, Berger B, Müller C, Flügge U-I (2007) The R2R3-MYB transcription factor HAG1/MYB28 is a regulator of methionine-derived glucosinolate biosynthesis in *Arabidopsis thaliana*. Plant J 51: 247-261

Giri AP, Wunschke H, Mitra S, Zavala JA, Muck A, Svatos A, Baldwin IT (2006) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. VII. Changes in the plant’s proteome. Plant Physiol 142: 1621-1641

Grienenberger E, Besseau S, Geoffroy P, Debayle D, Heintz D, Lapierre C, Pollet B, Heitz T, Legrand M (2009) A BAHD acyltransferase is expressed in the tapetum of *Arabidopsis* anthers and is involved in the synthesis of hydroxycinnamoyl spermidines. Plant J 58: 246-259

Halitschke R, Gase K, Hui DQ, Schmidt DD, Baldwin IT (2003) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. VI. Microarray analysis reveals that most herbivore-specific transcriptional changes are mediated by fatty acid-amino acid conjugates. Plant Physiol 131: 1894-1902

Halitschke R, Schittko U, Pohnert G, Boland W, Baldwin IT (2001) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. III. Fatty acid-amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specific plant responses. Plant Physiol 125: 711-717

Howe GA, Jander G (2008) Plant immunity to insect herbivores. Ann Rev Plant Biol 59: 41-66

---

www.plantphysiol.org on August 13, 2017 - Published by Downloaded from Copyright © 2010 American Society of Plant Biologists. All rights reserved.
Hu W, Hesse M (1996) Synthese der p-Cumaroylspermidine. Helv Chim Acta 79: 548-559

Imai A, Matsuyama T, Hanzawa Y, Akiyama T, Tamaoki M, Saji H, Shirano Y, Kato T, Hayashi H, Shibata D, Tabata S, Komeda Y, Takahashi T (2004) Spermidine synthase genes are essential for survival of Arabidopsis. Plant Physiol 135: 1565-1573

Jassbi AR, Gase K, Hettenhausen C, Schmidt A, Baldwin IT (2008) Silencing geranylgeranyl diphosphate synthase in Nicotiana attenuata dramatically impairs resistance to tobacco hornworm. Plant Physiol 146: 974-986

Jenkins GI (2009) Signal transduction in responses to UV-B radiation. Ann Rev Plant Biol 60: 407-431

Jin H, Cominelli E, Bailey P, Mehrtens F, Jones J, Tonelli C, Weisshaar B, Martin C (2000) Transcriptional repression by AtMYB4 controls production of UV-protecting sunscreens in Arabidopsis. EMBO J 19: 6150-6161

Kasukabe Y, He L, Nada K, Misawa S, Ihara I, Tachibana S (2004) Over-expression of spermidine synthase enhances tolerance to multiple environmental stresses and up-regulates the expression of various stress-regulated genes in transgenic Arabidopsis thaliana. Plant Cell Physiol 45: 712-722

Kazan K, Manners JM (2008) Jasmonate signaling: Toward an integrated view. Plant Physiol 146: 1459-1468

Keinanen M, Oldham NJ, Baldwin IT (2001) Rapid HPLC screening of jasmonate-induced increases in tobacco alkaloids, phenolics, and diterpene glycosides in Nicotiana attenuata. J Agri Food Chem 49: 3553-3558

Kessler A, Baldwin IT (2004) Herbivore-induced plant vaccination. Part I. The orchestration of plant defenses in nature and their fitness consequences in the wild tobacco Nicotiana attenuata. Plant J 38: 639-649

Koornneef A, Pieterse CMJ (2008) Cross talk in defense signaling. Plant Physiol 146: 839-844

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

Legay S, Lacombe E, Goicoechea M, Briere C, Seguin A, MacKay J, Grima-Pettenati J (2007) Molecular characterization of EgMYB1, a putative transcriptional repressor of the lignin biosynthetic pathway. Plant Sci 173: 542-549

Leubner-Metzger G, Amrhein N (1993) The distribution of hydroxycinnamoyl-putrescines in different organs of Solanum tuberosum and other solanaceous species. Phytochem 32: 551-556

Li J, Ou-Lee TM, Raba R, Amundson RG, Last RL (1993) Arabidopsis flavonoid mutants are hypersensitive to UV-B irradiation. Plant Cell 5: 171-179

Lippold F, Sanchez DH, Musialak M, Schlereth A, Scheible W-R, Hincha DK, Udvardi MK (2009) AtMyb41 regulates transcriptional and metabolic responses to osmotic stress in Arabidopsis. Plant Physiol 149: 1761-1772

Loake G, Grant M (2007) Salicylic acid in plant defence—the players and protagonists. Curr Opin Plant Biol 10: 466-472

Luo J, Fuell C, Parr A, Hill L, Bailey P, Elliott K, Fairhurst SA, Martin C, Michael AJ (2009) A novel polyamine acyltransferase responsible for the accumulation of spermidine conjugates in Arabidopsis seed. Plant Cell 21: 318-333
Machado A, Wu Y, Yang Y, Llewellyn DJ, Dennis ES (2009) The MYB transcription factor GhMYB25 regulates early fibre and trichome development. Plant J 59: 52-62

Martin-Tanguy J (1985) The occurrence and possible function of hydroxycinnamoyl acid amides in plants. J Plant Growth Regul 3: 381-399

Martin-Tanguy J (1997) Conjugated polyamines and reproductive development: Biochemical, molecular and physiological approaches. Physiol Plant 100: 675-688

Martin-Tanguy J (2001) Metabolism and function of polyamines in plants: recent development (new approaches). J Plant Growth Regul 34: 135-148

Matsuno M, Compagnon V, Schoch GA, Schmitt M, Debayle D, Bassard J-E, Pollet B, Hehn A, Heintz D, Ullmann P, Lapierre C, Bernier F, Ehlting J, Werck-Reichhart D (2009) Evolution of a novel phenolic pathway for pollen development. Science 325: 1688-1692

McKey D (1974) Adaptive patterns in alkaloid physiology. Am Nat 108: 305-320

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

Mellway RD, Tran LT, Prouse MB, Campbell MM, Constabel CP (2009) The wound-, pathogen-, and ultraviolet B-responsive MYB134 gene encodes an R2R3 MYB transcription factor that regulates proanthocyanidin synthesis in poplar. Plant Physiol 150: 924-941

Mengiste T, Chen X, Salmeron J, Dietrich R (2003) The BOTRYTIS SUSCEPTIBLE1 gene encodes an R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in Arabidopsis. Plant Cell 15: 2551-2565

Muroi A, Ishihara A, Tanaka C, Ishizuka A, Takabayashi J, Miyoshi H, Nishi T (2009) Accumulation of hydroxycinnamic acid amides induced by pathogen infection and identification of agmatine coumaroyltransferase in Arabidopsis thaliana. Planta 230: 517-527

Ohnmeiss TE, Baldwin IT (2000) Optimal defense theory predicts the ontogeny of an induced nicotine defense. Ecology 81: 1765-1783

Pandey SP, Baldwin IT (2008) Silencing RNA-directed RNA polymerase 2 increases the susceptibility of Nicotiana attenuata to UV in the field and in the glasshouse. Plant J 54: 845-862

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

Petroni K, Falasca G, Calvenzani V, Allegra D, Stolfi C, Fabrizi L, Altamura MM, Tonelli C (2008) The AtMYB11 gene from Arabidopsis is expressed in meristematic cells and modulates growth in planta and organogenesis in vitro. J Exp Bot 59: 1201-1213

Ponchet M, Martintanguy J, Poupet A, Marais A, Beck D (1982) Separation and quantification of basic hydroxycinnamic amides and hydroxycinnamic acids by reversed-phase high-performance liquid-chromatography. J Chromatogr 240: 397-404

Purrington, CB (2000) Costs of resistance. Curr Opin Plant Biol 3: 305-308
Rabiti AL, Betti L, Bortolotti C, Marini F, Canova A, Bagni N, Torrigiani P (1998) Short-term polyamine response in TMV-inoculated hypersensitive and susceptible tobacco plants. New Phytol 139: 549-553

Rodríguez-Kessler M, Ruiz OA, Maiale S, Ruiz-Herrerac J, Jiménez-Bremonta JF (2008) Polyamine metabolism in maize tumors induced by Ustilago maydis. Plant Physiol Biochem 46: 805-814

Rogers SO, Bendich AJ (1985) Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol Biol 5: 69-76

Saedler R, Baldwin IT (2004) Virus-induced gene silencing of jasmonate-induced direct defences, nicotine and trypsin proteinase-inhibitors in Nicotiana attenuata. J Exp Bot 55: 151-157

Santner A, Estelle M (2007) The JAZ proteins link jasmonate perception with transcriptional changes. Plant Cell 19: 3839-3842

Santner A, Estelle M (2009) Recent advances and emerging trends in plant hormone signalling. Nature 459: 1071-1078

Shinya T, Gális I, Narisawa T, Sasaki M, Fukuda H, Matsuoka H, Saito M, Matsuoka K (2007) Comprehensive analysis of glucan elicitor-regulated gene expression in tobacco BY-2 cells reveals a novel MYB transcription factor involved in the regulation of phenylpropanoid metabolism. Plant Cell Physiol 48: 1404-1413

Sønderby IE, Hansen BG, Bjarnholt N, Ticconi C, Halkier BA, Kliebenstein DJ (2007) A systems biology approach identifies a R2R3 MYB gene subfamily with distinct and overlapping functions in regulation of aliphatic glucosinolates. PLoS ONE 2: e1322

Stamp N (2003) Out of the quagmire of plant defense hypotheses. Quart Rev Biol 78: 23-55

Steppuhn A, Baldwin IT (2007) Resistance management in a native plant: nicotine prevents herbivores from compensating for plant protease inhibitors. Ecol Lett 10: 499-511

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

Stracke R, Werber M, Weisshaar B (2001) The R2R3-MYB gene family in Arabidopsis thaliana. Curr Opin Plant Biol 4: 447-456

Sudha G, Ravishankar GA (2002) Involvement and interaction of various signaling compounds on the plant metabolic events during defense response, resistance to stress factors, formation of secondary metabolites and their molecular aspects. Plant Cell Tiss Org 71: 181-212

Tebayashi SI, Horibata Y, Mikagi E, Kashiwagi T, Mekuria DB, Dekebo A, Ishihara A, Kim CS (2007) Induction of resistance against the leaf miner, Liriomyza trifolii, by jasmonic acid in sweet pepper. Biosci Biotech Biochem 71: 1521-1526

Teng S, Keurentjes J, Bentsink L, Koornneef M, Smeekens S (2005) Sucrose-specific induction of anthocyanin biosynthesis in Arabidopsis requires the MYB75/PAP1 gene. Plant Physiol 139: 1840-1852

Tohge T, Nishiyama Y, Hirai MY, Yano M, Nakajima J-i, Awazuara M, Inoue E, Takahashi H, Goodenowe DB, Kitayama M, Noji M, Yamazaki M, Saito K (2005) Functional genomics by integrated analysis of metabolome and transcriptome of Arabidopsis plants over-expressing an MYB transcription factor. Plant J 42: 218-235

Tong Geon L, Cheol Seong J, Jae Yoon K, Dong Sub K, Jae Han P, Dae Yeon K, Yong Weon S (2007) A Myb transcription factor (TaMyb1) from wheat roots
is expressed during hypoxia: roles in response to the oxygen concentration in root environment and abiotic stresses. Physiol Plant 129: 375-385

Vailleau F, Daniel X, Tronchet M, Montillet J-L, TriantaphylidÁ’s C, Roby D (2002) A R2R3-MYB gene, AtMYB30, acts as a positive regulator of the hypersensitive cell death program in plants in response to pathogen attack. Proc Natl Acad Sci USA 99: 10179-10184

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

Wada N, Shinozaki M, Iwamura H (1994) Flower induction by polyamines and related compounds in seedlings of morning glory (Pharbitis nil cv. Kidachi). Plant Cell Physiol 35: 469-472

Walling, LL (2009) Adaptive Defense Responses to Pathogens and Insects. In LC Van Loon, eds, Advances in Botanical Research: Plant innate immunity, Vol 51. Elsevier Ltd., London, pp 551-612

Walters D, Cowley T, Mitchell A (2002) Methyl jasmonate alters polyamine metabolism and induces systemic protection against powdery mildew infection in barley seedlings. J Exp Bot 53: 747-756

Walters DR (2003) Polyamines and plant disease. Phytochem 64: 97-107

Wang CY, Chen C-T, Wang SY (2009) Changes of flavonoid content and antioxidant capacity in blueberries after illumination with UV-C. Food Chem 117: 426-431

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

Wilkins O, Nahal H, Foong J, Provat NJ, Campbell MM (2009) Expansion and diversification of the Populus R2R3-MYB family of transcription factors. Plant Physiol 149: 981-993

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

Wyss-Benz M, Streit L, Ebert E (1990) Feruloylputrescine and caffeoylputrescine are not involved in growth and floral bud formation of stem explants from Nicotiana tabacum L. var Xanthi nc. Plant Physiol 92: 924-930

Yan J, Zhang C, Gu M, Bai Z, Zhang W, Qi T, Cheng Z, Peng W, Luo H, Nan F, Wang Z, Xie D (2009) The Arabidopsis CORONATINE INSENSITIVE1 protein is a jasmonate receptor. Plant Cell 21: 2220-2236

Zangerl, AR (2003) Evolution of induced plant responses to herbivores. Basic Appl Ecol 4: 91-103

Zhang Y, Cao G, Qu L-J, Gu H (2009) Involvement of an R2R3-MYB transcription factor gene AtMYB118 in embryogenesis in Arabidopsis. Plant Cell Rep 28: 337-346

Zhao J, Davis LC, Verpoorte R (2005) Elicitor signal transduction leading to production of plant secondary metabolites. Biotech Adv 23: 283-333

Zhao J, Zhang W, Zhao Y, Gong X, Guo L, Zhu G, Wang X, Gong Z, Schumaker KS, Guo Y (2007) SAD2, an importin -like protein, is required for UV-B response in Arabidopsis by mediating MYB4 Nuclear Trafficking. Plant Cell 19: 3805-3818
FIGURES LEGENDS

Figure 1
*NaMYB8* responds differentially to *Manduca sexta* OS-elicitation in *N. attenuata.*

(A) Technical replicate means (± SE) of *NaMYB8* transcript relative abundances quantified with RT-qPCR from pooled samples using 5 independent control, W+W- and W+OS-elicited plants. Inset shows a detail of rapid transient accumulation of transcripts between 15 and 120 min after elicitation. (B) Mean (± SE) levels of *NaMYB8* transcripts in unelicited and 1h W+OS-elicited local leaves of two independent homozygous ir-MYB8 and WT plants (n=3). Asterisks represent significantly different transcript abundances between genotypes within same treatment group at $P < 0.001$ (***; nd = not detected).

Figure 2
*NaMYB8* regulates the accumulation of CP and DCS in *M. sexta* attacked leaves.

*M. sexta* caterpillars were allowed to feed on *N. attenuata* WT and *NaMYB8* transgenic plants for 4 days before harvesting samples for analysis. (A) HPLC-chromatograms obtained from WT and ir-MYB-810 *M. sexta*-fed rosette leaf methanolic extracts detected at 254 nm wavelength. (B) Mean (± SE) concentration of CP, (C) DCS, (D) CGA, (E) rutin, (F) nicotine and (G) diterpene glycosides (DTGs) in WT, ir-MYB8-818 and ir-MYB8-810 leaves directly attacked by herbivores quantified by HPLC (nd = not detected). Asterisks represent significant differences amongst the genotypes within the treatment group at $P < 0.001$ (***; n = 5; FM, fresh mass).

Figure 3
Young systemically induced *N. attenuata* leaves and reproductive tissues accumulate high levels of CP upon OS-elicitation of rosette leaves.

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 three days later the samples from representative plant parts were collected and analyzed by HPLC. The dotted arrow shows the position of the locally W+OS-elicited rosette leaf at different stages of development. *NaMYB8* transcripts, shown in gray color as inset figure, were analyzed from sample tissues used for determination of CP at elongated stage. 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; 1SL, 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; nd = not detected; FM, fresh mass.

Figure 4
NaMYB8-silenced plants show normal growth and reproductive fitness. (A) CP and (B) DCS contents in reproductive tissues of WT and ir-MYB8-810 transgenic lines determined by HPLC; nd = not detected; B, flower buds; EB, elongating flower buds; F, open flowers; GC, green capsules with seeds. (C) Means (± SE) of stalk lengths measured throughout the plant development in the glasshouse, starting from day 41 after germination of plants. Asterisks represent significantly different growth parameters between WT and two ir-MYB8 homozygous lines at specific time points at P < 0.01 (**) and P < 0.001(***; n = 12). (D) Mean (± SE) lifetime seed capsules produced by plants determined 98 days after germination; no statistically significant differences were observed. (E) Schematic representation of the seed capsule position on the uppermost lateral branch used for seed mass determinations: T0, seed capsule located nearest to the branching point on the top most lateral branch; T1, most distal seed capsule on the same branch. (F) Mean (± SE) seed mass in representative seed capsules at T0 and T1 positions measured in 98 day-old plants; no statistically significant differences were observed.

Figure 5
Silencing NaMYB8 in N. attenuata makes plants vulnerable to insect herbivores. (A) Mean (± SE) of CP and DCS accumulation in locally W+OS-induced rosette leaves and systemically induced young stem leaves in N. attenuata analyzed 3 days after induction (n = 4); control plants remained unelicited. Neither CP nor DCS were detected (nd) in ir-MYB8 plants. (B) A pattern wheel-wounded rosette leaf of WT plants was treated either with OS-specific elicitor C18:3-glutamic acid dissolved in 0.02% tween or was treated with 0.02% tween (mock treatment), and both local and systemic accumulation of CP and DCS were analyzed as above. (C) Mean (± SE) mass gained by N. attenuata generalist herbivore (S. littoralis) and specialist herbivore (M. sexta) caterpillars when fed on systemically pre-induced young stem leaves of ir-MYB8-810 and WT plants, whose rosette leaves were elicited with C18:3-glutamic acid (FAC). A single rosette leaf was elicited every 4-th day to
enhance the CP accumulation in the young stem leaves. Asterisks represent significantly different growth responses of herbivores that fed WT and homozygous ir-MYB8-810 plants at specific time points at $P < 0.01$ (** and $P < 0.001$ (**; $n = 16$; FM, fresh mass).

**Figure 6**

CP functions as indispensable part of direct defense against *M. sexta* in *N. attenuata*.

(A) Means ($\pm$ SE) concentrations of CP sprayed on ir-MYB8-810 leaves over a period of 48 hours. (B) Mean ($\pm$ SE) *M. sexta* caterpillar mass gain 4 days after feeding on CP-sprayed or water-sprayed ir-MYB8-810 leaves. Asterisk represents significantly different *M. sexta* caterpillars growth responses between the treatments at $P < 0.05$ (*; $n = 16$; FM, fresh mass).

**Figure 7**

NaMYB8-regulated metabolic pathways in *N. attenuata* plants.

NaMYB8 transcription factor controls transcriptionally the accumulation of CP and DCS metabolites in *N. attenuata* plants; NaMYB8-dependent genes determined by microarray analysis are enclosed in rectangles, PAL, phenylalanine ammonia lyase; C4H, cinnamic acid-4-hydroxylase, C3H, cinnamic acid-3-hydroxylase; 4CL, 4-coumaroyl-CoA: ligase; CHS, chalcone synthase; HQT, hydroxycinnamoyl-CoA quinate transferase; SPS, spermidine synthase; ADC, arginine decarboxylase; ODC, ornithine decarboxylase; PMT, putrescine methyltransferase; COMT, caffeic acid O-methyltransferase; CoA, coenzyme A.
SUPPLEMENTAL MATERIALS LEGENDS

Supplemental Figure S1
Deduced NaMYB8 protein sequence aligned with its homolog from *N. tabacum* (NtMYBJS1).

Sequence of NaMYB8 gene was originally obtained using PCR and cDNA template from *N. attenuata*, and primers designed according to NtMYBJS1 coding sequence. 3’-end of NaMYB8 gene was obtained by 3’RACE, and sequence was finally verified using 454-new generation sequencing of cDNAs from *N. attenuata*. The two R2R3-MYB repeats are highlighted in gray with dashed outlined boxes.

Supplemental Figure S2
The map of NaMYB8 transformation vector pSOL8MYB8 and Southern blot analysis of the two independently transformed ir-MYB8 lines.

(A) Map of the transformation vector pSOL8MYB8. (B) Southern blot analysis of two independently transformed ir-MYB8 lines. A 10 µg of genomic DNA from each genotype was digested with EcoRI and Hind III and the hybridization was performed with 32P-labeled *hptII* (*hygromycin* phosphotransferase) gene probe. Both lines harbored a single insertion of *hptII* gene, indicating that these transformed lines contained only a single copy of the T-DNA insertion. (C) Southern blot analysis with NaMYB8-specific probe shows that NaMYB8 is present as a single copy gene in *N. attenuata*’s genome.

Supplemental Figure S3
NaMYB8 is required for the accumulation of CP and DCS in *M. sexta* attacked leaves.

(A) Mean (± SE) silencing efficiency of NaMYB8 in VIGS-silenced plants. The concentrations of CP (B), DCS (C), CGA (D), rutin (E), nicotine (F) in the EV-VIGS and NaMYB8-VIGS leaves that were directly attacked by *M. sexta* caterpillars for 4 days were determined by HPLC coupled to a PDA detector. Asterisks represent significantly different concentrations of secondary metabolites accumulated between the EV-VIGS and NaMYB8-VIGS plants after similar treatments at P < 0.05 (*) and P < 0.01 (**: n = 5; FM, fresh mass). Inset represents the UV spectra of CP and DCS.
Supplemental Figure S4

**Silencing NaMYB8 does not change W+OS-elicited phytohormone concentrations in the leaves.**

A rosette leaf in (+1) position was wounded with a pattern wheel and 20 µL of either water or OS was applied to the wounds. Means (± SE) represent JA levels in (A) stably-silenced ir-MYB8 plants and (D) transiently-silenced NaMYB8-VIGS plants; JA-Ile levels in (B) stably-silenced and (E) transiently-silenced NaMYB8-VIGS plants, in the leaves harvested at indicated time points determined with LC-MS/MS (n = 5). The closed symbols represent W+W treatments and open symbols represent W+OS treatments. Mean (± SE) ethylene (ET) emissions from W+OS treated leaves of (C) stably-silenced ir-MYB8 plants and (F) transiently-silenced NaMYB8-VIGS plants, enclosed in 250-mL flasks and measured after 5 h with a photoacoustic laser spectrometer (n = 3; FM, fresh mass).

Supplemental Figure S5

**M. sexta caterpillars perform better on NaMYB8-VIGS relative to the EV-VIGS plants.**

*M. sexta* neonates were placed on the stem leaf of either NaMYB8-VIGS or EV-VIGS plants and were allowed to feed without restricting the movement of caterpillars. Initial *M. sexta*’s caterpillars mass was recorded on 4th day of feeding, followed by recording mass every second day of the experiment (P < 0.05 (*); n = 15).

Supplemental Table S1

Silencing *NaMYB8* suppresses the accumulation of CP and DCS by down-regulating the expression of genes involved in phenylpropanoid and polyamine metabolism.
**Figure 1**

*NaMYB8* responds differentially to *Manduca sexta* OS-elicitation in *N. attenuata*.

(A) Technical replicate means (± SE) of *NaMYB8* transcript relative abundances quantified with RT-qPCR from pooled samples using 5 independent control, W+W- and W+OS-elicited plants. Inset shows a detail of rapid transient accumulation of transcripts between 15 and 120 min after elicitation. (B) Mean (± SE) levels of *NaMYB8* transcripts in unelicited and 1h W+OS-elicited local leaves of two independent homozygous ir-MYB8 and WT plants (n=3). Asterisks represent significantly different transcript abundances between genotypes within same treatment group at *P* < 0.001 (***) ; nd = not detected.)
Figure 2

NaMYB8 regulates the accumulation of CP and DCS in *M. sexta* attacked leaves.

*M. sexta* caterpillars were allowed to feed on *N. attenuata* WT and *NaMYB8* transgenic plants for 4 days before harvesting samples for analysis. (A) HPLC-chromatograms obtained from WT and ir-MYB-810 *M. sexta*-fed rosette leaf methanolic extracts detected at 254 nm wavelength. (B) Mean (± SE) concentration of CP, (C) DCS, (D) CGA, (E) rutin, (F) nicotine and (G) diterpene glycosides (DTGs) in WT, ir-MYB8-818 and ir-MYB8-810 leaves directly attacked by herbivores quantified by HPLC (nd = not detected). Asterisks represent significant differences amongst the genotypes within the treatment group at P < 0.001 (***; n = 5; FM, fresh mass).
M. sexta’s oral secretions (OS) elicited rosette leaf

---

CP (µg g⁻¹ FM⁻¹)

---

Early elongated

---

Elongated

---

Flowering

---

Mature

---

WT Control

WT W+OS

---

Buds (B)

Elongating buds (EB)

Green capsules (GC)

Ripe capsules (RC)

Flowers (F)

Young leaf (YL)

First stem leaf (1SL)

Local leaf (LL)

Systemic leaf (SL)

Old leaf (OL)

Stem basal (SB)

Stem upper (SU)

Senescent leaf (SeL)

Roots (R)

---

*Figure description:*

- **CP (µg g⁻¹ FM⁻¹):** Graph showing the concentration of CP in different plant parts.
- **Early elongated:** Graph displaying elongation stages.
- **Elongated:** Graph showing elongation stages.
- **Flowering:** Graph illustrating flowering stages.
- **Mature:** Graph depicting maturation stages.

---

*Legend:*

- WT Control
- WT W+OS

*Note:*

- The graph labels indicate various stages of plant development and growth, highlighting the impact of M. sexta’s oral secretions on different plant parts.

---

*Source:*

- www.plantphysiol.org (August 13, 2017) - Published by www.plantphysiol.org.
Figure 3
Young systemically induced N. attenuata leaves and reproductive tissues accumulate high levels of CP upon OS-elicitation of rosette leaves.

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 three days later the samples from representative plant parts were collected and analyzed by HPLC. The dotted arrow shows the position of the locally W+OS-elicited rosette leaf at different stages of development. NaMYB8 transcripts, shown in gray color as inset figure, were analyzed from sample tissues used for determination of CP at elongated stage. 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; 1SL, 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; nd = not detected; FM, fresh mass.
NaMYB8-silenced plants show normal growth and reproductive fitness.

(A) CP and (B) DCS contents in reproductive tissues of WT and ir-MYB8-810 transgenic lines determined by HPLC; nd = not detected; B, flower buds; EB, elongating flower buds; F, open flowers; GC, green capsules with seeds. (C) Means (± SE) of stalk lengths measured throughout the plant development in the glasshouse, starting from day 41 after germination of plants. Asterisks represent significantly different growth parameters between WT and two ir-MYB8 homozygous lines at specific time points at P < 0.01 (**) and P < 0.001(***; n = 12). (D) Mean (± SE) lifetime seed capsules produced by plants determined 98 days after germination; no statistically significant differences were observed. (E) Schematic representation of the seed capsule position on the uppermost lateral branch used for seed mass determinations: T0, seed capsule located nearest to the branching point on the top most lateral branch; T1, most distal seed capsule on the same branch. (F) Mean (± SE) seed mass in representative seed capsules at T0 and T1 positions measured in 98 day-old plants; no statistically significant differences were observed.
Intracellular expression of AvrMYB8 transgene in Arabidopsis responses to W+OS and W+FAC treatments.

A. CP and DCS were not detected in ir-MYB8 plants.

Day 0 = (+1) rosette leaf
Day 1 = (2nd) stem leaf

CP (µg g FM⁻¹) #

Control W+OS Control W+OS
T0 (local) T1 (systemic)

DCS (µg g FM⁻¹)

Control W+OS Control W+OS
T0 (local) T1 (systemic)

B. W+FAC (C18:3-Glutamic acid) locally induced rosette leaf (position)

Day 0 (+3) (+2) (+1)

CP (µg g FM⁻¹)

W+Tween W+FAC W+Tween W+FAC
T0 (local) T1 (systemic)

DCS (µg g FM⁻¹)

W+Tween W+FAC W+Tween W+FAC
T0 (local) T1 (systemic)

C. W+FAC (C18:3-Glutamic acid) locally induced rosette leaf (position)

Day 0 (+3) (+2) (+1)

2nd instar feeding on systemically induced young leaves

Neonate feeding on systemically induced young leaves

Caterpillar mass (g)

WT ir-MYB8-810

Spodoptera littoralis

Manduca sexta

Days after hatching
Figure 5
Silencing NaMYB8 in *N. attenuata* makes plants vulnerable to insect herbivores.

(A) Mean (± SE) of CP and DCS accumulation in locally W+OS-induced rosette leaves and systemically induced young stem leaves in *N. attenuata* analyzed 3 days after induction (n = 4); control plants remained unelicited. Neither CP nor DCS were detected (nd) in ir-MYB8 plants. (B) A pattern wheel-wounded rosette leaf of WT plants was treated either with OS-specific elicitor C18:3-glutamic acid dissolved in 0.02% tween or was treated with 0.02% tween (mock treatment), and both local and systemic accumulation of CP and DCS were analyzed as above. (C) Mean (± SE) mass gained by *N. attenuata* generalist herbivore (*S. littoralis*) and specialist herbivore (*M. sexta*) caterpillars when fed on systemically preinduced young stem leaves of ir-MYB8-810 and WT plants, whose rosette leaves were elicited with C18:3-glutamic acid (FAC). A single rosette leaf was elicited every 4-th day to enhance the CP accumulation in the young stem leaves. Asterisks represent significantly different growth responses of herbivores that fed WT and homozygous ir-MYB8-810 plants at specific time points at P < 0.01 (**) and P < 0.001 (***, n = 16; FM, fresh mass).
Figure 6
CP functions as indispensable part of direct defense against *M. sexta* in *N. attenuata*.

(A) Means (± SE) concentrations of CP sprayed on ir-MYB8-810 leaves over a period of 48 hours. (B) Mean (± SE) *M. sexta* caterpillar mass gain 4 days after feeding on CP-sprayed or water-sprayed ir-MYB8-810 leaves. Asterisk represents significantly different *M. sexta* caterpillars growth responses between the treatments at P < 0.05 (*; n = 16; FM, fresh mass).
Figure 7
NaMYB8-regulated metabolic pathways in *N. attenuata* plants.
NaMYB8 transcription factor controls transcriptionally the accumulation of CP and DCS metabolites in *N. attenuata* plants; NaMYB8-dependent genes determined by microarray analysis are enclosed in rectangles, PAL, phenylalanine ammonia lyase; C4H, cinnamic acid-4-hydroxylase, C3H, cinnamic acid-3-hydroxylase; 4CL, 4-coumaroyl-CoA: ligase; CHS, chalcone synthase; HQT, hydroxycinnamoyl-CoA quinate transferase; SPS, spermidine synthase; ADC, arginine decarboxylase; ODC, ornithine decarboxylase; PMT, putrescine methyltransferase; COMT, caffeic acid O-methyltransferase; CoA, coenzyme A.