Jasmonic acid and ethylene crosstalk: Regulation of growth, defense and metabolisms in native tobacco *Nicotiana attenuata* in response to herbivory

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Abbreviations

1-MCP	1-methylcyclopropane
4-CL	4-coumarate: coenzyme A ligase
as	antisense silencing
ABA	abscisic acid
cDNA	complementary deoxyribonucleic acid
CoA	coenzyme A
CP	caffeoyl putrescine
CS	caffeoyl spermidine
d	day
DCS	dicaffeoyl spermidine
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra aectic acid
ET	ethylene
ETR1	*ETHYLENE RESPONSE1*
EV	empty vector
EXP	expansins
FAC	fatty acid amino acid conjugated
HCA	hydroxycinnamic acid
HCAAs	hydroxycinnamic acid amides
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
IAA	indole-3-acetic acid, auxin
ir	inverted-repeat silencing
JA	jasmonic acid
LC	liquid chromatography
LC-TOFMS	liquid chromatography-tandam mass spectrometry
LOX3	lipoxygenase3
m/z	mass-to-charge ratio
MCS	monocaffeoyl spermidine
MeJA	methyl jasmonate
MeOH	methanol

mRNA	messenger ribonucleic acid
MS	mass spectrometry
NO	nitric oxide
OS	oral secretion
PAGE	polyacrylamide gel electrophoresis
PAL	phenylalanine-ammonia-lyase
PCR	polymerase chain reaction
PDA	photodiode array
PME	pectin methylesterase
RT	retention time
RT-qPCR	real time quantitative polymerase chain reaction
SDS	sodium dodecyl sulphate
VIGS	virus induced gene silencing
WT	wild type

1. Introduction

1.1 Plant hormones: key regulators of growth, development and defense

Plant hormones or phytohormones are small signaling molecules that function in regulating growth, development, and defense against abiotic and biotic stresses in plants. Naturally, phytohormones are synthesized in very small quantities, which are perceived by specific receptors and generated signals are transduced by complex signaling pathways to control downstream gene expression and translation machineries in the plant cells.

Functioning as growth regulators, phytohormones are often found in young developing tissues such as root tips, young leaves, shoot meristems and flowers. Exposure to biotic (pathogens, arthropod herbivores, mammals) or abiotic (high temperature, salinity, UV-irradiation, etc.) stresses makes plants increase the production of specific class of hormones related to plant defenses, which act as specific signals to activate plant defense against particular stress. While generally divided into developmental and defense hormones, the interactions between these two classes of regulators, often mediating very contradictory trends in plant behavior, are inevitable. As one essential feature, both growth and defense depend and therefore must compete for the same, often limited plant resources.

There are more than one million insect species that feed on plants (herbivores; Howe and Jander, 2008). Therefore, it is crucial for plants to have effective and diverse defense mechanisms to protect themselves from such a variety of potential enemies. Moreover, plants require sophisticated regulatory systems to control the elicitation of proper defenses, and phytohormone networks are considered to be an important part of these systems. Amongst various hormones, jasmonates and ethylene are two main hormone groups which well documented function in regulating plant defense against insect herbivores.

1.1.1 Jasmonates

Jasmonate group of plant hormones consists of jasmonic acid (JA) and other JA-derivates, such as methyl jasmonate (MeJA), cis-jasmonate, and JA-amino acid conjugates. Linolenic acid is converted by several enzymes into 12-oxophytodienoate (12-OPDA) in the chloroplasts, which is later transported to

peroxisomes where JA biosynthesis is completed (Delker et al., 2006; Delker et al., 2007; Wasternack, 2007). Physical damage can increase JA production; however, plants can distinguish herbivore attack from the mechanical tissue damage, eliciting much larger amounts of JA within an hour after herbivore attack, resulting in so called JA-burst (McCloud and Baldwin, 1997; Ziegler et al., 2001; Schmelz et al., 2003, Stork et al., 2009).

To activate herbivory defense, a specific JA-derivate, JA-Ile (JA conjugated to isoleucine) is required to bind to CORONATINE INSENSITIVE 1 (COI1) receptor protein (Yan et al., 2009). After binding to JA-Ile, COI1 interacts with JAZ repressor proteins (named after jasmonate-ZIM-domain) that normally form inhibitory complexes with the downstream transcription factors responsible for regulation of JA-responsive genes. Once counteracted by COI1/JA-Ile complex, JAZ proteins become ubiquitinated and degraded in 26S proteasome complex, which then relieves the negative pressure on transcription factors, which then bind to JA-regulated defense genes, eliciting defense against insect herbivores: production of toxic secondary metabolites, proteinase inhibitors and volatile organic compounds (Kessler and Baldwin, 2002; Halitschke and Baldwin, 2003; Zavala and Baldwin, 2006; Chini et al., 2007; Thines et al., 2007; Wasternack, 2007; Browse and Howe, 2008; Howe and Jander, 2008). Apart from its function in regulating plant defense, JA and its derivates are known to function as regulators of plant growth and development: for instance, root growth, leaf expansion, and flower and tuber development (Creelman and Mullet, 1995; Wasternack 2007; Zhang and Turner, 2008).

1.1.2 Ethylene

Ethylene (ET) can be considered as the only known gaseous plant hormone, while the role of nitric oxide (NO) as plant hormone has not yet been fully established. ET has been well characterized as a potent modulator of plant growth and development at various stages of plant life-cycle, including seed germination, root hair development, flower development, fruit ripening and leaf senescence (Wang et al., 2002 ; Binder et al., 2004; Chen et al., 2005; Yang et al., 2008). It has been shown that ET functions in plant resistance to necrotrophic pathogens, a pathway normally regulated by JA and therefore overlapping with the plant defense against herbivores (Lund et al., 1998; Penninckx et al., 1998; Knoester et al., 2001;

van Loon et al., 2006; Kavroulakis et al., 2007; Van der Ent et al., 2009). S-adenosylmethionine (S-AdoMet), a biosynthetic precursor of ET, is converted to ET by two enzymes, 1-aminocyclopropane-1-carboxylate synthase (ACS) and 1-aminocyclopropane-1-carboxylate oxidase (ACO) (Yang and Hoffman., 1984; Kende, 1993; Wang et al., 2002).

Plants perceive ET by multiple protein receptors. Interestingly, a specific dominant mutation in just one of the ET-receptors can make plants completely unable to perceive ET (ET-insensitive), and these plants even overproduce ET due to a defective feedback loop that normally controls ET-biosynthesis. Ethylene insensitive genotypes have been extensively used to elucidate the biological functions of ET in plants (Bleecker et al., 1988; Chang et al., 1993; Wilkinson et al., 1997; Cui et al., 2004; von Dahl et al., 2007). Binding of ET to its receptors inactivates a negative regulator of ET signaling, *CTR1,* which allows transcription of downstream genes regulating ET responses (Ecker, 1995; Alonso and Stepanova, 2004; Klee, 2004; Chen et al., 2005; Stepanova and Alonso, 2009). ET has been shown to modulate plant defense against herbivores by co-regulating production of toxic metabolites, for example nicotine, together with the main inducer of anti-herbivore defenses; JA (Kahl et al., 2000; Shoji et al., 2000; Winz and Baldwin, 2001).

1.1.3 JA-ET crosstalk

JA- and ET-bursts after herbivore attack occur in an intrigue overlapping manner, suggesting that JA-ET crosstalk could be strongly involved in regulating plant responses to herbivores. O'Donnell et al. (1996) reported that ET was required to optimize the expression of *proteinase inhibitor (pin)* gene that is strongly up-regulated by JA in tomato. Recently, Lorenzo et al. (2003) reported that JA and ET signaling pathways interconnect through *ETHYLENE REPOSIVE FACTOR 1 (ERF)* during plant response to pathogenic infections. These two examples indicate that the interaction between JA and ET involves in controlling plant defenses; however, it remains largely unknown whether the interactions between JA and ET in mediating plant defense against herbivores occur in mainly synergistic or antagonistic manner. ET showed positive effect on *pin* expression but ET seemed to suppress the expression of genes involved in nicotine biosynthesis, an alkaloid metabolite important for defense against herbivores in *Nicotiana* species (O'Donnell et al.,

1996; Shoji et al., 2000). Therefore, more studies are required to reveal the interactions between JA-ET in plant defense against herbivore before clear conclusions can be drawn.

1.2 Secondary metabolites: plant arsenals against attacking herbivores

Plants have developed a broad range of defenses against herbivores. Production of toxic metabolites, for example alkaloids, trypsine protease inhibitors, or glucosinolates is one of the common plant direct induced defense strategies. Terpenoids, alkaloids, and phenolic compounds are the most common groups of toxic metabolites that plants use for their defense (Harborne, 1999).

Hydroxycinnamic acid amides (HCAAs) are ubiquitous nitrogen-containing compounds that are synthesized from hydroxycinnamic acids (HCA) and polyamines in many plant species. There are two types of HCAAs: basic and neutral HCAAs, the basic forms having a free proton that may easily interact with other biomolecules, and changing their biological properties (Martin-Tanguy, 1985; Facchini et al., 2002; Edreva et al., 2007). HCAAs have first been proposed to function in plant growth and development, mainly because they preferentially accumulate in the young and reproductive tissues; however, no solid proof exists to support the growth regulatory function of HCAAs. Lately, several studies suggested that HCAAs might function as antiviral, antifungal and antioxidant agents in plants (Martin-Tanguy, 1985; Facchini et al., 2002; Walters et al., 2001; Walters, 2003; Edreva et al., 2007; Zacarés et al., 2007). Moreover, Kaur et al. (2010) has recently reported that HCAAs are indispensable for plant defense against herbivores.

Although HCAAs have been extensively studied at biochemical level, the information about signal transduction pathways and transcription factors that regulate their biosynthesis is still lacking. As HCAAs function in defense against herbivores, this information is essential for gaining novel insights and understanding of plant defense networks against herbivores.

1.3 Trade-off between growth and defense

Plants grow in very diverse environments and therefore, the encounters with herbivores are very common events in the plant's life cycle. To survive herbivore attack, plants developed sophisticated direct and indirect induced defense

mechanisms, discussed above, to fend-off herbivores. However, a major drawback of activating defense mechanisms is their cost. Plants have to often survive with very limited resources, and during each herbivore attack plants have to divert some of these resources from growth and development to their defense. It is vital for plants, which need to remain competitive and in acceptable fitness even during herbivore stress conditions, to tightly regulate their resource allocation between growth and defense, as essential feature for their survival.

JA-ET crosstalk represents one of the possible ways to regulate trade-off between growth and defense, mainly because both JA and ET are involved in control of both, growth and defense. Plant hormone crosstalk represents a powerful tool for plants to rapidly elicit the effective defenses in an economical manner. The models involving phytohormone crosstalk to regulate the trade-off between growth and defense have already been suggested by several studies that mainly involved plant-pathogen interactions (Heil, 2002; Walters and Boyle, 2005; Walters and Heil, 2007). Although several studies showed that plant growth appears to be negatively affected by herbivore attack (Baldwin, 1998; Redman et al., 2001; Zavala and Baldwin, 2004; Zavala et al., 2004), it remains to be determined how JA-ET crosstalk influences plant fitness and growth during herbivory.

1.4 Nicotiana attenuata: a model system for ecological studies

To understand JA-ET crosstalk and its function in redirecting resource allocation between growth and defense during herbivory stress, a suitable ecological model system with developed molecular toolbox for genetic manipulations is highly desirable. *N. attenuata* has been now studied at Max Planck Institute for Chemical Ecology for over a decade, having well known physiology and highly developed molecular toolbox, including construction of plants silenced in target gene expression. Importantly, it also produces large amounts of JA and ET in response to herbivore attack, and the plants respond differentially to herbivore elicitors found in the oral secretions of attacking larvae. These elicitors have been identified as fatty acid amino acid conjugates (FACs) in case of *N. attenuata* specialist herbivore *Manduca sexta*.

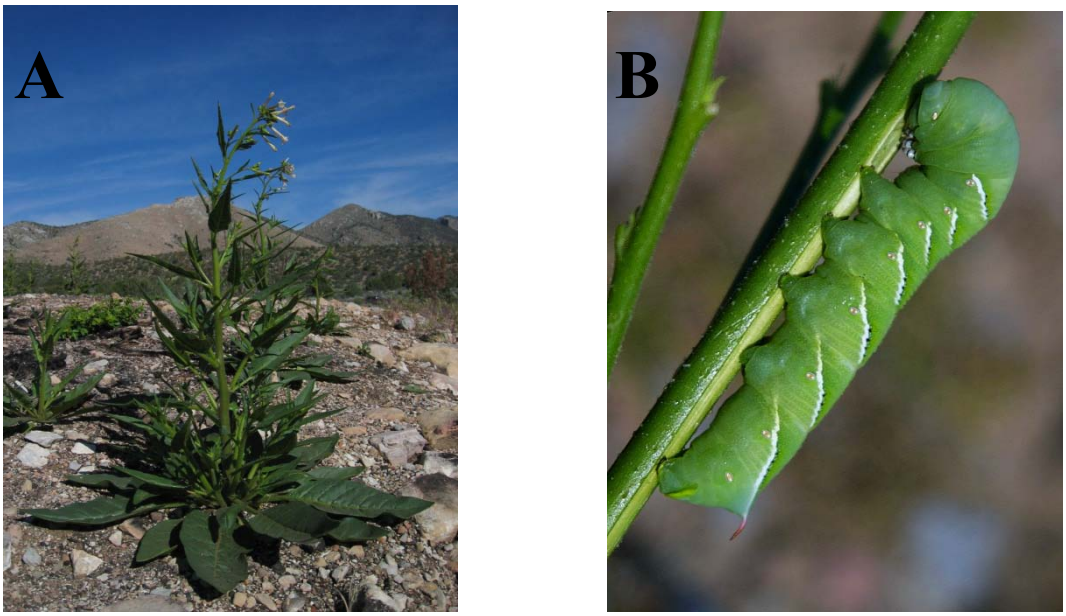


Figure 1 *Nicotiana attenuata* plants in their natural habitat (Great Basin Desert, USA) (A) and one of *N. attenuata*'s most damaging natural enemies, herbivore *Manduca sexta* (B). Photo courtesy: Danny Kessler and Celia Diezel

The tobacco species *N. attenuata* Torr.ex Watson (synonymous to *Nicotiana torreyana*) is a native plant found for example in the Great Basin Desert, USA. The seeds of *N. attenuata* synchronically germinate in the nitrogen-rich post-fire environments, responding to specific germination cues contained in wood smoke (Baldwin and Morese, 1994). Due to the short generation time, diploid genome and self-pollination character, *N. attenuata* plants represent an excellent model for molecular and ecological studies. In the natural habitat, more than 20 taxa of herbivores are known to feed on *N. attenuata* plants. Amongst them, tobacco hornworms (*M. sexta*; Lepidoptera, Sphingidae), are one of the most damaging herbivores in *N. attenuata*. JA and ET-burst are the two events that precede elicitation of direct and indirect induced defense responses against herbivores in *N. attenuata* plants (Kahl et al., 2000; Halitschke and Baldwin, 2003; Kang et al., 2006; Pluskota et al., 2007; von Dahl et al., 2007; Wu et al., 2007), and transgenic plants defective in both of these traits have recently become available. Moreover, transcritopmic and metabolomic information for *N. attenuata* has been rapidly accumulating in the last years, assisting the reconstruction of novel connections between hormone signals, gene expression and physiology/ecology of *N. attenuata* plants.

1.5 Objectives of the study

Herbivore attack is a common biotic stress in plants, which therefore developed effective induced defenses to repel the herbivores. The question of how plants manage and balance the allocation of their resources between growth and defense has been long asked. Many studies demonstrated that during stress conditions, plants tend to grow less than during normal conditions; however, the regulatory mechanisms behind these observations remain elusive.

The existence of plant hormone crosstalk has been a center point of attention for many years. Based on current understanding, phytohormone crosstalk is a very complex signaling event that involves receptors, transcription factors and downstream regulated genes, integrated in crosstalk signaling output. In plant response to herbivores, JA-ET crosstalk is likely to gain more interest in the future, in particular as part of complex mechanisms that regulate plant growth during defense. In addition, current data suggest that auxin (plant growth hormone) receptor shares strong similarity to JA receptor (*COI 1*), indicating a possible evolutionary connection between these two plant signals and therefore close proximity of plant growth and defense processes.

The aim of this study was to address the JA-ET crosstalk during herbivore stress, mainly its function in regulating plant growth and plant defense at the same time, and determine some of the molecular mechanisms underlining JA-ET crosstalk in *N. attenuata*. Specifically, two main objectives of this study have been outlined:

1) How does JA-ET crosstalk affect plant growth under herbivore stress, by characterizing morphological changes and determining possible molecular mechanisms responsible for morphological alteration in transformed plants that contain defects in JA and ET signaling pathways

2) How does JA-ET crosstalk affect the biosynthesis of plant secondary metabolites during herbivore attack, by determining the connectivity between JA-ET signal transduction pathways and molecular mechanisms that regulate biosynthesis of hydroxycinnamic acid amides in herbivore attacked plants

2. Crosstalk between jasmonic acid and ethylene regulates growth and morphogenesis during simulated herbivory in *Nicotiana attenuata*

2.1 Introduction

Herbivores substantially affect the accumulation of plant biomass and consequently threaten plants' survival and reproductive success in their natural environment. Plants have countered herbivore attack by evolving a suite of defense and tolerance responses, which has resulted in what is referred to as the "co-evolutionary arms race" in plant-herbivore interactions (Ehrlich and Raven, 1964; Kareiva, 1999). Even though essential for survival, defenses are thought to be costly for plants, negatively affecting their reproductive fitness in natural environments (Baldwin, 1998; Redman et al., 2001; Zavala and Baldwin, 2004; Zavala et al., 2004; Bostock 2005). It is likely that defense processes directly compete for plant resources allocated to growth and repair after mechanical stress (wounding), and plant defense-related signals are therefore thought to play important roles in optimizing a plant's resource allocation decisions.

It is well established that plant responses to biotic and abiotic stress stimuli are controlled by specific plant hormones (Pieterse et al., 2009); amongst them, jasmonic acid (JA) is largely responsible for mediating the elicitation of defenses against attack from herbivores and necrotrophic pathogens (Kessler and Baldwin, 2002; Turner et al., 2002; Weber, 2002; Wasternack, 2007). In addition to inducing plant defenses, jasmonates have been shown to play roles in growth and development, including flower development, tuber formation, tendril coiling, nyctinastic movements, trichome formation and senescence (Wasternack, 2007). While JA accumulation generally stimulates the above-mentioned processes, it inhibits root and leaf growth, and seed germination (Wasternack, 2007; Zhang and Turner, 2008). In *Arabidopsis*, JA biosynthesis is initiated by a wound-mediated release of α-linolenic acid (18:3) from chloroplastic membranes, followed by the activity of several chloroplast-located enzymes including 13-lipoxygenase (LOX) (Delker et al., 2006). Silencing of LOX3, a major enzyme responsible for JA biosynthesis in *Nicotiana attenuata*, has been shown to reduce JA levels and impair

both direct and indirect defenses in LOX3-silenced plants (Halitschke and Baldwin, 2003), providing an excellent tool for examination of the jasmonate function in defense as well as its role in growth and development, especially in plants under herbivore attack.

Ethylene (ET) gas, a small molecule with hormonal activity in plants, is known to be involved in mediating plant defense responses against herbivores (O'Donnell et al., 1996; Stotz et al., 2000; Ludwig et al., 2005, and von Dahl et al., 2007). Like JA, ET is also required for normal plant growth and development, including germination, fruit ripening, senescence and regulation of cell expansion and elongation (Binder et al., 2004; Chen et al., 2005; Stepanova et al., 2007). In *Arabidopsis*, ET signal transduction is initiated by perception through multiple membrane-bound receptors ETR1, ETR2, ERS1, ERS2 and EIN4 (Ecker, 1995; Alonso and Ecker, 2001; Chen et al., 2005). Ectopic heterologous expression of the mutated *Arabidopsis* ET receptor, *etr1-1*, has been shown to dominantly repress ET signaling in several plant species, including tomato, petunia, *Nemesia* and the native tobacco, *N. attenuata* (Bleecker et al., 1988; Chang et al., 1993; Wilkinson et al., 1997; Cui et al., 2004; von Dahl et al., 2007). Recently, the ethylene-insensitive *N. attenuta* plants have been used to examine the role of ET and its crosstalk with JA in plant defense against specialist herbivore *Manduca sexta* (von Dahl et al., manuscript in preparation for 2010).

In contrast to the defense-related hormones JA and ET, auxin, indole-3-acetic acid (IAA), is a pleiotropic regulator of plant growth and development, primarily targeting cell division, cell expansion and cell elongation (Teale et al., 2006). The partially overlapping functions of auxin and JA-ET in controlling growth and development suggest that some of the plant responses to JA and ET could be mediated by controlling auxin concentrations and/or perception machinery (reviewed by Woodward and Bartel, 2005; Stepanova and Alonso, 2009). In several reports, ET has been shown to influence IAA biosynthesis (Stepanova et al., 2005; 2007; 2008; Ruzicka et al., 2007), sensitivity to IAA (Visser et al., 1996), and IAA transport in plants (Vandenbussche et al., 2003; Negi et al., 2008; Ivanchenko et al., 2008; Konieczny et al., 2009). These interactions indirectly point to the existence of an active crosstalk between JA, ET and IAA during plant responses to herbivores: JA and ET are known to rapidly accumulate in herbivore-attacked tissues, while the concentrations of IAA in the leaves rapidly decline after wounding (Thornburg and

Li, 1991), possibly due to effects of ET and JA on IAA metabolism.

As defense and growth are both essential for plants, the crosstalk between growth- and defense-related signals should be tightly controlled to allow efficient coordination of growth and development in the natural environment (Shoji et al., 2000; Rojo et al., 2003; Sun et al., 2006; von Dahl and Baldwin, 2007; Shishi, 2008; Koornneef and Pieterse, 2008). To examine more closely the hormone crosstalk in mechanically-wounded and herbivore-attacked plants, namely interactions between JA, ET and IAA, we used *N. attenuata* plants silenced in JA production (asLOX3) and ET perception (mETR1), and their genetic cross (mETR1asLOX3), and compared their growth and local response to wounding and simulated herbivore attack. The combination of JA-deficiency and ET-insensitivity resulted in a novel growth phenotype observed in mETR1asLOX3 plants, characterized by callus-like cell development around puncture wounds or wounds inflicted by *M. sexta* larvae feeding on the leaves. The emergence of callus-like structures in mETR1asLOX3 correlated with the elevation of free IAA levels in the leaves, suggesting that both JA and ET suppress IAA accumulation and thereby prevent callus-like cells formation and healing in wild type (WT) *N. attenuata* leaves after wounding and herbivore attack.

2.2 Results

2.2.1 Hemizygous mETR1asLOX3 cross combines the properties of both parental lines

Homozygous lines of the native tobacco *N. attenuata* silenced for *lipoxygenase3* (asLOX3) and lines ectopically expressing the mutated *ETHYLENE RESPONSE1* (mETR1) have been characterized previously (Halitschke and Baldwin, 2003; von Dahl et al., 2007). To examine the effects of JA and ET crosstalk, we created a new hemizygous cross between these two lines, which was designated in this study as mETR1asLOX3 (Fig. 1A). Because mETR1asLOX3 plants contained only a single allele of the parental transgenes, *asLOX3* and *mETR1*, we first compared the cross to original homozygous parental lines.

First, JA concentrations were determined after wounding the plants with a pattern wheel (W) and directly applying oral secretions (OS) from *M. sexta* larvae. The peak of JA accumulation occurred 1 h after elicitation in wild type (WT) and

mETR1 plants, reaching more than 1000 ng JA g^{-1} FM in both lines (Fig. 1B). In contrast, JA was found at much lower concentrations, ~ 200 g^{-1} FM in asLOX3 and in mETR1asLOX3 cross, suggesting that LOX3 silencing was still as effective in the cross as it was in the parental line (Fig. 1B).

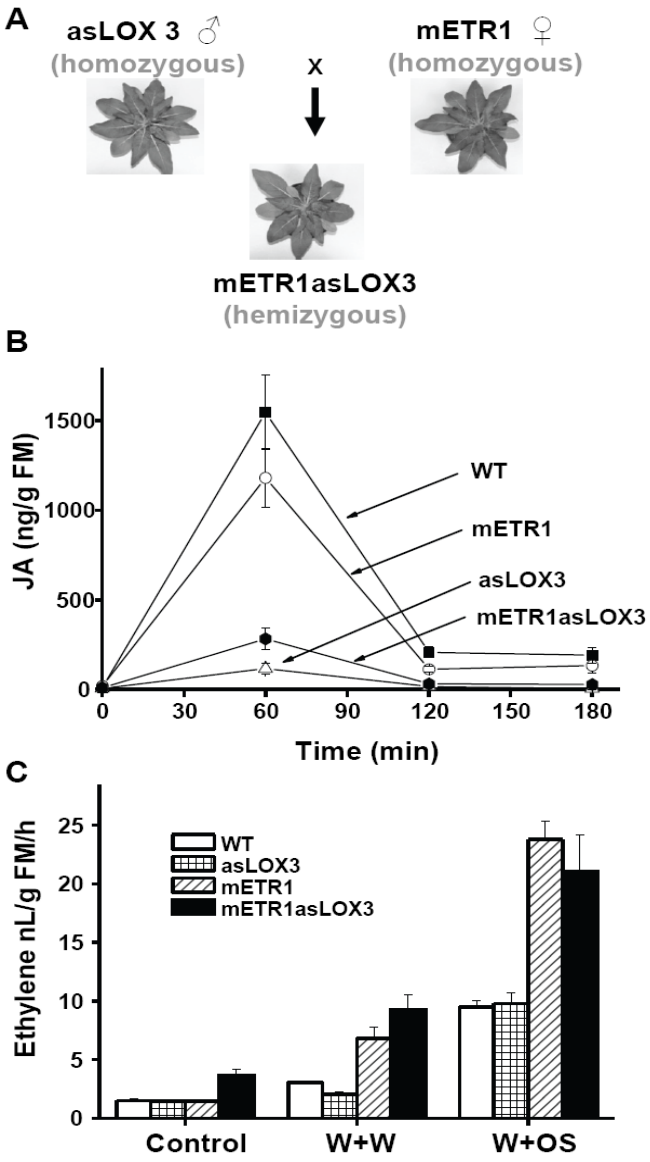


Figure 1: **Genetic cross between JA-deficient and ET-insensitive plants shows combined phenotypes of both parental lines.** (A) Homozygous mETR1 flowers were pollinated with asLOX3 pollen to produce a hemizygous mETR1asLOX3 cross. (B) JA levels in WT, mETR1, asLOX3 and mETR1asLOX3 at the indicated time points after wounding with a pattern wheel and applying oral secretions from *M. sexta* (W+OS treatment). (C) ET emissions from WT (open bars), mETR1 (striped), asLOX3 (checked) and mETR1asLOX3 (filled) cut leaves collected over 5 hour after wounding with a pattern wheel and applying water (W+W) or W+OS treatment; control leaves remained untreated. FM, fresh mass

ET accumulation is known to be controlled via ET perception and a feedback loop with ET biosynthesis (Kende, 1993; Zhang et al., 2009); ectopic expression of

the mutated ET receptors thus not only affects ET perception but also increases ET production due to the lack of feedback control, which is often used as an indirect indicator of ET-insensitive phenotypes (Wilkinson et al., 1997; von Dahl et al., 2007). Prior to elicitation, isolated leaves from all genotypes emitted less than 5 nL g^{-1} FM h^{-1} ethylene (Fig. 1C); wounding with a pattern wheel and directly applying water to the wounds (W+W) showed only moderate effect on ET production in WT and asLOX3, however ET increased to much higher levels in mETR1 and mETR1asLOX3 plants, suggesting that both genotypes are ET insensitive (Fig. 1C).

Replacing water with OS had a similar but much more pronounced effect on the ET emissions from wounded *N. attenuata* leaves (Fig. 1C), consistent with the previous reports of OS strongly potentiating the ET burst in *N. attenuata* (Kahl et al., 2000; von Dahl et al., 2007). In summary, despite containing only a single allele of the transgenes, mETR1asLOX3 hemizygous plants showed combined phenotypes, each of them comparable to the parental homozygous lines; we therefore used WT, asLOX3, mETR1 and mETR1asLOX3 plants to examine the crosstalk between JA and ET during wounding and herbivory.

2.2.2 Callus-like cells development in mETR1asLOX3 leaves after wounding and OS-elicitation

JA and ET have been previously implicated in regulation of plant growth and development (Pierik et al., 2006; Dugardeyn and Van der Straeten, 2008; Zhang and Turner, 2008); we investigated whether JA or ET, alone or together, could affect plant responses after wounding and/or simulated herbivory, using WT, mETR1, asLOX3 and mETR1asLOX3 plants. Surprisingly, we observed an extensive callus-like cells formation around the wounds, which was specific to mETR1asLOX3 plants (Fig. 2A). While simple wounding was sufficient to induce callus-like structures on the leaves (see margins on the leaf discs in Fig. 3A), simulated herbivory treatment, which is reportedly associated with significantly higher levels of the putative callus-suppressing signals, JA and ET (Fig. 1B, C), showed a comparable phenotypic response and callus-like development.

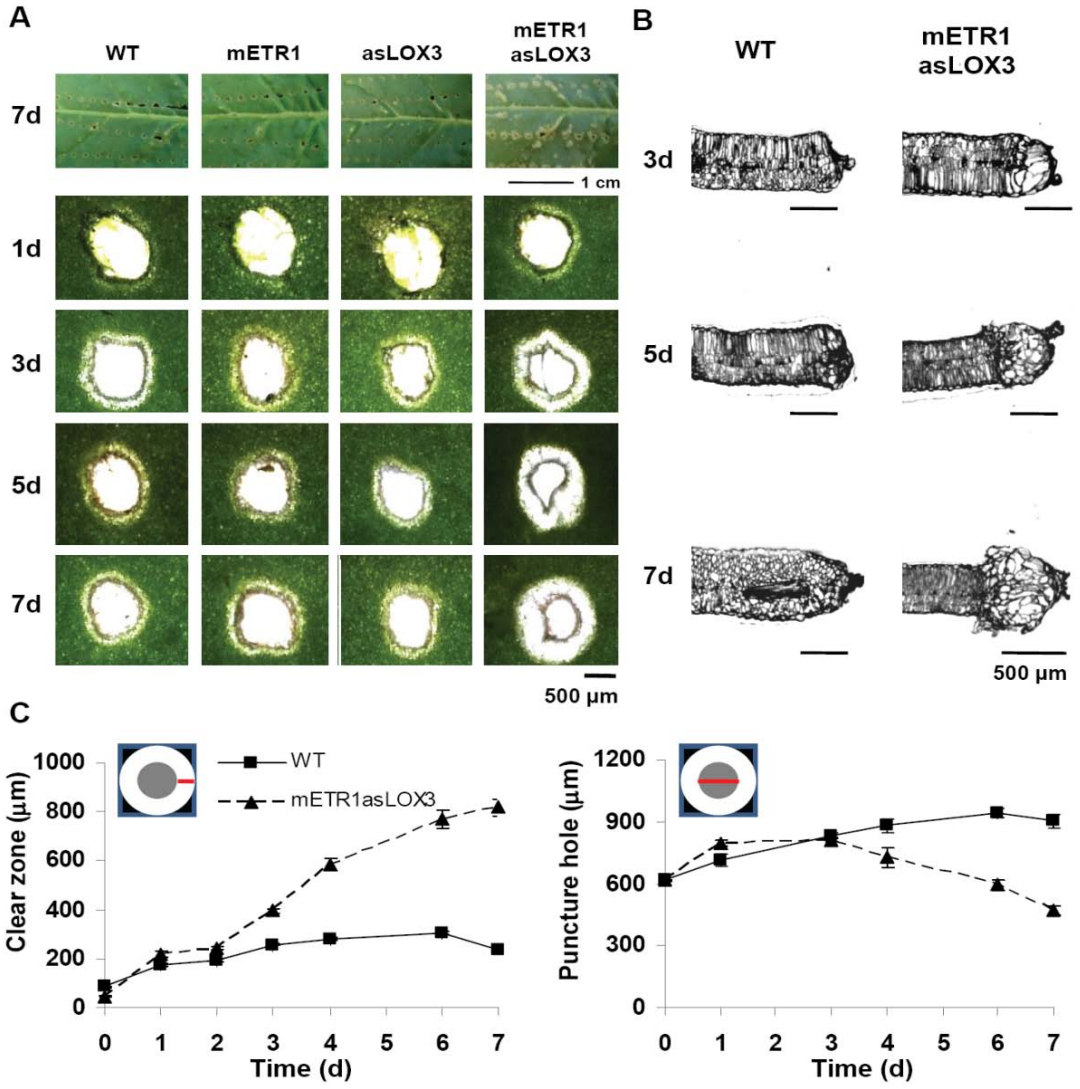


Figure 2: **mETR1asLOX3 leaves develop callus-like cells around puncture wounds after W+OS treatment.** (A) Transparent cells around wounds develop into a large zone of expanded callus-like cells in mETR1asLOX3 leaves. (B) Microscopic analysis of toluidine blue-stained transversal sections of WT and mETR1asLOX3 leaf lamina capturing individual puncture wounds at 3, 5 and 7 d after W+OS treatment. (C) Perimeter size of clear cell zone in OS-treated WT and mETR1asLOX3 leaves (left; mean from 20 measurements ± SE) and puncture holes measured as indicated by the red line in the schematics (right; mean from 20 measurements ± SE).

We therefore decided to use OS-elicitation in all following experiments because it allowed for a starker contrast among responses of elicited genotypes. Moreover, OS-elicitation faithfully simulates most responses elicited by the *N. attenuata-M. sexta* feeding. When we closely inspected the wounds caused by a short feeding bout by *M. sexta* larvae on the leaves, a virtually identical response to those seen in OS-elicited leaves was observed (data not shown). In contrast, only a very limited development of transparent cells around the wounds and no expansion of these cells were observed in WT, mETR1 and asLOX3 leaves, both with simulated

(Fig. 2A) and natural herbivory (data not shown).

Time-course microscopic analysis showed that callus-like cells in mETR1asLOX3 leaves developed from a transparent cell layer around the wound edges (designated as the clear zone), which became easily visible on the second and/or third day (d) after treatment with W+OS (Fig. 2C, left). Interestingly, the size of pattern wheel wounds (designated as puncture hole) began to decrease in mETR1asLOX3 after 3 d (Fig. 2C, right), suggesting that the transparent cells expanded inwards, covering the open areas as they do during normal tissue healing processes. We further dissected the wound sites from mETR1asLOX3 and WT plants to determine the architecture of plant cells surrounding the wounds. In transversal leaf cross-sections, the transparent cells of mETR1asLOX3 appeared as large, expanded, irregularly shaped cells that were completely absent in similarly treated and dissected WT leaves (Fig. 2B). These results suggest that W+OS-induced callus-like cells in mETR1asLOX3 are composed of abnormally expanded cells, whose development depends on a combination of low JA production and a lack of ET signaling after wounding, as demonstrated in the following complementation experiments.

2.2.3 JA and ET deficiency are both indispensable for callus-like cells formation in mETR1asLOX3 plants

Our data suggested that either JA or ET is sufficient to suppress the development of callus-like cells around puncture wounds in *N. attenuata* plants. To test this hypothesis, we excised discs from WT, mETR1, asLOX3 and mETR1asLOX3 leaves and placed them on agar-containing media supplemented with two different concentrations of methyl jasmonate (MeJA; 5 μM and 50 μM).

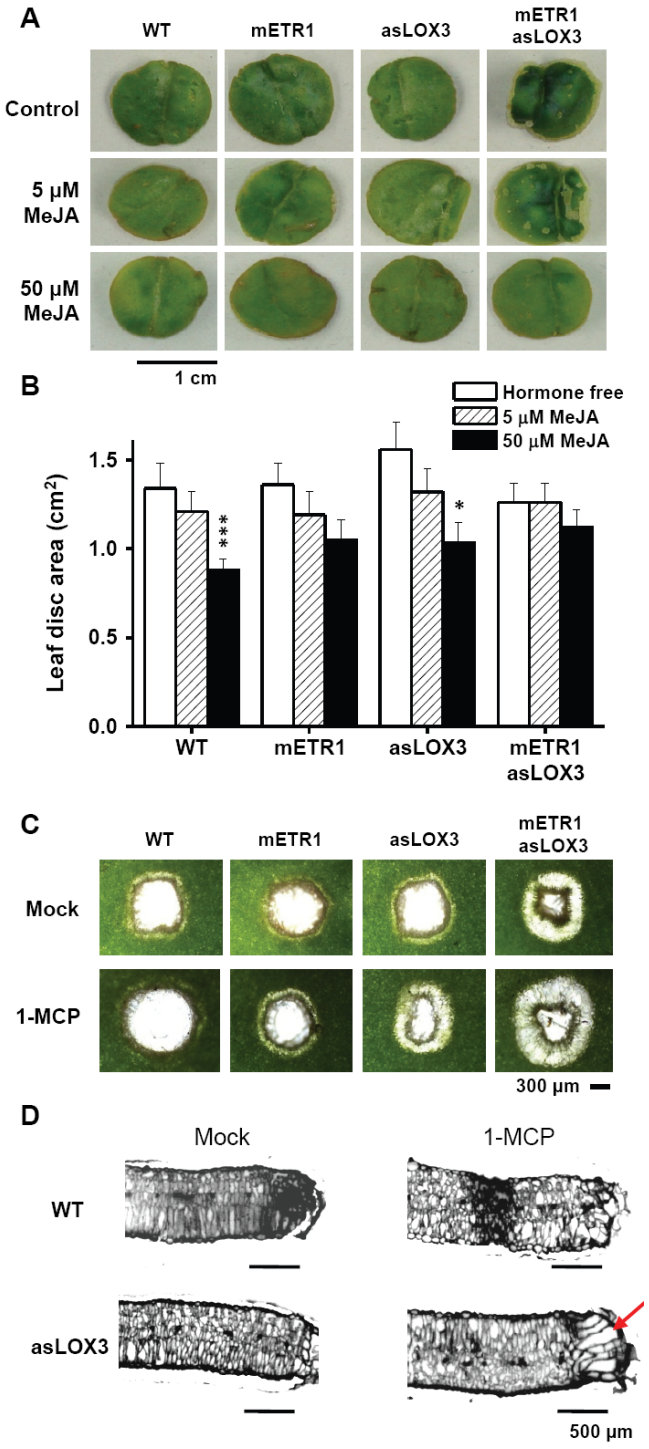


Figure 3: **Crosstalk between JA and ET mediates ectopic callus-like formation in mETR1asLOX3.** (A) Discs from WT, mETR1, asLOX3 and mETR1asLOX3 leaves were placed on media containing 0, 5 and 50 µM MeJA, which gradually suppressed callus-like formation in mETR1asLOX3 genotype. (B) Final leaf disc areas measured after 10 d in culture; values are the means of 20 leaf discs for each 0, 5 and 50 µM MeJA-treated WT, mETR1, asLOX3 and mETR1asLOX3 genotypes with SE indicated. Asterisks indicate means that differed significantly between MeJA-treated and untreated leaf discs in respective genotypes (Student's *t*-test, *** $P < 0.001$; * $P < 0.05$). (C) Leaves from WT, mETR1, asLOX3 and mETR1asLOX3 plants were exposed overnight to the ET receptor antagonist 1-MCP (see Materials and Methods) and treated with W+OS on the next morning; a large transparent zone and callus-like cells developed in asLOX3 leaves, phenocopying the mETR1asLOX3 phenotype. (D) Dissection of 1-MCP-treated leaves from W+OS-elicited WT and asLOX3 leaves as described above, stained with toluidine blue and observed under a microscope (red arrow shows enlarged cells in W+OS-elicited asLOX3 leaves + 1-MCP).

In control plates without MeJA, isolated mETR1asLOX3 leaf discs could still develop callus-like structure on their cut edges, as observed around the puncture wounds on intact leaves. In contrast, WT, mETR1 and asLOX3 leaves only rarely formed spontaneous callus-like on agar plates (Fig. 3A). Corroborating our hypothesis, callus-like development in mETR1asLOX3 leaf discs was substantially reduced by treatment with 5 μM MeJA and completely absent in the presence of 50 μM MeJA (Fig. 3A). The final size of the discs from all genotypes placed on MeJA-containing media was reduced compared to leaf discs cultivated without MeJA (Fig. 3B), showing that JA not only inhibited cell proliferation on cut-edges in mETR1asLOX3 but also reduced leaf disc expansion in all genotypes. To verify the negative role of ET signaling in development of the mETR1asLOX3 phenotype, we pre-treated WT, mETR1, asLOX3 and mETR1asLOX3 leaves with 1-methylcyclopropane (1-MCP), a known ET receptor antagonist (Sisler and Serek, 1997). The leaves were subsequently elicited with W+OS, and observed for 7 d, while being maintained in clip-cages supplemented with fresh 1-MCP in 2-d intervals. No callus-like developed around wounds on WT and mETR1, in both control and 1-MCP-treated leaves. In contrast, more callus-like cells appeared in mETR1asLOX3, and also in asLOX3 leaves but only after 1-MCP application (Fig. 3C). The callus-like cells developing around asLOX3 puncture wounds closely resembled those previously observed in mETR1asLOX3 plants, which was further confirmed by dissecting the leaves and observing the wound sites under the microscope (Fig. 3D). From the complementation experiments above, ET insensitivity and low JA content in the leaves after wounding are both indispensable for the appearance of the mETR1asLOX3 phenotype in transgenic *N. attenuata* plants.

2.2.4 Leaf expansion in WT plants is reduced after simulated herbivory

Because 50 μM MeJA (~11 μg mL^{-1}) in the cultivation media inhibited the expansion of leaf discs *in vitro* (Fig. 3B), we speculated that endogenous JA accumulation after wounding (~1-2 μg g^{-1} FM) could also inhibit the leaf growth at the whole plant level. We used repeated W+OS elicitations on young developing transition leaves (see Materials and Methods) to maximize the effects of JA and ET in WT, mETR1, asLOX3 and mETR1asLOX3, and compared the expansion of

elicited leaves to the corresponding leaves from untreated control plants (Fig. 4).

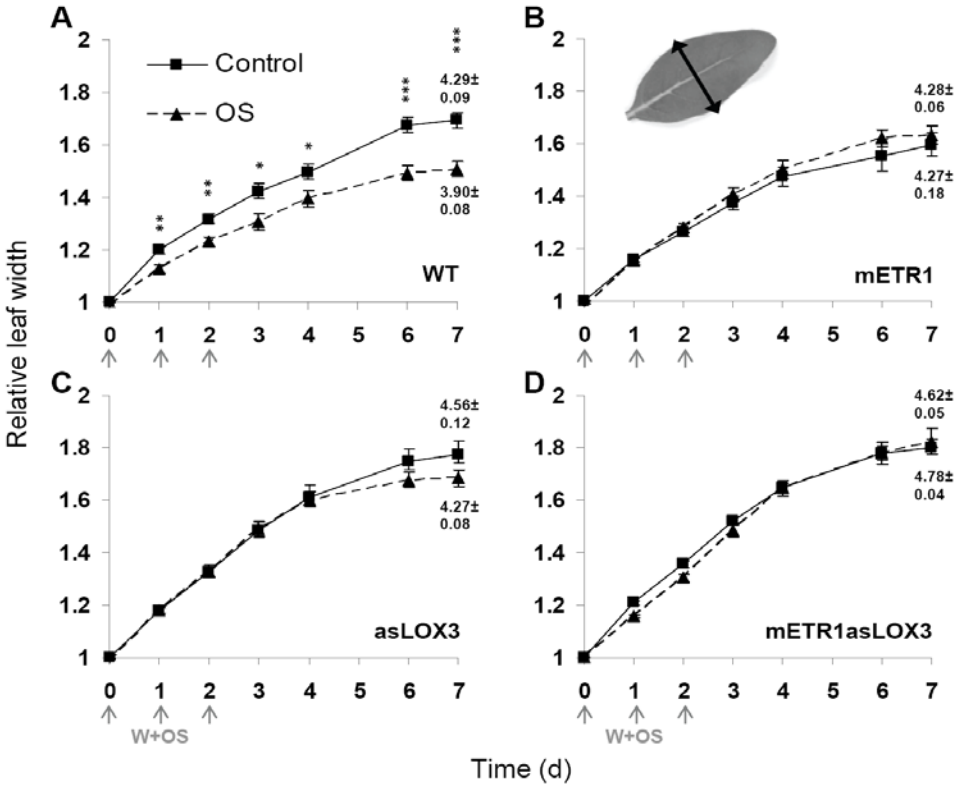


Figure 4: **JA and ET negatively affect leaf growth after W+OS treatment.** A transition leaves of rosette-stage WT (A), mETR1 (B), asLOX3 (C) and mETR1asLOX3 (D) plants were treated three times with W+OS and relative leaf widths (actual width/width at the start of experiment) were measured in 1 d intervals until 7 d after the first treatment; control leaves on separate set of plants remained untreated. Values are the means of 7-10 leaves from individual OS-elicited and control WT, mETR1, asLOX3 and mETR1asLOX3 plants with SE indicated. Asterisks indicate means that differed significantly between OS-elicited and control leaves in respective genotypes and at respective time points (Student's *t*-test, *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$). Numbers in graphs show the final leaf width ± SE at 7 d in cm. Arrows indicate days when leaves were treated with W+OS.

We chose relative leaf width (actual width/width at the start of experiment), measured in 1-d-intervals, as main descriptive parameter to determine leaf expansion after W+OS treatment. Locally treated WT leaves with W+OS showed significantly less expansion compared to untreated WT leaves (Fig. 4A), supporting the previous notion that JA and ET may negatively influence leaf growth *in planta.* In direct support, the leaves from asLOX3 plants were not affected by W+OS treatment during the first 4 d; however, towards the end of the experiment their expansion became more comparable to that of WT leaves, even though the differences in relative leaf width between OS-treated and untreated asLOX3 leaves at 6 and 7 d appeared not to be statistically significant (Fig. 4A and 4C; Student's *t*-test, $P = 0.213$ at 6 d; $P = 0.143$ at 7 d). There were no statistically significant differences in the relative leaf widths between W+OS-treated and control leaves of mETR1 and

mETR1asLOX3 plants (Fig. 4B, D). A partial loss of growth inhibition in asLOX3 but its complete loss in mETR1 plants, which elicit normal levels of JA (Fig. 1B), suggests that the negative effect of JA on leaf expansion could be mediated via ET-dependent signaling. In this case, ET would play a dominant role in leaf growth inhibition after wounding.

2.2.5 IAA content is higher in W+OS-treated mETR1asLOX3 leaves

IAA is known to regulate growth and development in plants (Chen, 2001; Swarup et al., 2002; Woodward and Bartel, 2005) and the exogenous application of auxin is sufficient to induce callus proliferation in isolated plant tissues cultivated *in vitro* (Skoog and Tsui, 1948). The spontaneous callus-like on wound edges of mETR1asLOX3 leaf discs and the leaf expansion of mETR1asLOX3 plants after W+OS treatment led to our hypothesis that the changes in endogenous IAA content could mediate these atypical growth responses. We thus measured IAA concentrations in WT, mETR1, asLOX3 and mETR1asLOX3 transition leaves before and 3, 5 and 7 d after single treatment with W+OS. Before treatment, there were no statistically significant differences in IAA content amongst the genotypes ($P \leq 0.05$), even though the levels of IAA tended to be higher in mETR1asLOX3 leaves (Fig. 5A). IAA concentrations uniformly decreased in all genotypes up to 3 d after W+OS treatment; however, while IAA concentrations continued to decline in WT plants at 5 and 7 d after treatment, IAA content began to rise again in asLOX3, mETR1 and mETR1asLOX3 plants and most markedly in mETR1asLOX3 plants (Fig. 5A).

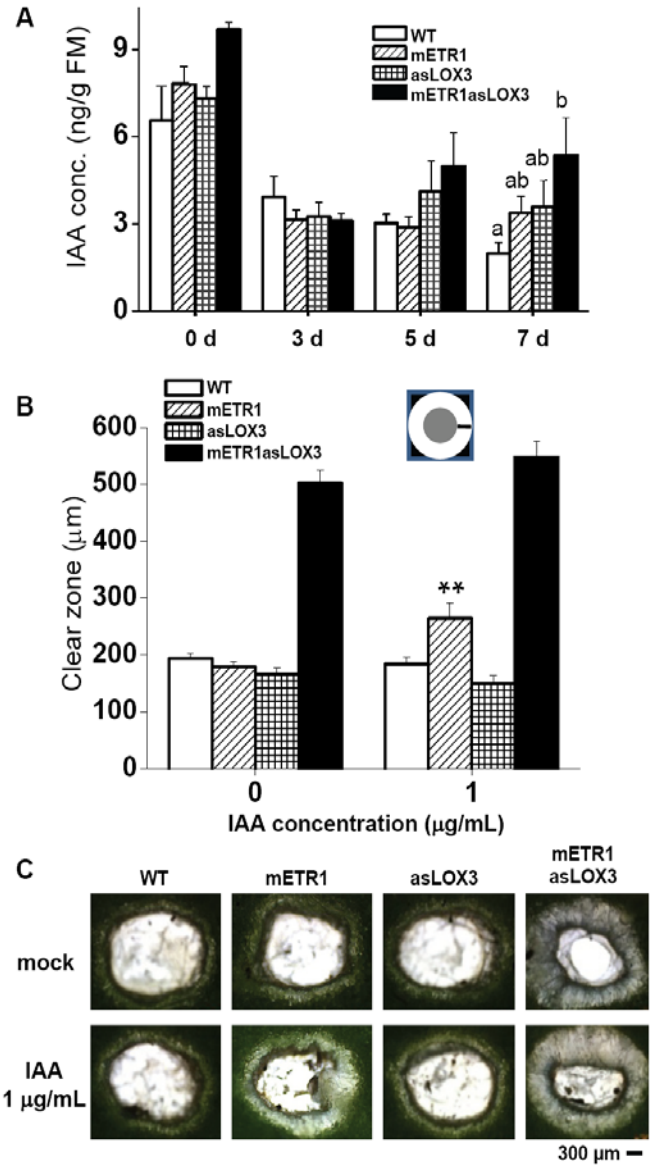


Figure 5: **IAA levels are higher in mETR1asLOX3 leaves after W+OS treatment and mETR1 plants show increased sensitivity to IAA.** (A) IAA concentrations in WT, mETR1, asLOX3 and mETR1asLOX3 leaves measured before, 3, 5 and 7 d after W+OS treatment. Values are the means of three biological replicate measurements with SE indicated. Different letters indicate means that differed significantly between genotypes at 7 d post treatment (Mann-Whitney test, $P < 0.05$). (B) WT, mETR1, asLOX3 and mETR1asLOX3 plants were treated with W+OS and supplemented with 50 µL IAA in water (1 µg mL^{-1}; repeated in 2 d interval) or water without IAA (control).The size of clear zone was measured after 7 d; values are the means of 20 measurements with SE indicated. Asterisks indicate means that differed significantly between IAA-treated and untreated leaves in respective genotypes (Student's *t*-test, ** $P < 0.01$). (C) Microscopic image of puncture wounds 7 d after treatment with W+OS in WT, mETR1, asLOX3 and mETR1asLOX3 leaves maintained in the presence or absence of IAA (1 µg mL^{-1}).

The kinetic of IAA concentrations correlated with the time-resolved growth of callus-like cells on mETR1asLOX3 leaves (Fig. 2A), suggesting that higher IAA concentrations could play an important role in maintaining callus–like cells growth in mETR1asLOX3 plants. The whole leaf analysis of IAA levels does neither take into account local distribution of IAA across the leaf lamina nor the local sensitivity of

cells to IAA, which may have differed and significantly contributed to the penetrance of the expanded cell phenotype around wounds in mETR1asLOX3 leaves.

We therefore tested whether the exogenous application of IAA could promote callus-like formation in otherwise callus-suppressed genotypes: WT, asLOX3 and mETR1. While the first two genotypes showed no response to IAA, statistically larger clear zone around puncture wounds (Fig. 5B) and more expanded callus-like cells (Fig. 5C) developed in mETR1 plants treated with 1 μM IAA, suggesting that ET insensitivity may have increased the sensitivity to IAA in mETR1 leaves, promoting cell expansion even in the presence of higher wound-induced JA levels. Alternatively, IAA could have reduced the effect and/or levels of JA in wounded mETR1 leaves.

2.2.6 Transcriptional changes in mETR1asLOX3 leaves

We already demonstrated that the lack of JA and ET signaling is responsible for ectopic cell expansion and callus-like cells proliferation in mETR1asLOX3 leaves after W+OS elicitation; however, the genetic basis of this process remained unknown. To address this question, we used a custom cDNA microarray spotted with more than 16,000 cDNA probes from cultivated tobacco cells (Matsuoka et al., 2004; Galis et al., 2006). We chose to compare labeled cDNA probes from mETR1asLOX3 and WT leaves at 60 h after W+OS treatment, a time coincident with the first cell expansion around mETR1asLOX3 wounds. In addition, the expression profiles of mETR1 and asLOX3 plants relative to those of WT plants were compared to distinguish between a single hormone- and JA-ET crosstalk-mediated transcriptional change.

We found 96 genes that were up-regulated more than 1.5-fold in mETR1asLOX3 compared to WT plants, but only 1 and 7 genes, respectively, which were induced in mETR1 and asLOX3 plants (Fig. 6). Interestingly, the genes with function in cell wall remodeling were enriched in the up-regulated gene list of W+OS-treated mETR1asLOX3 plants (18/96 genes; Table 1), suggesting that these genes could be responsible for ectopic cell expansion and callus-like development in mETR1asLOX3 leaves.

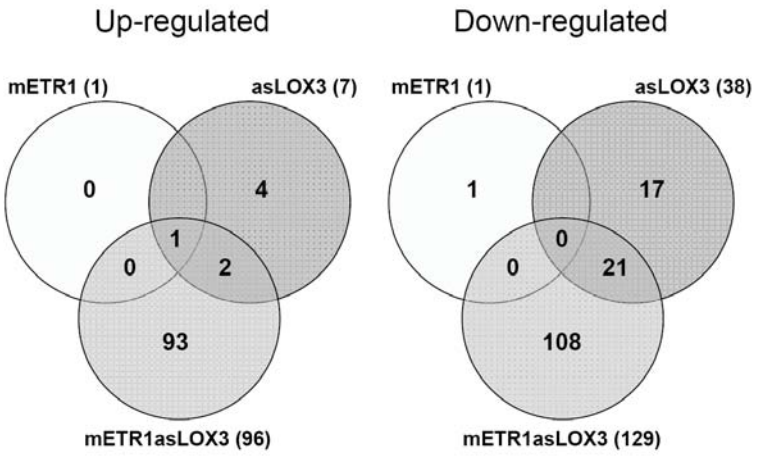


Figure 6: **cDNA microarray analysis revealed a large number of genes specifically regulated in mETR1asLOX3 leaves.** cDNA samples from mETR1, asLOX3 and mETR1asLOX3 were hybridized against WT leaf samples prepared at 60 hours after W+OS treatment. Venn diagrams of up- (left) and down-regulated (right) transcripts show a large group of genes specifically regulated in mETR1asLOX3 leaves ($\geq$ 1.5 fold change, $P \leq 0.05$; n=3).

Selected up-regulated genes were further analyzed by quantitative real-time PCR (qRT-PCR) performed with samples at 0, 6, 20 and 60 h after W+OS treatment. Representatives of two classes of genes, pectin methylesterase (PME, +2.7-fold change; BP131988) and expansin (+1.9-fold change; BP531140), previously shown to mediate cell expansion in other plant species (Micheli, 2001; Pien et al., 2001; Gao et al., 2008), were selected and the analysis confirmed that the transcripts in mETR1asLOX3 were more abundant compared to other transgenic lines and WT (Fig. 7). Furthermore, transcripts of the auxin-inducible *RSI-1* gene (+2.8-fold change; BP530317) with a proposed function in regulation of auxin-induced cell division (Cheong et al. 1999; Kwon et al. 1999) were more abundant in mETR1asLOX3 leaves after W+OS, suggesting that RSI-1 might be involved in the regulation of cell expansion in mETR1asLOX3 leaves (Fig. 7). In contrast, the up-regulation of two selected genes (BP533791 and BP131199) involved in photosynthesis (Supplemental Table S1) could not be confirmed by

Table 1: **Microarray analysis of gene expression in mETR1LOX3 leaves.** List of selected up-regulated genes with potential function in cell wall remodeling in mETR1asLOX3 plants relative to WT plants, observed 60 hours after wounding with a pattern wheel and applying OS from *M. sexta* (≥ 1.5 fold change, P ≤ 0.05, n=3).

		BlastX				mETR asLOX		asLOX		mETR	
NCBI acc.	EST	e-value	NCBI acc.	Org.	Definition	Avg fold	p-value	Avg fold	p-value	Avg fold	p-value
BP530317	21327	1.00E-29	EEF31200	Rc	Putative RSI-1 protein precursor	2.81	0.05	0.83	0.26	1.06	0.42
BP531226	24117	2.00E-25	BAF46299	In	extensin like protein	2.78	0.03	0.85	0.36	1.13	0.19
BP131988	4113	5.00E-53	BAB08634	At	pectinesterase like protein	2.68	0.05	0.92	0.56	1.24	0.32
BP130526	2505	3.00E-22	EEF31200	Rc	Putative RSI-1 protein precursor	2.67	0.03	0.85	0.36	0.95	0.21
BP534536	33116	2.00E-42	XP_002325592	Pt	fasciclin-like arabinogalactan protein 9.2	2.56	0.02	1.31	0.10	1.32	0.10
BP527758	12568	2.00E-30	ACN50176	Ac	pectinesterase	2.55	0.00	0.89	0.33	1.19	0.32
BP534152	32109	6.00E-55	AAL30453	Nt	endo-beta-1,4-glucanase precursor	1.97	0.02	0.98	0.42	0.98	0.42
BP529145	14031	3.00E-88	AAC96079	Nt	alpha-expansin precursor	1.95	0.00	1.24	0.04	1.02	0.79
BP535434	35274	2.00E-48	EEF29809	Rc	Putative multicopper oxidase	1.87	0.00	1.13	0.26	1.33	0.14
BP531140	24029	2.00E-23	AAC63088	Le	expansin	1.86	0.04	1.06	0.57	0.98	0.42
BP531385	24282	6.00E-33	AAF99833	At	Putative pectinesterase	1.84	0.03	0.92	0.50	1.15	0.27
BP529559	14461	7.00E-25	AAL26478	Nt	RALF precursor	1.80	0.01	1.14	0.23	0.95	0.42
BP535453	35296	2.00E-95	CAH17984	Nt	secretory peroxidase N1	1.78	0.03	1.20	0.04	0.98	0.85
BP534996	34205	1.00E-05	AAB63341	Nt	extensin	1.68	0.01	1.11	0.29	0.83	0.30
BP136388	8982	7.00E-14	EEF43915	Rc	Putative xyloglucan endotransglucosylase	1.67	0.02	1.12	0.49	1.05	0.74
BP131567	3624	6.00E-26	EEF29809	Rc	Putative multicopper oxidase	1.62	0.02	0.99	0.85	1.29	0.42
BP530744	23001	9.00E-59	BAA33065	Nt	beta-D-glucan exohydrolase	1.55	0.02	0.99	0.82	0.99	0.71
BP135392	7901	2.00E-13	CAB08077	Sl	pectinesterase	1.52	0.00	1.03	0.62	1.11	0.42

qRT-PCR, using cDNA samples prepared from an experiment carried out independently from the microarray induction; this result suggests that the up-regulation of these genes in mETR1asLOX3 plants was not directly associated with the callus-like cell formation but with other unidentified conditions, most likely light conditions in the glasshouse or slightly different age of the leaves at the time of treatment (data not shown). In summary, the transcription profiles of mETR1asLOX3 plants indicated that crosstalk between JA and ET negatively regulates the transcript accumulation of genes functionally related to cell wall modification, consistent with the unusual cell expansion and development of callus-like cells in mETR1asLOX3 plants after W+OS treatment.

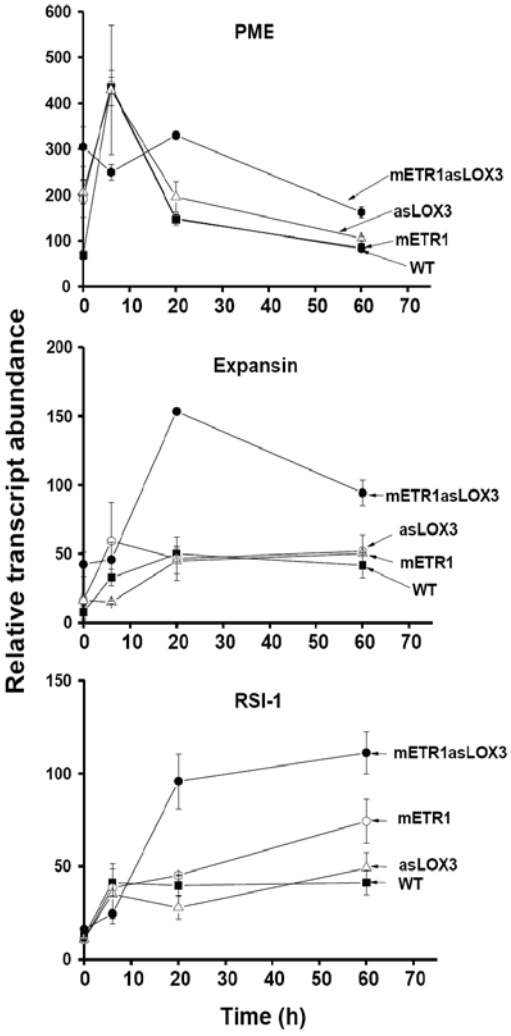


Figure 7: **qRT-PCR transcript abundance of selected cell wall-related genes.** Relative transcript abundance in WT, mETR1, asLOX3 and mETR1asLOX3 leaves was measured by qRT-PCR before, 6, 20 and 60 h after W+OS treatment. *PME* (BP131988), *expansin* (BP531140) and *RSI-1* (BP530317) genes show higher transcript abundance at 20 and 60 h after treatment, confirming the initial microarray hybridization data at 60 h (relative values are means of three biological replicates of the measurement ± SE).

2.2.7 Natural callus-like cells formation after wounding in Brassica species

The ectopic callus-like formation in *N. attenuata* plants was initiated by genetically manipulating the JA biosynthesis and ET perception in *N. attenuata*, resulting in a novel mETR1asLOX3 genotype. However, similar responses exist in WT plants in nature: *Brassica oleracea* L. var. geminifera "Rosella" plants cultivated in the glasshouse showed a phenotype comparable to the artificial mETR1asLOX3 cross. In particular, young leaves of *B. oleracea* responded to wounding by formation of large transparent zones around wounds, followed by visible callus-like proliferation (Fig. 8A).

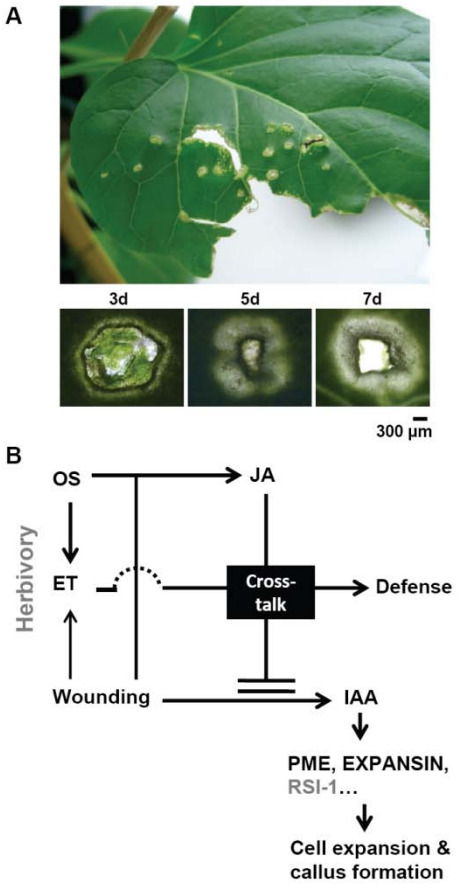


Figure 8: **Crosstalk between JA and ET tunes herbivory defense and growth in plants.** (A) A wounded WT *Brassica oleracea* leaf showing extensive callus-like formation around wounds, which resembles the response of genetically modified *N. attenuata* mETR1asLOX3 plants (top). Pattern wheel wounded young leaves of *B. oleracea* at 3, 5 and 7 d after W+OS elicitation observed under the microscope (bottom). (B) Proposed role of JA, ET and auxin in response to wounding: wounding and herbivore-associated elicitors (fatty acid-amino acid conjugates; FACs) activate JA and ET biosynthesis, which activates defense against herbivores, while suppressing IAA levels and growth, mediated by cell expansion and cell proliferation. In the absence of both negative signals in genetically modified mETR1asLOX3 plants, the leaves of *N. attenuata* are able to initiate healing, resulting in cell expansion and callus-like cells proliferation around puncture wounds.

It remains to be determined whether these plants confer reduced ET-sensitivity; however, relatively low levels of JA in the herbivore-attacked leaves have been recently reported for *B. oleracea* plants (Bruinsma et al., 2009).

2.3 Discussion

JA and ET are essential for plant defense against insect herbivores, as well as they strongly influence plant growth and development. Given the pleiotropic effects of these hormones, it is important to determine how JA and ET interact at a molecular level, in order to understand how plants adjust their growth and defense in responses to herbivores in the natural environment. In this study, the presence of JA and ET was found to determine the local response to wounding in herbivore attacked *N. attenuata*; plants lacking both defense signals responded by previously unreported ectopic cell expansion and callus-like formation around the puncture wounds, suggesting a shift from defense to growth in ET- and JA-signaling deficient plants.

2.3.1 Growth inhibitory effects of JA and ET in plants

When we challenged plants with simulated herbivory treatment, the absence of JA and ET signals in *N. attenuata* allowed greater leaf expansion in treated leaves of transgenic lines compared to WT plants (Fig. 4). This finding is consistent with the previous reports that JA and ET inhibit growth, including isolated cells, seedlings and mature plants, in particular when present at higher concentrations (Pierik et al., 2006). Interestingly, the root growth inhibition assay is routinely used to screen seedlings for JA insensitivity (Staswick et al., 1992; Ellis et al., 2002); however, the mechanism(s) of this inhibition is not completely understood. As a potential explanation, Swiatek et al., (2002) showed that exogenously applied JA inhibits tobacco Bright Yellow-2 (BY-2) cells proliferation by arresting the cells in the G1 phase of their cell cycle. Recently, Zhang and Turner (2008) demonstrated that increasing endogenous JA levels by continuously wounding *Arabidopsis* plants reduced their growth by suppressing mitosis in the youngest leaves, which is compatible with our current findings and young leaf expansion patterns in W+OS-elicited *N. attenuata* plants (Fig. 4). In a closer comparison of the two systems, ten leaves of each plant were wounded in the *Arabidopsis* experiment, one leaf per day over a period of ten days, while we treated -- once per day -- the same *N. attenuata* leaf with W+OS at days 0, 1 and 2 (Fig. 4). The negative effect of wounding in *Arabidopsis* plants was completely abolished in JA biosynthesis deficient mutants; however, in tobacco leaves the expansion of leaf laminas seemed to be only partially restored in JA-deficient asLOX3 genotype (Fig. 4C) after W+OS treatment, although

the differences were not statistically significant. In contrast, the leaves remained completely unaffected by W+OS in mETR1 and mETR1asLOX3 plants (Fig. 4B and 4D).

This suggests that OS-induced ET accumulation may play a major inhibitory role in leaf expansion after wounding, and that the JA effects could have been mediated by an ET-dependent signaling pathway. Previously, excessive ET production and dominant mutations in ET signal transduction, for example *ctr1*, resulted in dwarfism and a smaller leaf phenotype in *Arabidopsis* plants (Kieber et al., 1993; Knoester et al., 1997). In contrast, ET-insensitive plants such as *etr1* and *ers1* had larger leaves (Bleecker et al., 1988; Hua et al., 1995; Grbic and Bleecker, 1995), consistent with the proposed dominant role of ET in control of leaf expansion.

2.3.2 Restriction of growth by rigid plant cell walls

By completely removing the growth inhibitory signals, JA and ET, from the local wound response of *N. attenuata*, we found a novel leaf phenotype, callogenesis-like process, in the double deficient mETR1asLOX3 plants. Callus formation, defined as proliferation of undifferentiated cells around wounds, is normally absent in wounded *N. attenuata* leaves in the glasshouse as well as in the natural environment. A microscopic analysis of puncture wounds in mETR1asLOX3 plants revealed abnormally large chlorophyll-deprived cells around the wounds (Fig. 2a), which overgrew the puncture holes. This response is typical of healing processes in some herbaceous plants and trees, which use cell proliferation and deposition of lignin, suberin and waxes to make the wounds impenetrable, preventing further damage caused by opportunistic pathogen infections and excessive water loss (Lulai and Corsini, 1998; Bucciarelli et al., 1999; Ibrahim et al., 2001; Leon et al. 2001; Paris et al., 2007).

Cell expansion in plants is normally counteracted by the existence of rigid cell walls, composed of complex polysaccharide network and structural proteins in differentiated plant cells (Cosgrove, 2005). However, the lack of flexibility would be obviously detrimental to plants living in the extremely variable environments; for example, rice seedlings have to avoid water submergence by rapid coleoptile elongation (Vriezen et al., 2003) that would be naturally disabled by the rigid cell walls. Therefore, plants evolved specific mechanisms that allow rapid and reversible

modifications of the plant cell walls in response to rapid environmental change (Cosgrove, 2005). Because the cells around wounds in mETR1asLOX3 plants significantly expanded (Fig. 2C), we speculated that the cell wall loosening mechanisms could have been involved in the unusual response of mETR1asLOX3 leaves. Indeed, a comparison of global transcript profiles of asLOX3, mETR1 and mETR1asLOX3 leaves to those of WT plants in response to W+OS elicitation revealed that ectopic cell expansion in mETR1asLOX3 is indeed associated with a preferential accumulation of transcripts related to cell wall loosening, including expansins, extensins, endo-beta-1,4-glucanase, putative xyloglucan endotransglucosylase/hydrolase protein and several pectin methylesterases (Table 1; Cosgrove, 2005; Irshad et al., 2008). In particular, expansins are well-known primary cell wall-loosening agents that disrupt non-covalent bonds of polysaccharides, making cell walls more flexible and expandable (McQueen-Mason and Cosgrove, 1995; Cho and Kende, 1997; Cosgrove et al., 2002; Cosgrove, 2005). Importantly, a strong correlation between transcript accumulation of expansins and growth responses has already been demonstrated (Cosgrove, 2000), supporting the idea that transcriptional changes in tobacco expansin observed in mETR1asLOX3 leaves (Fig. 7) could be functionally relevant to ectopic cell wall expansion in these plants.

2.3.3 IAA levels are controlled by JA-ET crosstalk after W+OS elicitation

While the potential mechanisms involved in cell expansion in mETR1asLOX3 leaves have been proposed by the microarrays, the actual signal(s) responsible for callus-like cells initiation after wounding in mETR1asLOX3 remained elusive. We examined the IAA content as a potential mediator of callogenesis-like process and leaf expansion in mETR1asLOX3 plants, finding higher levels of IAA to be associated with cell growth in W+OS-treated mETR1asLOX3 leaves. In *Arabidopsis*, wounding has been previously shown to down-regulate a number of genes that are positively associated with response to the auxin (Cheong et al., 2002), supporting our observations and proposed negative role of the wound-induced signals, ET and JA, in controlling auxin signaling in wounded leaves. ET is known to interact with IAA at multiple levels, including IAA biosynthesis, transport and perception (Woodward and Bartel, 2005); however, in most of these interactions, ET exerted a synergistic effect on IAA function and/or

biosynthesis examined in the roots and young seedlings, most frequently hypocotyls (Visser et al., 1996; Vandenbussche et al., 2003; Stepanova et al., 2005; Stepanova et al., 2007; Ivanchenko et al., 2008). In contrast, our double deficient mETR1asLOX3 plants showed increased auxin levels and callus-like formation in leaves, indicative of JA-ET crosstalk playing a negative regulatory role in accumulation of IAA in wounded WT leaves. It is possible, that synergistic effects of ethylene on auxin are tissue specific, limited to roots and seedlings, and leaves may be exerting an opposite response similar to the antagonistic effect of ET on IAA during abscission of fruits and flowers (Brown, 1997). Recently, tryptophan conjugates with JA (JA-Trp) have been shown to function as endogenous auxin inhibitors in *Arabidopsis*, offering an alternative model for negative contribution of JA to auxin signal transduction after wounding (Staswick, 2009). However, the amounts of JA-Trp found in wounded *Arabidopsis* leaves were relatively small, making JA-Trp conjugate unlikely to be a strong negative regulator of IAA-mediated responses in these plants. JA, ET and auxin crosstalk in mETRasLOX3 plants was not limited to sole changes in IAA concentrations in the wounded leaves: treating mETR1 plants with exogenous IAA produced more callus-like cells around the wounds compared to the two IAA non-responsive genotypes, asLOX3 and WT (Fig. 5C). It suggests that apart from contributing to increased IAA content in the mETR1asLOX3 plants, ET-insensitivity may have promoted the response to already higher IAA levels after wounding, triggering ectopic callus-like cell development.

Recently, a short-lived ET and auxin-inducible tomato transcriptional regulator SI-IAA3, a member of the auxin/indole-3-acetic acid (Aux/IAA) gene family, has been shown to intersect with the auxin and ET signal transduction pathways (Chaabouni et al., 2009), representing one of the first known functional modules involved in ET and IAA crosstalk. Similarly, ERF1, an EREB family transcription factor and ORA59, an AP2/ERF domain transcription factor, have been proposed to integrate JA and ET signaling pathways in plants (Lorenzo et al., 2003; Pre et al., 2008). Functional analysis of the proteins that integrate JA, ET and auxin signaling in tobacco, and the quantification of JA-Trp conjugates in the wounded tobacco leaves should be carried out in the future to help unraveling the molecular basis of ectopic callus-like cells formation and leaf expansion in mETR1asLOX3 plants, contributing to better understanding of crosstalk between defense and growth-related signals in plants interacting with the environment.

2.3.4 Crosstalk in evolution of plant defenses and growth

Parallel evolution of specialist and generalist insect herbivores with plants provoked an accelerated development of defenses and novel attack strategies in plants and herbivores, respectively (Karieva, 1999). Here, we propose that the putatively evolutionary older IAA-mediated process of wound healing has been independently concealed in *N. attenuata* plants by two defense signaling pathways, mediated ET and JA, ensuring that local regrowth would not take place in *N. attenuata*. Instead, these strongly defended plants redirect their available resources into the production of toxic defensive metabolites that function as direct defenses (nicotine, TPIs) to repel and/or eliminate herbivores. Although direct support for this hypothesis is still missing, we show the first experimental evidence that an (ancient) ability of *N. attenuata* plants to heal is silently resident in these plants, and can be reactivated by simultaneously suppressing JA and ET signaling pathways with transgenic technology (Figs. 2A; 8B). Our findings provide a strong support to the existence of persistent active crosstalk between growth and defense, cost and benefits that are proposed to optimize plant resources during wounding and herbivore attack.

2.4 Materials and Methods

2.4.1 Plant material and growth conditions

WT and transformed lines of *N. attenuata* were germinated on Gamborg's B5 media (Duchefa; http://www.duchefa.com) as previously described by Krügel et al. (2002). The transformed plants used were 35s-etr1a, harboring a mutated form of *Arabidopsis* ET receptor *etr1-1* as described in von Dahl et al. (2007; mETR), antisense (as) LOX3 as described in Halischke and Baldwin (2003; asLOX3) and the genetic cross between 35s-etr1a and asLOX3, prepared in this study (mETR1asLOX3). Seeds were germinated and maintained at 26°C /16 h, 155 µmol m^{-2} s^{-1} light : 24°C/8 h dark cycle (Percival, Perry, IA, USA) for 10 d and young seedlings were planted individually in soil in Teku plastic pots. 10 d later, early rosette plants were transferred to soil in 1 L pots and grown in the glasshouse with day/night cycle of 16 (26-28°C)/8 (22-24°C) h under supplemental light from Master Sun-T PIA Agro 400 or Master Sun-T PIA Plus 600W high-pressure sodium lamps (Philips, Turnout, Belgium). Rosette-stage plants were approximately four weeks old when used for the experiments.

2.4.2 JA, ET measurements and plant treatments

JA was measured by 1200L quadrupole MS/MS system (Varian, Inc.; http://www.varianinc.com) as described in Diezel et al. (2009). ET emissions were measured with a photoacoustic spectrometer (INVIVO; https://www.invivo-gmbh.de) as described in Wu et al. (2008). For plant treatments, a transition rosette leaf from each plant was wounded with a fabric pattern wheel to produce three puncture rows on each side of the mid-vein. Fresh wounds were immediately treated with either 20 µL water or 20 µL of 1:5 (v/v) water-diluted oral secretions (OS) from *M. sexta.* Control plants remained untreated. *M. sexta* larval OS were collected after larvae were reared on *N. attenuata* WT plants until the 3rd to 5th instar; OS were collected after regurgitation of larvae through a Teflon tube connected to vacuum and stored under argon at -20°C.

For leaf expansion measurements, a transition rosette leaf from 7-10 independent WT and transformed plants was wounded with a fabric pattern wheel to produce one row of wounds on each side of the mid-vein. Fresh wounds were immediately treated with 20 µL 1:5 diluted (v/v) OS from *M. sexta*. The same treatment was repeated on days 1 and 2, producing one additional row of wounds each time. Control leaves remained untreated. The width of the treated leaves was measured every day for seven days after the first treatment and plotted in graphs as relative leaf widths (actual width/width at the start of experiment) against time.

For treatments of leaves with exogenous IAA, transition leaves of rosette-stage WT, mETR1, asLOX3 and mETR1asLOX3 plants were treated with W+OS and immediately applied 50 µL of IAA-containing solution (1 µg/mL) in water. 50 µL of IAA-containing solution was re-applied to leaves every 2 d; control leaves were W+OS elicited and 50 µL water was applied instead of IAA at the same time intervals.

2.4.3 Leaf micro-dissection and light microscopy

N. attenuata WT and transgenic leaves were wounded with a fabric pattern wheel to produce two puncture rows on each side of the mid-vein. Freshly wounded leaves were treated with OS as described above and leaves were harvested at 1, 3, 5, and 7 d after treatment. Each row of puncture wounds was observed under a light microscope (Leica Microsystem; http://www.leica-microsystems.com) and individual

puncture wound images were stored with LM Image Manager (Leica Microsystem; http://www.leica-microsystems.com). The diameter of puncture wounds and the size of the transparent zone around each puncture were measured with Adobe Photoshop CS version 8.0 software (http://adobe.com) using stored images; at least 20 punctures were examined per genotype and treatment.

Wounded parts of the leaves were fixed and embedded in hydroxyethylmethacrylate resin (Technovit 7100; Heraeus Kulzer GmbH; http://www.Kulzer-Technik.de) as recommended by the manufacturer. Embedded samples were dissected using a rotation microtome (MICRO International, Walldorf, Germany) and stained with toluidine blue to increase image contrast. Stained sections were observed with a ZEISS Stereo Discovery V.8 microscope (Carl-Zeiss; http://www.zeiss.de) and pictures created and stored with AxioVision 4.5 software.

2.4.4 1-methylcyclopropane (1-MCP) treatment

To inhibit ET perception, leaves were exposed to 1-methylcyclopropane (1-MCP) an ET receptor antagonist: 250 mg of SmartFresh (3.3% 1-MCP, AgroFresh, Rohm and Haas, Italia) was dissolved in 25 mL of alkaline solution (0.75% KOH + NaOH in 1:1 ratio) to release the active substance, 1-MCP. 500 uL of activated solution of 1-MCP was infiltrated into a small cotton bud and immediately placed inside of the clip-cages attached to the leaves. Leaves were pre-exposed overnight to 1-MCP before wounding with a pattern wheel and immediately applying 1:5 diluted (v/v) *M. sexta* OS. Leaves were continuously maintained in the clip-cages with 1-MCP, which was replaced every 2 d. Control leaves were kept in the identical clip-cages supplied with an equivalent amount of the solvent alkaline solution. Tissues from wounded areas were fixed, embedded and sectioned as described above.

2.4.5 In vitro *callus-like cells induction on leaf discs*

Five mature leaves from WT and transformed lines were sterilized with 2% (w/v) dichloroisocyanuric acid (Sigma Aldrich; http://www.sigmaaldrich.com) and washed three times in sterile water. Four discs per leaf were punched out from the middle part of the leaves avoiding tip and leaf basis that differed substantially in their morphogenetic potential. Leaf discs were placed on Murashige and Skoog medium (Duchefa; http://www.duchefa.com) supplied with 3% sucrose and 0, 5 or 50µM methyl jasmonate (Sigma Aldrich; http://www.sigmaaldrich.com). Plates with leaf

discs were incubated at 26°C /16 h, 155µmol m^{-2} s^{-1} light : 24°C/8 h dark cycle (Percival, Perry, IA, USA) for ten days before taking images and measuring leaf disc diameters and calculating corresponding disc areas.

2.4.6 IAA measurements

Three transition leaves from three individual WT and transformed line plants were treated with OS as described for the hormone analysis, and pooled for each measurement. Leaves were harvested before and 3, 5, and 7 d after treatment, snap-frozen in liquid nitrogen and kept at -80°C until analyzed. Approximately 1 g of liquid nitrogen-ground leaf powders was extracted over night by diffusion in the dark at -20°C using 10 ml 100% methanol supplemented with antioxidant 25 mM diethyldithocarbamic acid (DECT, Sigma Aldrich: http://www.sigmaaldrich.com) and 50 ng per sample of internal standard indole-3-acetic acid (phenyl-^{13}C6, 99%) (Cambridge Isotope Laboratories, Inc.; http://www.isotope.com). Samples were centrifuged at 3000 x g, 4°C for 30 min, and supernatants were collected before re-extracting the pellets with 5 ml 100% methanol/25 mM DECT on ice for 30 min. Samples were centrifuged as before and supernatants combined in single tubes. Water was added to adjust the final concentration of methanol in each sample to 50% methanol (v/v) and diluted samples were purified using preconditioned Supelclean™ LC-18 SPE columns (Supelco: http://www.sigmaaldrich). Flow-through fractions were immediately applied to activated diethylaminoethyl Sephadex (DEAE) A25 column (Amersham Pharmacia Biotech: http://www1.gelifesciences.com) equilibrated with 50% methanol and IAA was absorbed to the resin by gravity flow. Columns were additionally rinsed with 50 mL of 50% methanol (v/v) and new Supelclean LC-18 SPE columns were connected below the DEAE columns. IAA was then eluted from DEAE with 6% formic acid (v/v) (Riedel-de Haen: http://www.sigmaalrich.com) and trapped on SPE column. IAA retentate was subsequently eluted from the SPE columns with 5 mL of pure diethyl ether (Fluka: http://www.sigmaaldrich). The acidic water phase was quickly removed by pipetting and samples were immediately dried under a stream of nitrogen. Dry samples were quantitatively re-dissolved in 1.5 mL of 100% methanol, transferred to Eppendorf tubes and dried under vacuum in Eppendorf Concentrator 5301 (http://www.eppendorf.com).

Before measurements, samples were re-dissolved in 150 µL 70% (v/v)

methanol, centrifuged at 16,000 x g, 4°C for 30 minutes and supernatants applied to 1200L quadrupole MS/MS system (Varian, Inc.; http://www.varianinc.com). 10 µl were injected onto a Prodigy column (150x2 mm, 3 µm diameter, Phenomenex, USA) attached to a pre-column (C18, 4 x 2 mm, Phenomenex, USA). A mobile phase composed of 0.05% acetic acid and acetonitrile was used in a gradient mode for the separation. The mobile phase comprised solvent A (0.05% acetic acid) and solvent B (acetonitrile) used in a gradient mode time/concentration for (min/%B): 1.5'/20; 6'/97; 17'/97; 18'/20; 25'/20. IAA was detected as negative ions in a MRM mode: molecular ions M-H (-) at m/z 174 generated from endogenous auxin and from its internal standards 180 were fragmented under 35 V CE. The product ion of auxin and its internal standard was m/z 130 and 136, respectively. The ratio of ion intensities of the product ions was used to directly quantify auxin in the samples and normalized to fresh mass of the samples.

2.4.7 cDNA microarrays

To analyze transcriptional changes, WT and transformed plants were treated with W+OS as described above, and leaf material from 15 individual plants in each transgenic genotype and 45 corresponding WT plants were harvested at 60 hours after induction. For each microarray experiment, 5 leaves of 5 independent plants were pooled to obtain 3 biological replicates before total RNA was extracted from leaves. mRNA was isolated, reverse-transcribed and labeled as previously described in Halitschke and Baldwin (2003). To monitor relative transcriptional changes in each genotype, treated samples from transgenic lines were always hybridized against identically treated WT samples.

We used a custom-made 16K tobacco microarray spotted inhouse (VersArray, Bio-Rad: http://www.bio-rad.com), using PCR-amplified tobacco cDNA clones that was a gift from Plant Science Center RIKEN Institute, Japan, and described previously in Galis et al. (2006). Spotted arrays were hybridized and washed as described in Halitschke et al. (2003). Spot intensities for Cy3 (transgenic lines) and Cy5 (WT) signals were analyzed with AIDA software (Raytest; http://www.raytest.com), and global background-subtracted raw signal intensities (Supplemental Tables S3-S5) were LOWESS normalized using MIDAS software (http://www.tm4.org). Statistically significant up-regulated cDNA spots from three biological replicates with transgenic line to WT normalized signal ratios ≥ 1.5 or ≤

0.66 (Student's *t*-test, P≤ 0.05) were identified. Several specifically up-regulated gene signals from mETR1asLOX3 were validated with quantitative RT-PCR at 60 h and three additional time points (0, 6, 20 h) after W+OS treatment.

2.4.8 qRT-PCR analysis

Total RNA was isolated from approximately 150 mg leaf tissues using the Trizol reagent following the manufacturer's protocol (Invitrogen; http://www.invitrogen.com). Crude RNA samples were treated with RQ1 DNase (Promega; http://www.promega.com), followed by phenol-chloroform extraction and ethanol precipitation. DNA-free RNA samples were reverse-transcribed using oligo (dT_{18}) primer and Superscript II enzyme (Invitrogen; http://www.invitrogen.com) following the manufacturer's recommendations. qRT-PCR of the candidate genes selected from the microarray study were performed with Stratagene MX3005P instrument (Stratagene; http://www.stratagene.com) as recommended by the manufacturer. For normalization purpose, primers specific for the elongation factor-1α gene from tobacco (EF1-α; Acc. D63396) were used as an internal standard to adjust concentrations of cDNA in the samples. Specific primers in 5′-3′ direction used for SYBR Green-based analyses of up-regulated genes were:

PME3-F; CCCCGGAGTGTTCAGCTTGAAC
PME3-R; ATGCCTAGTAGGAGCGTCAACAAG
EXP-F; CATCGCTCTTCTACTTCATGGA
EXP-R; CTCTATTCTTCCCCTCCATTCAC
RSI-F; CCACAAACAAACATGCCCTTGC
RSI-R; CCGGTACGACAACTCATCTTAGAC

2.4.9 Statistical analysis

Data were analyzed with StatView software and statistical significance displayed where relevant to the measurements.

3. A novel dicaffeoyl sperimidine transferase is regulated by jasmonic acid and ethylene crosstalk in *Nicotiana attenuata*

3.1 Introduction

Hydroxycinnamic acid amide conjugates (HCAAs) belong to a diverse group of plant secondary metabolites, which are widely distributed in higher plants including *Arabidopsis thaliana*, maize, rice and numerous *Solanaceae* species (Martin-Tanguy, 1985; 1997; Bonneau et al., 1994; Facchini et al., 2002; Hagel and Facchini, 2005). Naturally, organ- and developmentally-specific accumulation and distribution of HCAAs suggest their intrinsic role in plant growth and development (Martin-Tanguy, 1985; Facchini et al., 2002; Edreva et al., 2007); however, the correlation between HCAAs accumulation and morphological changes remains to be elucidated. Recently, HCAAs gained more attention due to their possible roles in plant defense against biotic (pathogen infection and herbivore attack) and abiotic (mineral deficiency and UV-irradiation) stress factors (Martin-Tanguy, 1985; Camacho-Cristobal et al., 2005; Izaguirre et al., 2007; Kaur et al., 2010). Even though chemical structure, biosynthesis and distribution of HCAAs have been extensively studied, signal transduction pathways and transcription factors that control HCAAs biosynthesis and accumulation remain scarcely known (Martin-Tanguy, 1985; 1997; Back et al., 2001; Facchini et al., 2002; Kristensen et al., 2004; Hagel and Facchini, 2005).

Jasmonic acid (JA) and ethylene (ET) are well-known plant stress signals that mediate plant responses to biotic stress conditions, including pathogen infection and damage by insect herbivores (Halitschke and Baldwin, 2003; Broekaert et al., 2006; Adie et al., 2007; von Dahl et al., 2007; Howe and Jander 2008; Bari and Jones; 2009). Goossens et al., 2003 demonstrated that methyl jasmonate (MeJA), a JA-derivative, can induce expression of alkaloid and phenylproponoid biosynthetic genes that correlated with the elevated alkaloid and phenylpropanoids accumulation in tobacco Bright Yellow-2 (BY-2) cells.

In *Nicotiana attenuata*, the accumulation of phenylpropanoids and alkaloids during defense against herbivores has been shown to depend on active jasmonic acid signaling (Halitschke and Baldwin, 2003; Zavala et al., 2004; Pluskota et al., 2007; Paschold et al., 2008). Interestingly, JA burst in *N. attenuata* (and other plants)

overlaps with increased ET emission from the plants after herbivore attack, suggesting an existence of active crosstalk between JA and ET in regulation of plant defense metabolite accumulation. Indeed, a crosstalk between JA and ET signals has already been shown to modulate the production of alkaloid nicotine in *Nicotiana* species (Shoji et al., 2000; Winz and Baldwin; 2001).

Transcription factors bind selectively to the promoter DNA regions of genes, which results in increased or decreased transcription rates -- a mechanism that is frequently used by induced defense mechanisms in plants (Eulgem et al., 2000; Sugimoto et al., 2000; Singh et al., 2002; Zheng et al., 2007). Coordinating defenses, transcription factors are directly or indirectly controlled by defense hormones like JA, providing rapid and specific elicitation of plant defense against paticular stress. For example, an R2/R3 MYB-type transcription factor rapidly responded to MeJA elicitation in BY-2 cell cultures, which has correlated with the expression of core enzymes in phenylproponoid biosynthesis (e.g. PAL), and the accumulation of specific phenylproponoid-polyamine conjugates including caffeoyl putrescine (CP) in the cells. Recently, Kaur et al. (2010) demonstrated that a *N. attenuata* homolog of this transcription factor functions as major regulator of dicaffeoyl spermidine (DCS) and CP accumulation in response to herbivory in *N. attenuata* plants. While the MYB transcriptional activity has been already connected to upstream JA signaling cascade, and downstream to HCAAs biosynthesis, it remains elusive how this MYB transcription factor mediates HCAAs biosynthesis, as well as what role is played by ET in this near completion regulatory pathway.

The synthesis of HCAAs is conveyed by conjugation of polyamines (putrescine, spermidine and spermine) to acylated hydroxycinnmaic acid (HCA) (cinnamoyl-, coumaroyl-, caffeoyl-, feruloyl- and sinapoyl-CoA) by the activity of specific acyl transferases. The use of transcriptomic and biochemical approaches applied to model plant species allowed identification of a large number of acyl transferases, collectively named BAHD protein family, which was derived from initials of the first four characterised enzymes in the family; benzylalcohol *O*-acetyl transferase (BEAT) from *Clarkia breweri* (Dudareva et al., 1998); anthocyanin *O*-hydroxycinnamoyl transferase (AHCT) from *Gentiana triflora* (Fujiwara et al., 1998); hydroxycinnamoyl/benzoyl-CoA: anthranilate hydroxycinnamoyl/ benzoyl transferase (HCBT) from *Dianthus caryohyllus* (Yang et al., 1997); and deacetylvindoline 4-*O*-acetyl transferase (DAT) from *Cantharanthus roseus* (St-

Pierre et al., 1998). The two enzymes, HCBT (hydroxycinnamoyl-CoA: hydroxyanthranilate N-hydroxycinnamoyl transferase) from *Avena sativa* (AsHHT1) and agamatine coumaroyl transferase (ACT) from *Hordeum vulgare* are two examples of BAHD genes which acylate polyamines, leading to the formation of HCAAs after pathogen infection (Yang et al., 1997; Burhenne et al., 2003; Yang et al., 2004). Recently, three new spermidine acylating enzymes have been identified in *Arabidopsis thaliana*: spermidine disinapoyl transferase (SDT), spermidine dicoumaroyl transferase (SCT), and spermidine hydroxycinnamoyl transferase (SHT) (Luo et al., 2009; Grienenberger et al., 2009). While the acyl transferase activity towards formation of CP and DCS, two major HCAAs found in tobacco, has already been demonstrated at biochemical level in the purified plant extracts (Meurer-Grimes et al., 1989; Negrel et al., 1989, 1991, 1992), the responsible genes have yet to be identified.

Accumulated data from various study models and cell cultures show that JA is essential for triggering HCAAs biosynthesis in response to herbivory stress, which process is mediated by MYB-dependent transcriptional activity (Galis et al. 2006; Paschold et al. 2008; Kaur et al., 2010). By the analysis of MYB-dependent transcripts in *N. attenuata*, we identified an EST fragment named *DH29*, and show that it encodes a novel acyl transferase enzyme that is required for DCS biosynthesis in *N. attenuata*. Interestingly, ectopic silencing of *DH29* by posttranscriptional silencing method resulted in not only dramatic reduction in DCS content but also strongly increased accumulation of CP after herbivory, suggesting a strong interconnectivity between DCS and CP biosynthetic pathways. Using recombinant technology and protein activity assays *in vitro*, DH29 recombinant protein catalyzed formation of monocaffeoyl spermidine from spermidine and caffeoyl-CoA, a first and therefore limiting step in DCS biosynthesis. The expression profiling of spermidine monocaffeoyl-CoA transferase (*SmCT*) in JA-deficient and ET-insensitive *N. attenuata* plants revealed that in a signal transduction cascade, JA is used to activate MYB8, which then controls the expression of *SmCT*. Silencing of *SmCT* increased the accumulation of CP, which impaired caterpillar growth, confirming the defensive character of CP against insect herbivores as previously shown by Kaur et al. (2010) but disabling -- at present -- identification of the ecological function of DCS.

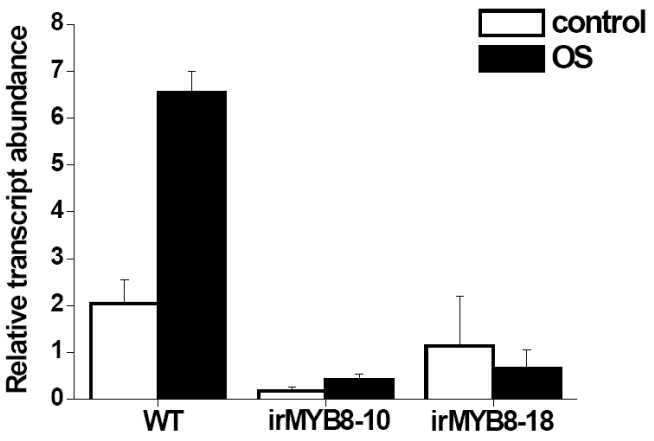
3.2 Results

3.2.1 DH29 encodes a novel acyl transferase gene from tobacco

In the previous microarray analysis using transformed *N. attenuata* plants silenced in the expression of MYB8 transcription factor (irMYB8; Kaur et al., 2010), an EST clone DH29 (Acc. No. CA59184; Hui et al. 2003) derived from a gene of unknown function, showed consistently lower expression in transgenic line at 90 minutes after simulated herbivory, that is wounding of the leaves with a fabric pattern wheel and immediately applying oral secretions from *Manduca sexta* to the wounds (W+OS), suggesting that DH29 may be under direct transcriptional control of MYB8. To confirm *DH29* expression pattern, relative transcript levels of DH29 in samples prepared from 1 h W+OS-induced leaf tissues from wild type (WT) and two independently transformed irMYB8 lines, irMYB8-10 and irMYB8-18, were analyzed by quantitative RT-PCR (qRT-PCR). DH29 showed strongly reduced accumulation of transcripts in both MYB8-silenced lines compared to that of the WT plants (Fig. 1A), confirming that MYB8 transcription factor is an upstream regulator of DH29 during herbivory.

Cloning of the full length clone of DH29 revealed that this fragment is part of a deduced gene consisting of 449 amino acids, which showed ~ 30%, sequence similarity to benzylalcohol *O*-acetyl transferase (BEAT) enzyme from *Clarkia beweri* (Acc. No. ACC18062), a member of the large BAHD acyl transferase gene family in plants (D' Auria, 2006). In a phylogenetic analysis using selected members of BAHD family (D' Auria, 2006), DH29 protein was classified in clade III of BAHD enzymes, which are proposed to use mostly acetyl-CoA as their acyl donor; however enzymes in BAHD family are very diverse and accept broad range of substrates and therefore, it is not possible to predict the actual substrate of DH29 enzyme based on sequence homology. Recently, Kaur et al. (2010) reported that the accumulation of two hydroxycinnamic acid amides (HCAAs), caffeoyl putrescine (CP) and dicaffeoyl spermindine (DCS) was strongly reduced in irMYB8 plants, both at constitutive level and after W+OS treatment.

A



B

```
NaDH29          MGFLCANLKNSLAVEIMSKKLVKPSSPTPTHLQSYKLSFFDQLAIRMHVPIVLIYHNLNN 60
CbBEAT          -----------MNVTMHSKKLLKPSIPTPNHLQKLNLSLLDQIQIPFYVGLIFHYETLSD 49
                          : * : ****:*** ***.***. :**::**: * ::* ::: *..*.:

NaDH29          --SITNELLEESLSKTLTHVYPSAGRINKDRRVVDCLDQGVEFIIAKVNCQLEDFLEQAR 118
CbBEAT          NSDITLSKLESSLSETLTLYYHVAGRYNGTDCVIECNDQGIGYVETAFDVELHQFLLGE- 108
                  .** . **.***:***  *  *** *    *::* ***: :: : .: :*.:**

NaDH29          KDIDLANHFWPQGIKDVDDNYDFAITPLVFVQVTRFECGGLALSVAAEHIAIDGFTNMKF 178
CbBEAT          ---ESNNLDLLVGLSGFLS--ETETPPLAAIQLNMFKCGGLVIGAQFNHIIGDMFTMSTF 163
                   :  *     *:... .  :   .**. :*:. *:****.:..  :**  * **  .*

NaDH29          IYEWAKVCRLGIPTSTTTDIFNYDLGDIFPARDTSRILKPLASLAIPKDTITYVAKRFVF 238
CbBEAT          MNSWAKACRVGIKEVAHP---TFGLAPLMPSAKVLNIPPPPS-----FEGVKFVSKRFVF 215
                : .***.**:**   : .   .:.*. ::*: .. .*  * :      : :.:*:*****

NaDH29          NEASISKLRNKIASG-------VLSFKPSRVEIVTALLWRALIRASQAKNG--RLRPSLM 289
CbBEAT          NENAITRLRKEATEEDGDGDDDQKKKRPSRVDLVTAFLSKSLIEMDCAKKEQTKSRPSLM 275
                ** :*::**:: :.          . :****::***:* ::**. . **:   : *****

NaDH29          SFPVNLRGKASLPKLSDTFGNFAVEVPVVFTPNETKMELHNLIALIRDATDKTMVSSAKA 349
CbBEAT          VHMMNLRKRTKLALENDVSGNFFIVVNAESK-ITVAPKITDLTESLGSACGEIISEVAKV 334
                 . :*** ::.*.  .*. *** : * .  .   .  :: :*   : .* .: : . **.

NaDH29          SNDELVS--MAANLYNMTQEWEANEEVDEFTCSSLCRFPMKEADFGLGKPCWMTFGLRQS 407
CbBEAT          DDAEVVSSMVLNSVREFYYEWGK-GEKNVFLYTSWCRFPLYEVDFGWGIPSLVDTTAVPF 393
                .: *:**  :  .: ::  **    * : *  :* ****: *.*** * *. :

NaDH29          -QVFWLYDADFGSSIAAQVDLNESLMHYFERDQDLNTFTILNN 449
CbBEAT          GLIVLMDEAPAGDGIAVRACLSEHDMIQFQQHHQLLSYVS--- 433
                  :. : :*  *..**.:. *.*  *  *::.::* ::.
```

Figure 1: **The expression of a novel acyl transferase DH29 from *N. attenuata* is strongly reduced in two independent irMYB8-silenced lines**. (A) A single rosette leaf from WT, irMYB8-10 and irMYB8-18 was induced with W+OS and the whole leaves were collected 1 h later. Graph shows mean (±SE) *DH29* relative transcription abundance quantified by qRT-PCR from control (n=5) and W+OS-treated (n=5) samples. (B) Full length DH29 protein sequence aligned with the most sequentially related protein benzylalcohol acetyl transferase (BEAT) from *Clarkia breweri.* Asterisk (*) represent identical amino acids, two dots (:) represents conserved substitution amino acids, and one dot (.) represents semi-conserved substitution amino acids in DH29 and CbBEAT proteins.

As we knew that the expression of a putative acyl transferase DH29 was considerably low in irMYB8 plants, we proposed that DH29 might be involved in transferring caffeoyl-CoA to either putrescine or spermidine, forming CP or DCS, respectively. Previously, acyl transferases catalyzing amide bond formation between hydroxycinnamoyl-CoA and polyamines to form HCAAs have been reported: for example, spermidine disinapoyl transferase (SDT) from *Arabidopsis thaliana*

catalyses formation of disinapoyl spermidine (Luo et al., 2009), and spermidine hydroxycinnamoyl transferase (SHT) from tobacco callus catalyzed coumaroyl spermidine formation (Negrel et al., 1991).

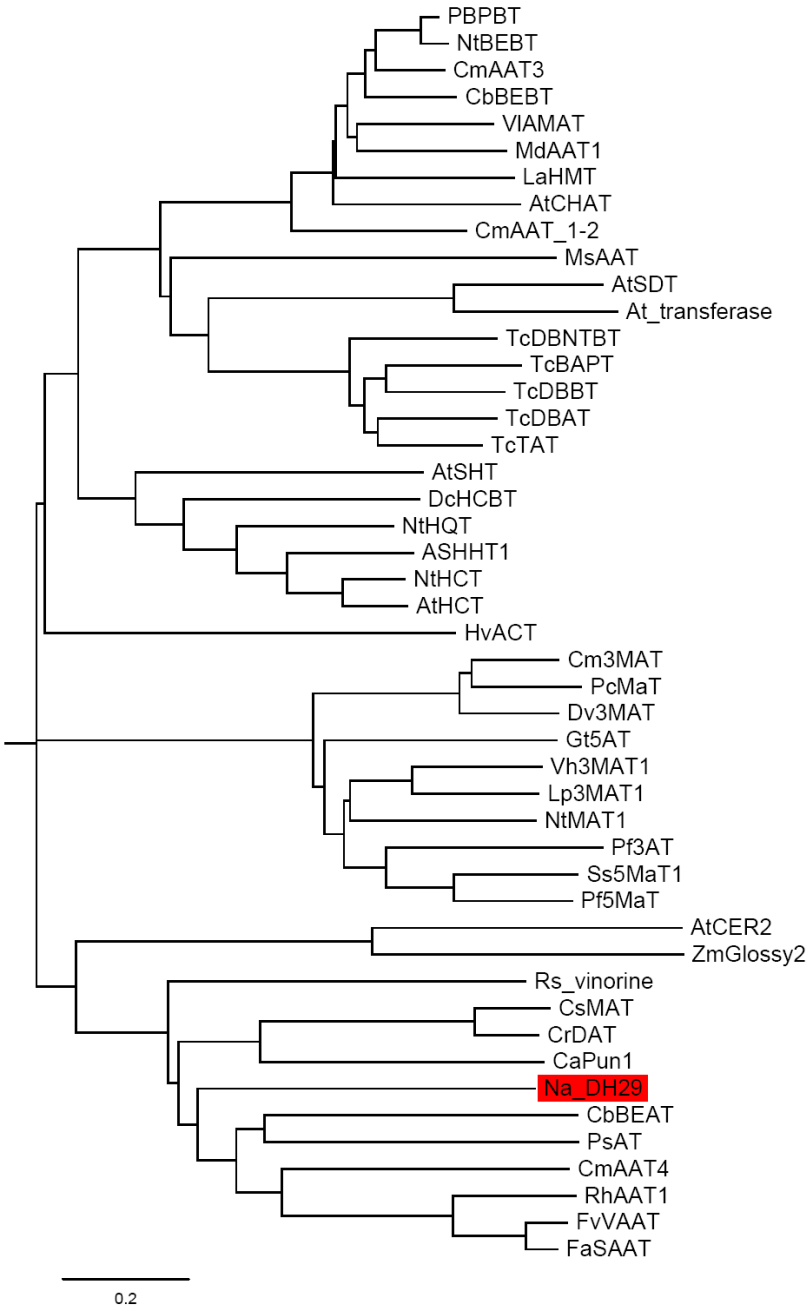


Figure 2: DH29 protein is a novel member of BAHD protein family. A phylogenic tree of selected protein sequences belonging to previously characterized members of BAHD protein family (D' Auria, 2006) and the protein sequence of DH29. Protein sequences were first aligned with ClustalX2 and the alignment results were subjected to neighbor joining and bootstrap analysis by MEGA4 software.

3.2.2 Silencing of DH29 by Virus Induced Gene Silencing (VIGS) impairs accumulation of DCS in N. attenuata

In irMYB8 *N. attenuata* plants, low expression of *MYB8* gene correlated with low accumulation of CP and DCS after W+OS treatment in the leaves. We thus examined the relationship between MYB8-regulated *DH29* expression and CP and DCS accumulation using DH29-VIGS-silenced plants. First, silencing efficiency of DH29 was examined by RT-qPCR, using a specific pair of primers located outside of the DH29 silencing region used in VIGS vector; *DH29* expression in DH29-VIGS plants was reduced by ~ 60% and ~80% relative to empty vector (EV) levels at 6 and 20 hours after W+OS treatment, respectively (Fig. 3A), indicating efficient silencing of DH29 expression by VIGS technology. A single rosette leaf from DH29- and EV-VIGS plants was then treated with W+OS and CP and DCS contents were analyzed by high performance liquid chromatography (HPLC) coupled to photodiode array (PDA) detector at 0, 24, 48 and 72 hours after W+OS treatment. Interestingly, DH29-VIGS plants contained much higher constitutive levels of CP but had strongly reduced levels of DCS compared to EV-VIGS plants (Fig. 3B, C). CP concentrations significantly increased in both DH29- and EV-VIGS plants after W+OS treatment; however, total CP concentrations in DH29-VIGS plants were significantly higher than those in EV-VIGS plants (Fig. 3C). In contrast, DCS concentrations in DH29-VIGS plants were constitutively low and did not increase upon W+OS treatments as they did in EV-VIGS plants (Fig. 3B,D), suggesting that DH29 was required for DCS biosynthesis.

In summary, we propose that DH29 encodes a spermidine dicaffeoyl transferase (SDT) enzyme, which regulates accumulation of DCS in *N. attenuata* during herbivory stress. Considering the biosynthetic pathways of CP and DCS, both routes require caffeoyl-CoA as an acyl donor and therefore, high accumulation of CP in DH29-VIGS plants could be explained by metabolic reconfiguration in favor of CP after partial or complete block in the DCS pathway. In this model, a strong substrate competition between CP and DCS synthases can be predicted in the respective biosynthetic pathways (Fig. 4).

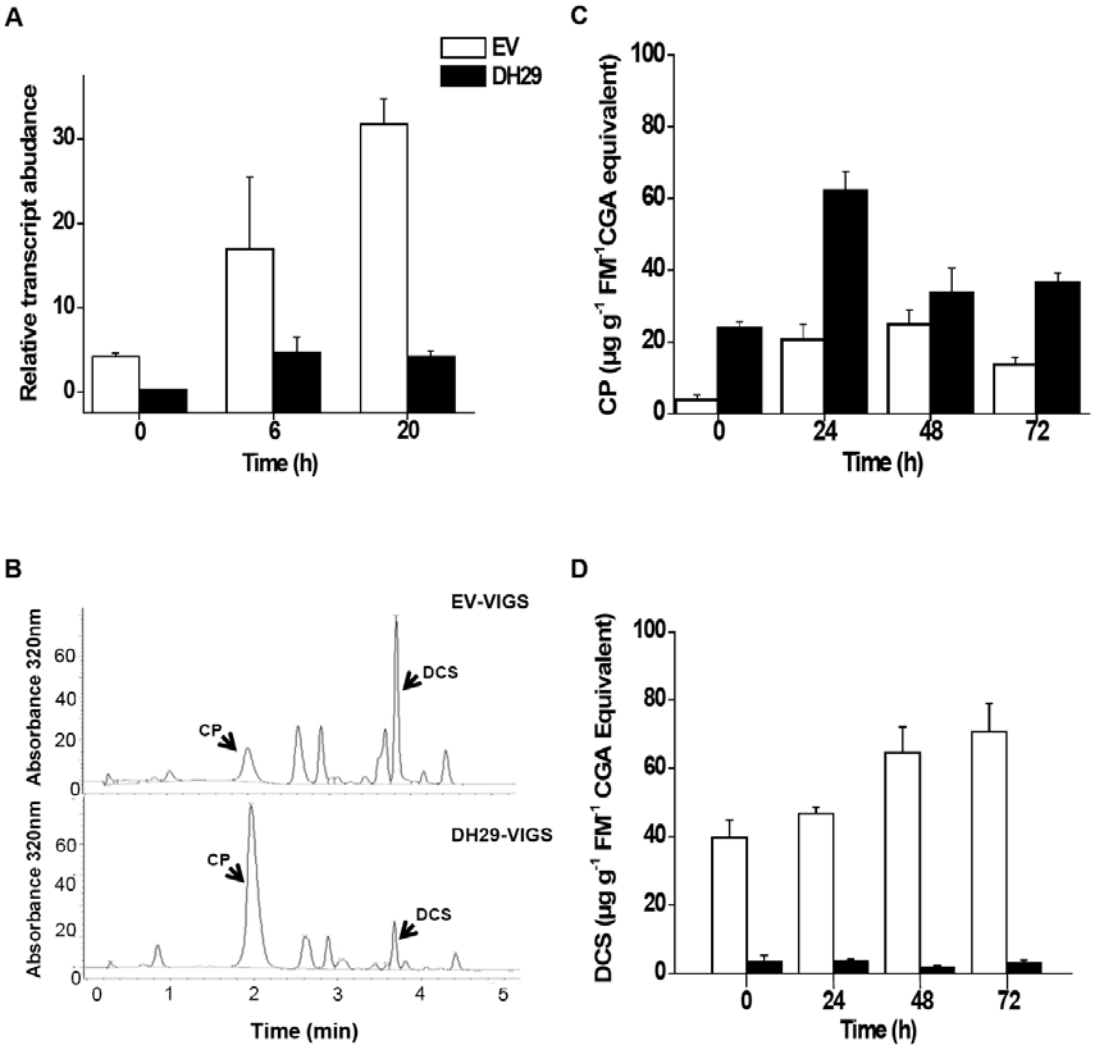


Figure 3: Silencing of *DH29* reduces the accumulation of DCS in *N. attenuata* leaves. *DH29* expression was silenced by VIGS; empty expression vector without inserted fragment (EV) was used as control. Individual leaves from elongated EV- and DH29-VIGS plants were elicited with W+OS and samples were collected at designated time points. Each sample was analyzed by qRT-PCR (transcripts) and HPLC (metabolites). (A) Mean (±SE) of *DH29* relative transcript abundances in control and W+OS-treated leaves from EV-and DH29-VIGS plants at 6 and 20 hours after W+OS elicitation; the constitutive transcript abundances (0 h) were obtained from untreated leaf samples harvested before elicitation. (B) HPLC chromatograms of EV (upper) and DH29-VIGS (lower) methanolic leaf extracts prepared from samples collected 24 h after W+OS elicitation and detected at 320nm wavelength; CP and DCS eluted at retention times 1.9 and 3.8 min, respectively. Concentrations of CP (C) and DCS (D) in EV- and DH29-VIGS leaves at 24, 48 and 72 h after W+OS were quantified based on calibration curves constructed for chlorogenic acid and expressed as CGA equivalents. The constitutive levels were obtained from untreated plants (0 h). All data points represent means (±SE) from 5 independent samples of each EV- and DH29-VIGS plants.

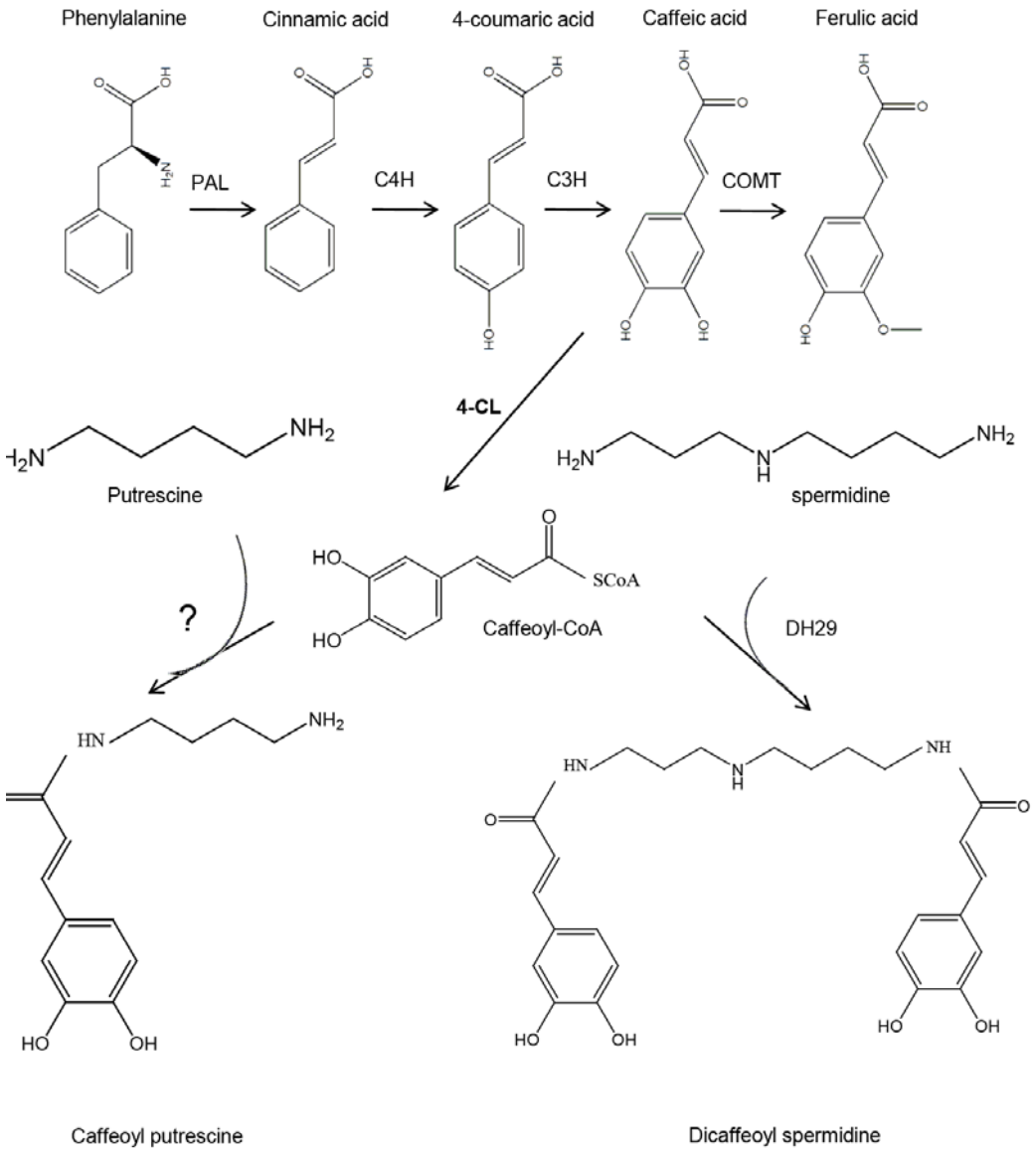


Figure 4: CP and DCS compete for caffeoyl-CoA as an acyl donor in their biosynthetic pathways. Caffeoyl-CoA derived from phenylpropanoid pathway can condense with either putrescine or spermidine to form CP and DCS, respectively. The excess of free caffeoyl-CoA in DH29-VIGS plants leads to enhanced CP biosynthesis and accumulation in *N. attenuata*.

3.2.3 DH29 encodes a functional spermidine caffeoyl-CoA transferase in N. attenuata

Because the accumulation of DCS after simulated herbivory attack in *N. attenuata* was impaired in DH29-silenced plants, we approached determination of the biochemical activity of *DH29 in vitro* by cloning full length *DH29* cDNA into *E. coli* expression vector as fusion with histidine tag (His-tag). His-tagged recombinant protein was purified using Ni-column and subjected to SDS-PAGE, showing a

dominant band at expected size of the recombinant protein (52kD). An enzyme activity assay was then performed using purified recombinant protein fraction, caffeoyl-CoA as acyl donor, and spermidine as acyl acceptor, and the enzymatic reaction was terminated at 15 minutes after addition of caffeoyl-CoA. Protein extract prepared and purified alongside with the DH29 fusion protein -- but using empty vector without insert -- was used in control reaction, showing no conversion of reaction substrates.

The reaction products after 15 min reaction with DH29 protein were subjected to HPLC and liquid chromatography-tandem mass spectrometry (LC-TOFMS) analysis. Caffeoyl-CoA and DCS, show their respective UV absorbance maxima at 360 nm and 320 nm, eluted from the column at close retention times (RT) 3.80 min and 3.83 min, respectively. However, while caffeoyl-CoA peak significantly reduced in 15 minutes reaction mixture compared to time zero (Fig. 5A), no increase in expected DCS peak at RT = 3.83 min could be detected. Surprisingly, a new reaction product peak with UV absorbance spectrum close to DCS appeared at RT 1.08 minutes (Fig. 5A). Using LC-TOFMS analysis and positive-ion electrospray mode, this compound showed a molecular ion $[M+H]^+$ at a mass-to-charge ratio (m/z) 308.20 (Fig. 5B, C), consistent with the expected molecular mass of monocaffeoyl spermidine (MCS). The difference in MCS RT in 5A (1.08 min) and 5B (0.82 min) was due to different chromatographic conditions used in HPLC-PDA and LC-TOFMS analysis. The kinetic of MCS accumulation was determined at different reaction times (0, 5, 10, 15, 20, 30 and 60 minutes), showing that MCS formation was linear up to 30 minutes (Fig. 6A), after which time point the reaction became saturated with a limited additional increase of MCS peak between 30 and 60 minutes (Fig. 6A).

Using LC-TOFMS, we examined whether MCS was the only HCAAs reaction product in the mixture. After calibration of LC-TOFMS system with a real plant sample containing DCS and its possible isomers (Fig. 7), we searched for these products in the DH29 *in vitro* protein reaction using selected ion monitoring mode of LC-TOFMS instrument; however, no traces of DCS isomers or possible higher structure, tricaffeoyl sperimidine (TCS), could be identified in the mixture, suggesting that MCS was the only reaction product of the recombinant DH29 enzyme.

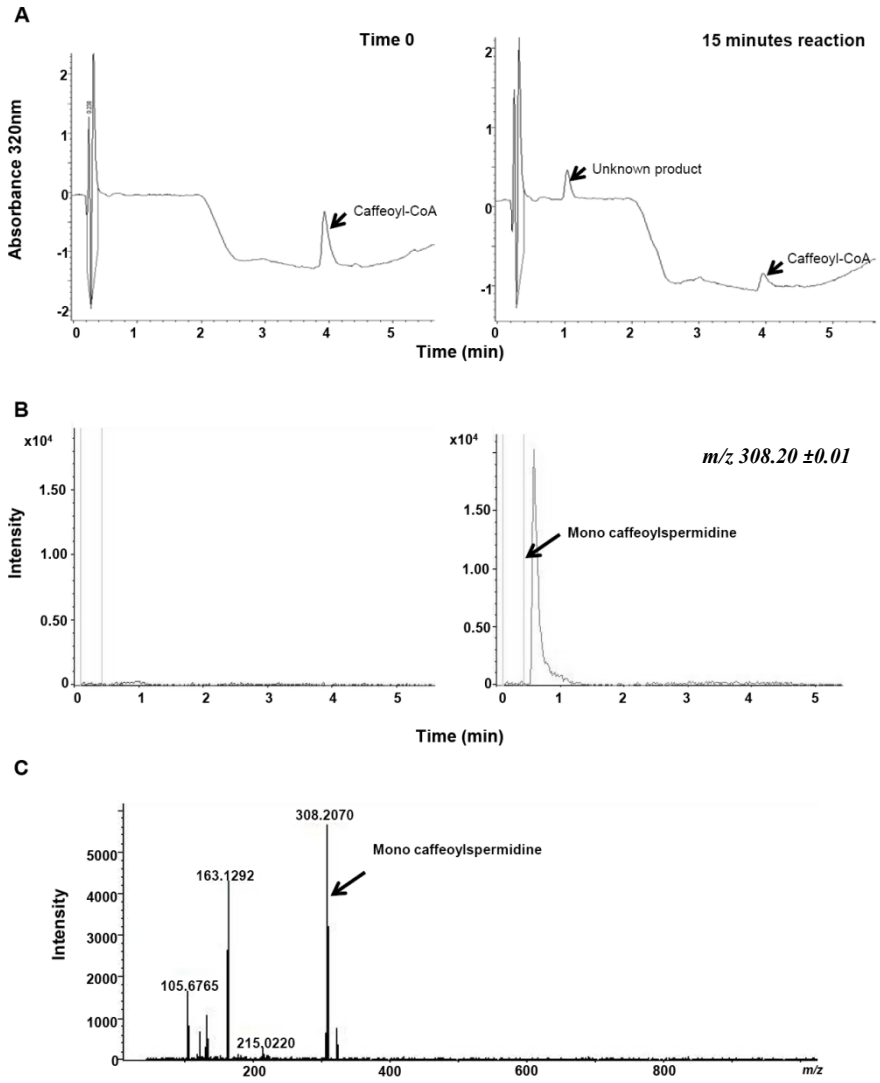


Figure 5: Recombinant DH29 protein shows acyl transferase activity and catalyzes MCS formation *in vitro*. The activity of recombinant DH29 protein was examined in reaction mixture composed of caffeoyl-CoA (acyl donor) and spermidine (acyl acceptor) in Tris-HCl based buffer. The reaction was initiated by addition of caffeoyl-CoA and reaction products were analyzed by HPLC and LC-TOFMS. (A) HPLC chromatograms (top lane) detected at 320nm wavelength show formation of a novel UV-absorbing product at RT 1.08 min and proportional decrease in caffeoyl-CoA concentrations at RT 3.80 min, 15 min after initiation of the reaction with DH29 enzyme. (B) LC-TOFMS selected ion monitoring identified a single reaction product in 15 min reaction mixture with $[M^+H^+]$ at *m/z* 308.20 +/- 0.01 and RT 0.82 min. (C) Mass spectrum of DH29 enzyme reaction product determined by LC-TOFMS at RT: 0.8 min showing MCS molecular ion at (m/z) 308.20 and its major fragmentation product at (m/z) 163.12.

To exclude the possibility that an excess amount of spermidine in the reaction was responsible for the preferential MCS formation, we conducted an experiment with serial dilutions of spermidine (up to 100-fold dilution relative to original reaction), matched with constant amounts of caffeoyl-CoA and DH29 recombinant enzyme added to *in vitro* reactions. While the amount of MCS progressively declined in reactions with the enzyme terminated at 30 min (Fig. 6B), reflecting decreasing

spermidine availability, no DCS or TCS were found as reaction products (data not shown).

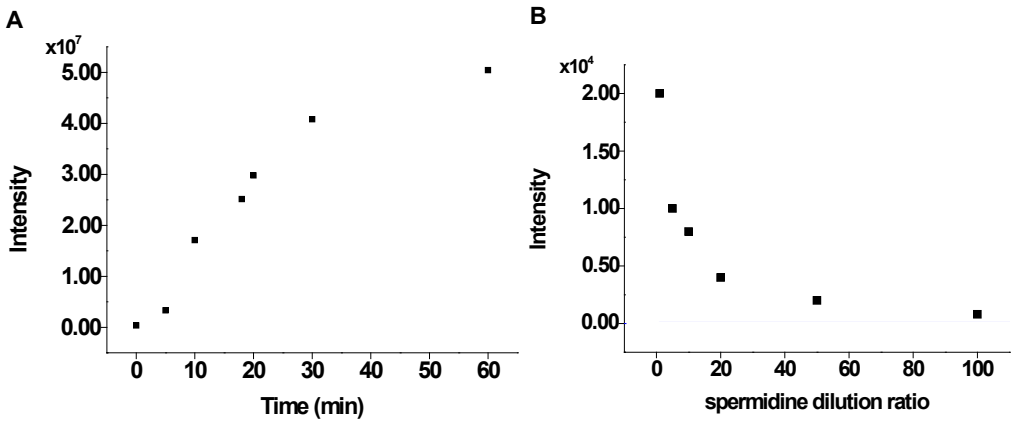


Figure 6: Formation of MCS by recombinant DH29 protein is time and substrate concentration dependent. (A) DH29 activity in recombinant protein assay was interrupted at 5, 10, 15, 20, 30 and 60min after caffeoyl-CoA addition and reaction products were analyzed for MCS formation by LC-TOFMS operating in positive ion monitoring mode; reaction showed saturation after 30 min for given concentrations of substrates and enzyme. (B) Acyl acceptor spermidine concentrations in the reaction were reduced 0-, 5-, 10-, 20-, 50- and 100-times, while caffeoyl-CoA and enzyme concentrations remained constant, and reaction products were analyzed by LC-TOFMS. Even in large excess of acyl donor (caffeoyl-CoA), formation of DCS was not observed.

Interestingly, the presence of MCS peak in the plant samples from EV-VIGS plants could be detected by LC-TOFMS, however DH29-VIGS plants lacked corresponding ion peak (Fig. 7), supporting the major function of DH29 in first acylation step in DCS synthesis, lending strong support to the hypothesis that DH29 functions as spermidine monocaffeoyl-CoA transferase (SmCT) in *N. attenuata* plants. We therefore proposed that DCS biosynthesis in *N. attenuata* occurs in two-step enzymatic reaction, in which SmCT (DH29) conjugates first caffeoyl-CoA molecule to spermidine, resulting in MCS, followed by an unknown second enzyme activity that conjugates additional caffeoyl-CoA to MCS, leading to formation of DCS and its isomers. Nevertheless, and alternative pathway involving a transamination of two MCS molecules catalyzed by an unknown enzyme cannot be excluded, and therefore more transcriptomic and metabolomic analysis is required to reconstruct the complete DCS biosynthetic pathway in *N. attenuata*.

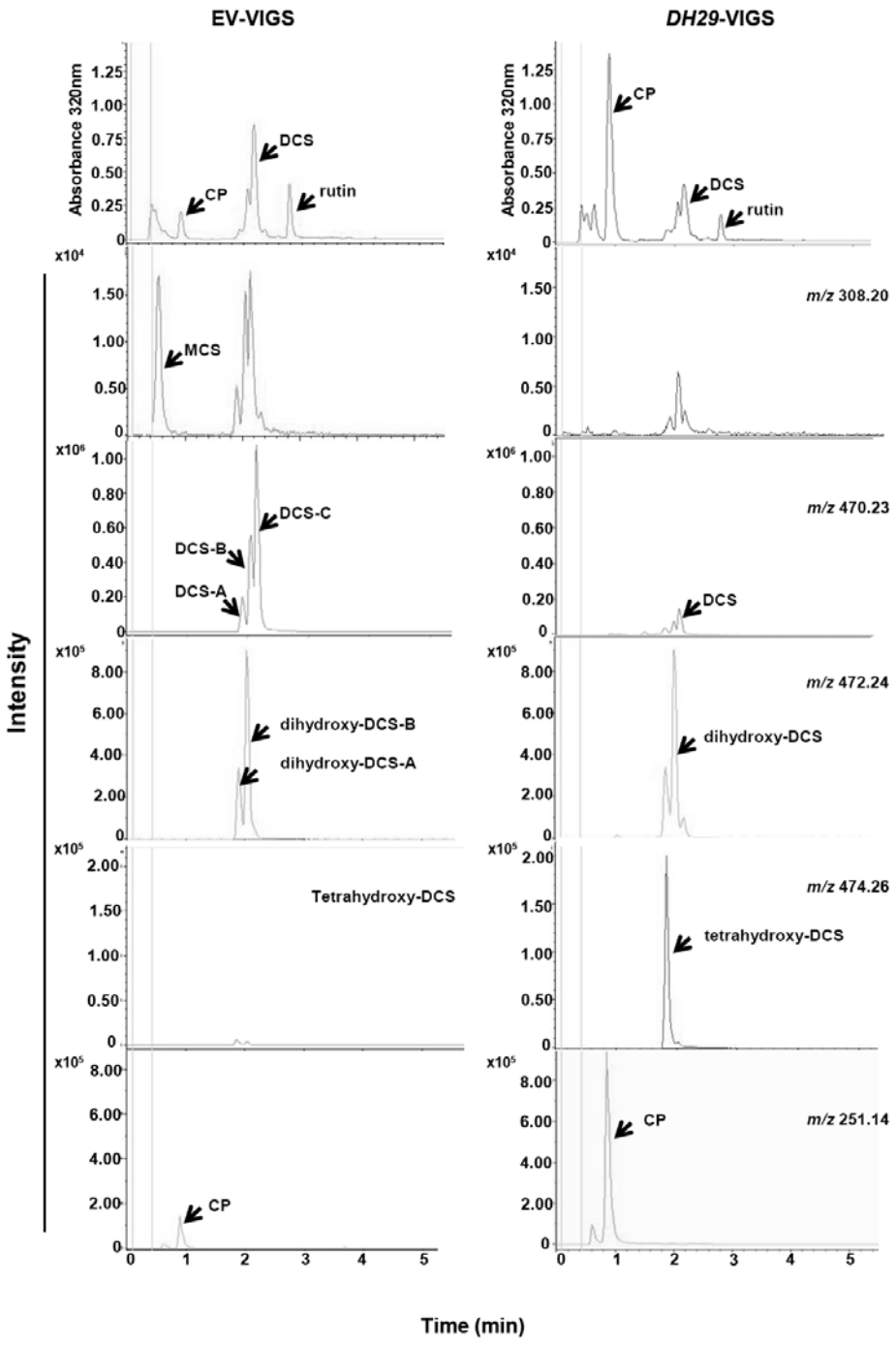


Figure 7: W+OS-treated DH29-VIGS plants show altered patterns of CP and DCS accumulation. The leaves of EV- and DH29-VIGS plants were induced by W+OS and whole leaves were collected 24 h later for LC-TOFMS analysis. UV absorbance chromatograms from EV (left panel) and DH29-VIGS (right panel) leaves extracted in 40% methanol and detected at 320 nm wavelength are shown in top line. Below, LC-TOFMS selected ion traces of EV (left panel) and DH29-VIGS (right panel) identified in positive selected ion monitoring mode are shown. Compounds were identified by their specific mass and retention time (RT), and where possible, in combination with observed UV-absorbing peaks and their known UV absorption spectra: MCS (*m/z* 308.20, RT: 0.82min), DCS-A, DCS-B and DCS-C (*m/z* 470.23, RT: 1.90, 2.05 and 2.18min, respectively), dihydroxy-DCS-A and dihydroxy-DCS-B (*m/z* 472.24, RT: 1.90 and 2.05min), tetrahydroxy-DCS (m/z 474.26, RT 2.0min) and CP (m/z 251.14, RT: 0.90min).

3.2.4 Accumulation of DCS in plant onthogeny

Revealing the identity of a biosynthetic enzyme for DCS in *N. attenuata*, we were interested in distribution and ecological function of this metabolite in plants. We therefore analyzed DCS accumulation throughout the plant development in different plant tissues, as well as in tissues of plants subjected to simulated herbivory treatment (W+OS). In the previous report, we showed that MYB8-controlled CP accumulation plays important role in direct plant defense against herbivores (Kaur at al., 2010). DCS accumulation followed similar pattern that was previously shown for CP: young rosette leaves contained highest levels of DCS, which further increased after W+OS elicitation (Fig. 8). In the flowering plants, the accumulation of DCS was no further elicited by DCS and the content of DCS in flowering and mature vegetative tissues remained very low. Similar to CP, reproductive organs of *N. attenuata* at mature stage of development accumulated higher levels of DCS (Kaur et al., 2010; Fig. 8). Interestingly, DCS was never detected in the roots at any developmental stage.

SmCT (DH29) expression was determined in the plants and their respective tissues at elongated stage, showing that *SmCT* expression coarsely correlated with the accumulation of DCS in different plant organs, as well as it followed the increased DCS accumulation after W+OS elicitation (data not shown). The inducible accumulation of DCS suggested that the enzyme is regulated by cues contained in *M. sexta* OS known to activate hormone signaling cascade in *N. attenuata* plants, including JA and ET as mediators of defense response.

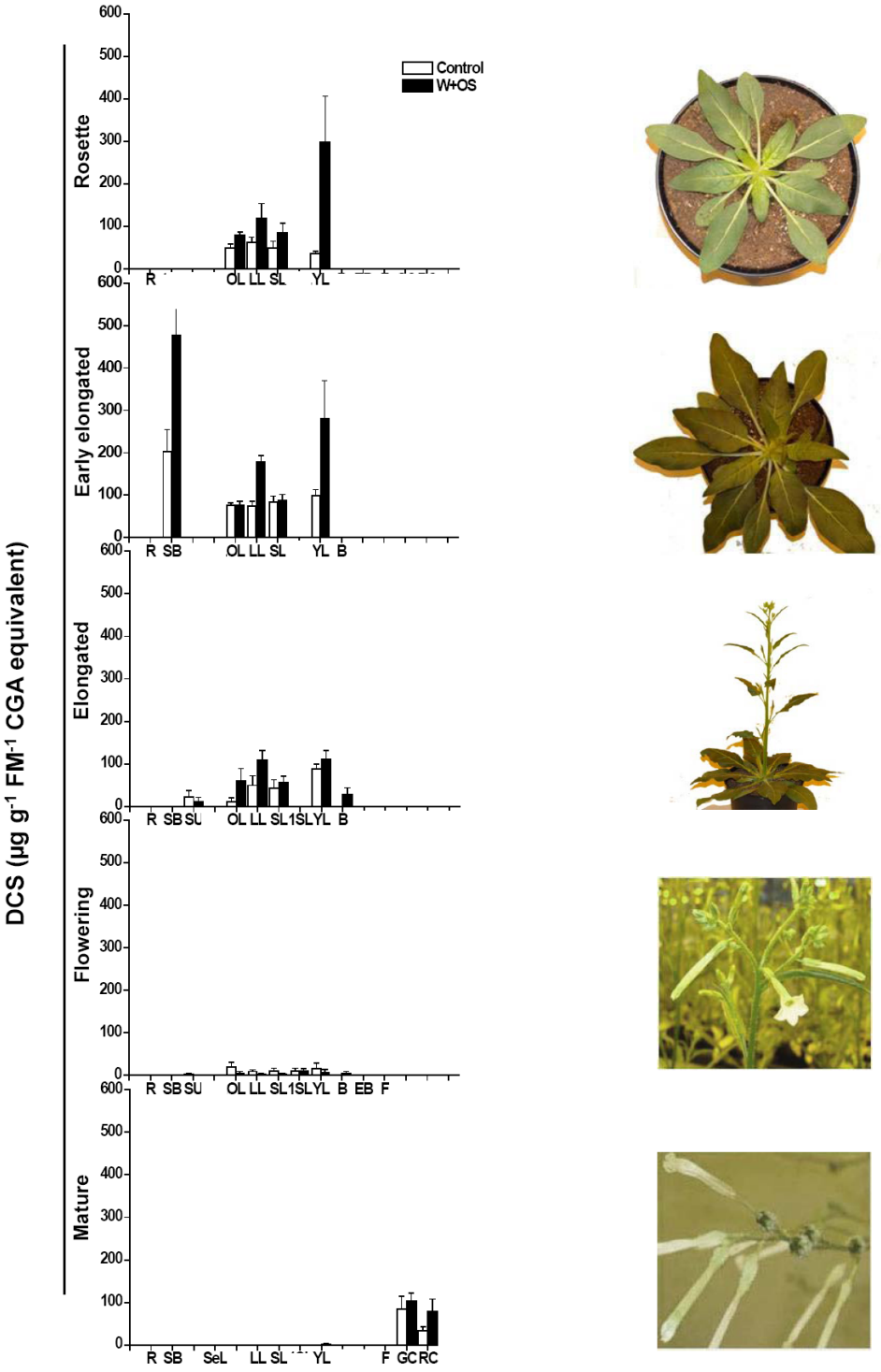


Figure 8: W+OS-elicited leaves and reproductive tissues of *N. attenuata* accumulate high levels of DCS. WT plants were germinated in sand and supplemented with nutrients dissolved in water to the roots. At each developmental stage, a single rosette leaf was treated by W+OS (black bars) and three days later the samples were collected (n=5); control samples (open bars) were collected from untreated plants at similar position on the plant. Samples from all available plant parts at specific stage of development were analyzed by HPLC for DCS content (only analyzed parts are labeled at *x-axis*). R, root; SB, stem basal; SU, stem upper; SeL, senescent leaf; OL, old leaf; LL, local rosette leaf (W+OS induced); SL, systemic rosette leaf; 1SL, first stem leaf; YL, young stem leaf; B, flower buds; EB, elongated flower buds; F, open flowers; GC, green capsules with seeds; RC, ripe capsules with seeds, FM, fresh mass.

3.2.5 SmCT transcript accumulation is controlled by JA and ET crosstalk

We demonstrated that *SmCT* transcript accumulation in *N. attenuata* in the previous paragraphs is under control of MYB8-transcription factor (Fig. 1A). Jasmonic acid (JA) and ethylene (ET) are well-known herbivory-associated signals that activate downstream defense responses in plants (Adie et al., 2007; von Dahl et al., 2007; Howe and Jander 2008; Bari and Jones; 2009), as well as CP accumulation in tobacco plants is known to be regulated by JA (Keinanen et al., 2001; Chen et al., 2006; Paschold et al., 2008). We therefore examined whether JA and ET could be directly involved in regulating *SmCT* expression. In our previous analysis of microarray data, we found that the expression of *SmCT* was strongly diminished in asLOX3 (low JA production) plants 6 h after W+OS treatment, while it did not change significantly from WT levels in mETR1 plants (ethylene-insensitive). Interestingly, the gene expression in the genetic cross between asLOX3 and mETR (referred to as mETRasLOX3; see Chapter 2) was intermediate between WT and asLOX3, suggesting that a crosstalk between ET and JA could play an important role in regulating *SmCT* gene transcription (microarray data not shown).

RT-qPCR was then used to confirm the signatures proposed by the microarray analysis, using asLOX3, mETR1 and mETR1asLOX3 transgenic lines and comparing them to WT plants. Moreover, because *MYB8* transcription factor functions as an upstream regulator of *SmCT* transcription, we also analyzed *MYB8* transcripts in the transformed lines and WT to determine whether *SmCT* is tightly co-expressed with *MYB8* transcription factor. As expected from MYB8-dependent metabolite accumulation, *MYB8* and *SmCT* transcripts were strongly reduced in asLOX3 plants at all measured time points (0, 1, 3, 6, 20 and 60 h) after W+OS treatment, consistent with the microarray data (Fig. 9A, B). While, *MYB8* transcripts in mETR1 plants were similar to WT, *SmCT* transcript accumulation in these plants was significantly reduced at 3h after induction relative to WT (Fig. 9A, B). Interestingly, mETR1asLOX3 plants showed significantly more transcripts of *SmCT* gene compared to asLOX3 plants at 6 h time point after W+OS treatment, with a similar but less pronounced trend also found in *MYB8* transcription. These results, which were further verified with samples from an independent biological replicate of the experiment (Fig. 9, insets), indicated that a complex JA-ET crosstalk is involved in integration of W+OS signals, and *MYB8* and *SmCT* transcription. While it was clear that *SmCT* and *MYB8* genes are both strongly dependent on JA, and SmCT

followed MYB8 transcriptional activity in most cases, ET seemed to differentially contribute to *SmCT* regulation on high (3h; positive regulator) and low (6h; negative regulator) JA background, which was partially uncoupled from the presence of MYB8 transcripts in the cells (3 h time point).

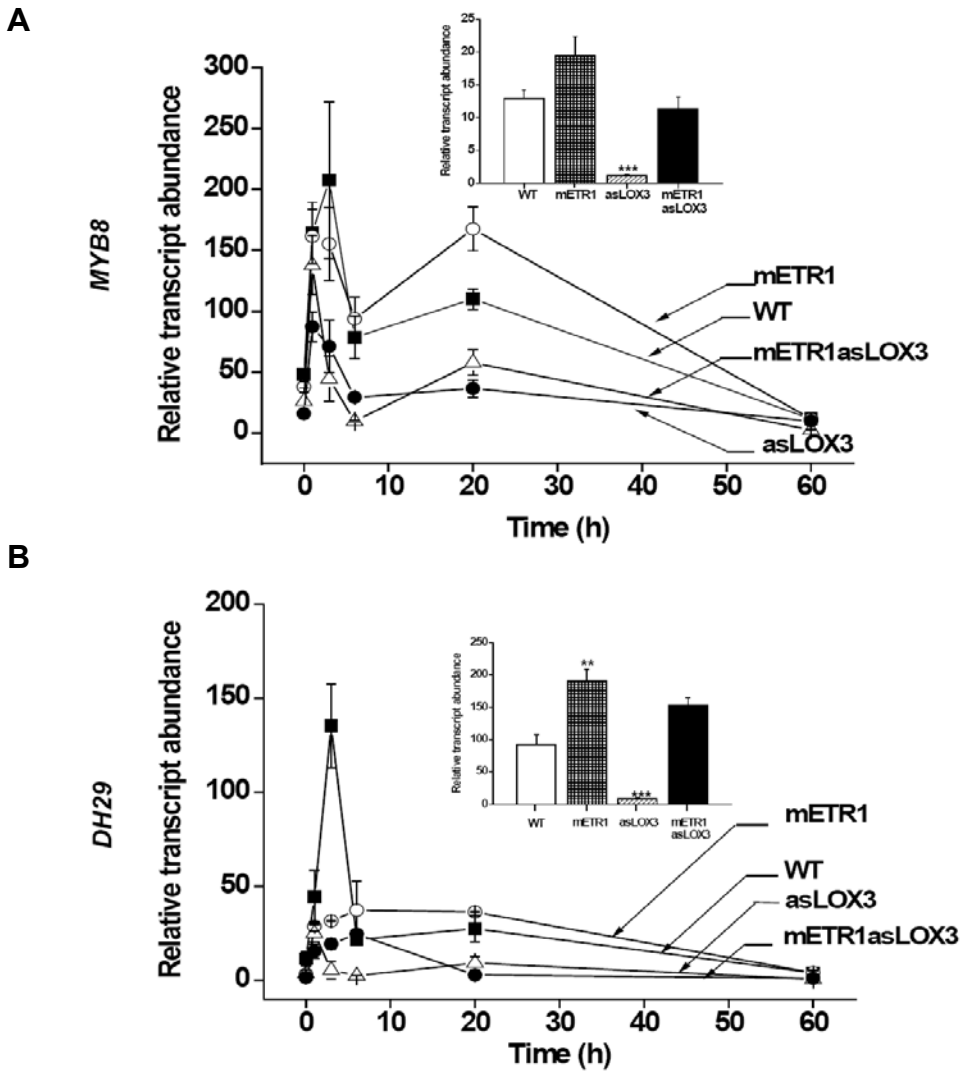


Figure 9: JA-ET crosstalk regulates expression of *MYB8* and *SmCT* in W+OS-treated *N. attenuata* leaves. (A) *MYB8* and (B) *SmCT* relative transcript abundances in WT, mETR1, asLOX3 and mETR1asLOX3 leaves at 0, 1, 3, 6, 20 and 60 h after treatment with W+OS. All data points are means (±SE) of 3 independent biologically replicated samples. Insets show detail of transcript accumulation of *MYB8* and *SmCT* at 6h after W+OS treatment from another independent experiment with transgenic lines; expression of both genes in mETR1asLOX3 is increased relative to asLOX3 plants, suggesting an alternative ET-suppressed pathway to regulate the expression of *MYB8* and *SmCT* genes in mETR1asLOX3 plants.

To confirm that JA-ET crosstalk, rather than different levels of JA in the mETRasLOX3 cross is responsible for the higher *SmCT* transcript accumulation in mETR1asLOX3 leaves, we chemically blocked ethylene receptors by 1-methylcyclopropane (1-MCP), an ethylene receptor antagonist, in asLOX3 plant background, and subsequently elicited these plants with W+OS. Mock-treated plants

with 0.75% NaOH+KOH, used to dissolve and activate the release of 1-MCP from Smart Fresh^{TM} commercial substrate, were used as controls. The *SmCT* gene showed significantly higher transcript accumulation in 1-MCP-treated leaves at 20 h after W+OS elicitation compared to mock-treated plants (Fig. 10B), consistent with the results obtained previously in transgenic plants (Fig. 9B).

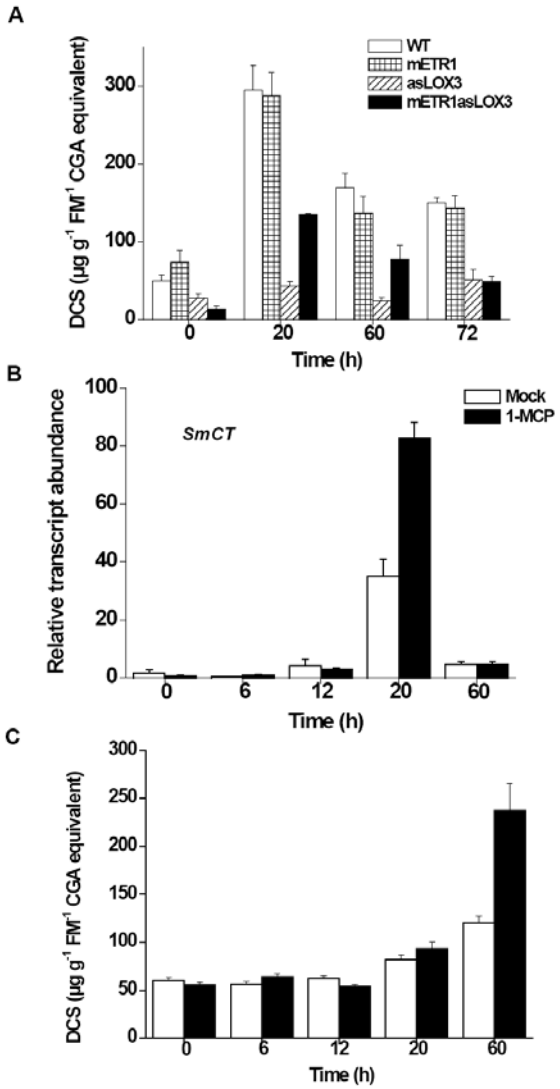


Figure 10: JA-ET crosstalk modulates DCS accumulation after W+OS treatment in *N. attenuata*. WT, mETR1, asLOX3 and mETR1asLOX3 rosette leaves were treated with W+OS and samples were collected at 0, 20, 60 and 72 hour later. (A) Mean (±SE) concentrations of DCS in transgenic and WT leaves (n=3) analyzed by HPLC; untreated leaves at time 0 h were used as controls. (B) *SmCT* relative transcript abundances in the leaves from asLOX3 plants that were pre-treated with ethylene receptor antagonist 1-MCP (10mg/mL in 0.75%NaOH+KOH; black bars) or with alkaline solution (mock treatment; open bars) overnight, and had the leaves elicited with W+OS (n=5). Samples were collected at 0, 6, 12, 20 and 60h after W+OS treatment and analyzed with qRT-PCR. (C) Mean (±SE) concentrations of DCS in mock (open bars) and 1-MCP treated (black bars) asLOX3 leaves (n=5) used for gene expression analysis above.

The results of transcript analysis, and higher transcript accumulation of *SmCT* in mETR1asLOX3 compared to asLOX3 plants in particular, led us to the analysis of DCS accumulation in mETR1, asLOX3 and mETR1asLOX3 transgenic lines and

WT. DCS accumulation was determined at 0, 24, 48 and 72 h after W+OS induction in rosette stage plants, using HPLC system with PDA detection as before. DCS strongly accumulated in WT and mETR1plants after W+OS elicitation; however, asLOX3 plants accumulated significantly less DCS in their leaves. In agreement with transcript accumulation data, mETR1asLOX3 plants still accumulated significant amounts of DSC in their leaves, despite being reportedly deficient in their JA production (see Chapter 2). In addition, we found significantly more DCS in 1-MCP-treated leaves of asLOX3 plants at 60 hours after W+OS treatment (Fig. 10C), consistent with the higher *SmCT* transcript levels in these plants (Fig. 10B).

3.2.6 Attempts to determine biological function DCS

JA and ET represent two early stress signals in plants that activate broad range of plant's direct and indirect defenses (Halitschke and Baldwin, 2003; Broekaert et al., 2006; von Dahl et al., 2007; Paschold et al., 2008; Howe and Jander 2008; Bari and Jones; 2009). Indeed, JA-dependent and MYB8-regulated accumulation of CP was recently shown to function as important defensive trait in *N. attenuata* plants that defend against herbivores (Kaur et al. 2010). Because DCS accumulation was co-silenced in irMYB8 plants, we speculated that this metabolite could play similar role as CP in anti-herbivore defense. To determine a possible ecological function of DCS in plants, we placed newly hatched *M. sexta* neonates on elongated/flowering EV-VIGS and SmCT (DH29)-VIGS plants, and determined caterpillar mass gain at 4, 7, 10 and 12 days after initiation of feeding. However, we found no significant difference in caterpillar growth between EV-VIGS and SmCT-VIGS-fed caterpillars (Fig. 11B).

Considering the data presented in Fig. 11B, this results was not unexpected. Even though SmCT-VIGS plants contained low levels of DCS, they also had high compensatory levels of CP. Therefore, the lack of DCS could have been compensated by an increase in CP in SmCT-VIGS plants, which, pending overlapping defense role of both metabolites may have resulted in no significant differences in caterpillar growth on these plants. To realistically estimate the biological function of DCS, we would have to create plants with low silenced DCS levels but normal (or no) CP content to eliminate the artifacts associated with CP over-accumulation. Recently, Kaur et al. (2010) demonstrated that CP accumulation in the leaves strongly deceased with plant age; based on these observations, we

decided to perform another herbivory bioassay with mature SmCT-VIGS and EV-VIGS plants. Surprisingly, *M. sexta* caterpillars performed significantly worse on SmCT-VIGS plants (Fig. 11C), which was in strong contrast with expected defensive role of DCS against herbivores.

We therefore performed a more detailed examination of CP and DCS accumulation profiles, using different developmental stages of EV- and SmCT-VIGS plants. Here, the early elongated/elongated plants, and their CP and DCS contents, followed our previous results with WT W+OS-elicited leaves (Fig. 11A and Fig. 3C,D), and the levels of CP expectedly decreased towards flowering and mature stages in EV-VIGS plants. While DCS levels in older plant leaves remained slightly higher than CP (Fig. 11A), the levels of DCS did not change significantly by VIGS silencing in the flowering and mature plants, suggesting a low biosynthetic rates and slow turnover of the metabolite in these plants. Unexpectedly, CP content in SmCT-VIGS plants again strongly increased to more than 300 µg per g FM when the plants entered flowering stage, and it reached almost 600 µg per g FM concentrations of CP in mature plant leaves that have been silenced in SmCT activity, explaining poor performance of *M. sexta* larvae on these plants (Fig. 11C). Inferring from defensive role of CP against herbivores (Kaur et al., 2010), we concluded that ectopic accumulation of high levels of CP in mature plants had major adverse effect on *M. sexta* caterpillar performance, further confirming the role of CP in anti-herbivore defense, but disabling the analysis of ecological function of DCS in *N. attenuata* plants.

In an alternative approach, we propose chemical synthesis of DCS and application of synthetic DCS to irMYB8 plants, deficient in accumulation of both CP and DCS, which should then enable determination of DCS function in *N. attenuata* plants. However, falling beyond the scope and time limits of this work, this task will be conducted and results reported in the future.

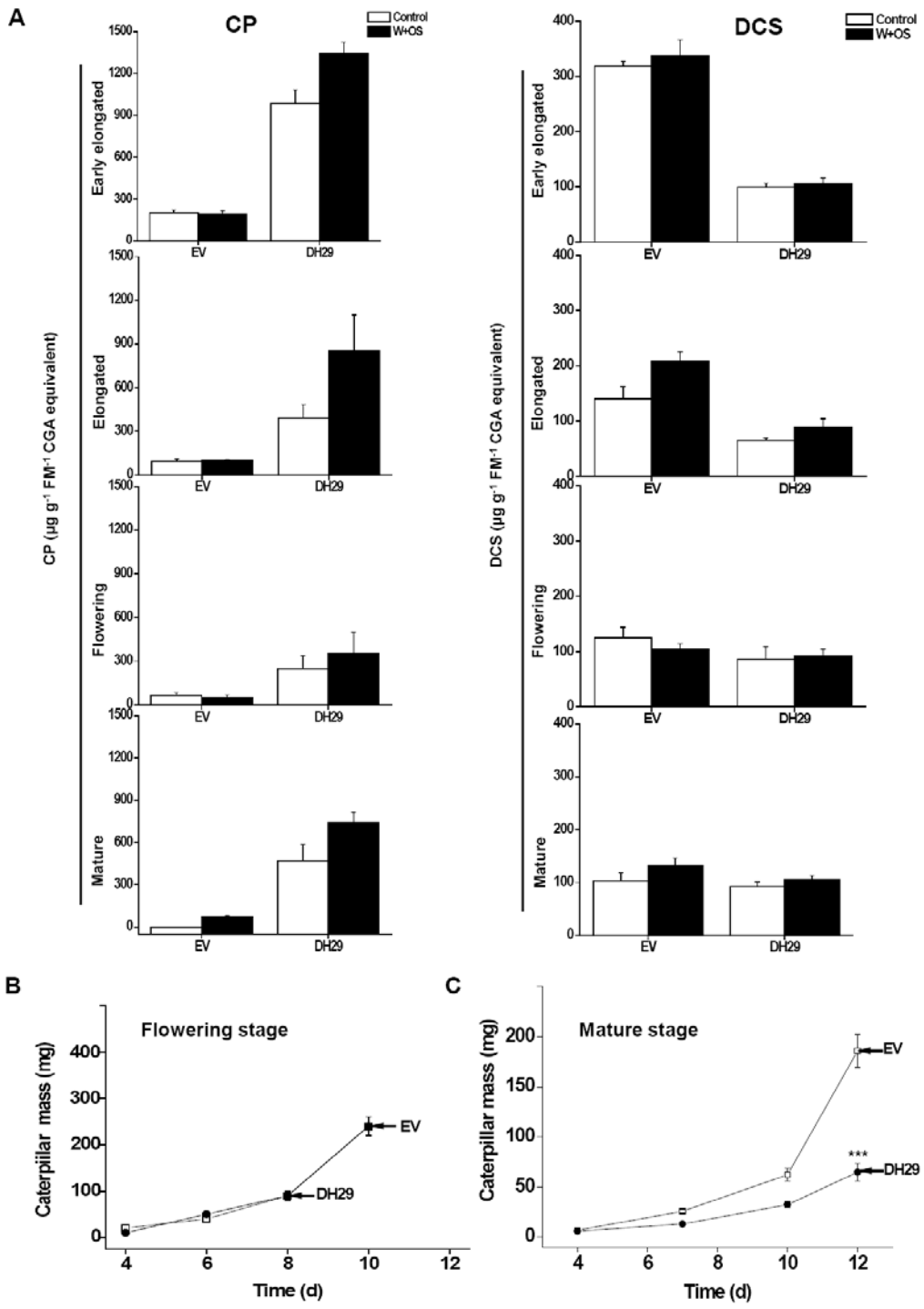


Figure 11: High CP accumulation in SmCT (DH29)-silenced plants makes *N. attenuata* more resistant to *M. sexta.* To monitor the accumulation of DCS and CP in the leaves, rosette leaves of EV- and DH29-VIGS plants were elicited with W+OS at different developmental stages and analyzed by HPLC for their CP and DCS contents before (control; open bars) and 1 day after elicitation (W+OS; black bars). (A) CP (left panel) and DCS (right panel) concentrations show dynamic changes which depend on plant age and silencing of *SmCT* gene. (B) Mean (±SE) mass of caterpillars feeding on 25 independent elongated/flowering stage EV and SmCT-VIGS plants at 4, 7, 10 and 12 d after feeding. (C) Caterpillars feeding on flowering/mature stage plants with highly increased content of CP. Asterisks represents significant differences of *M. sexta* caterpillar masses between caterpillars fed on EV and those fed on DH29-VIGS plants (Student's *t*-test, *** $P < 0.001$)..

3.3 Discussion

HCAAs, a group of abundant secondary metabolites in higher plants, accumulate to higher levels in response to biotic and abiotic stress factors. DCS, one of the major HCAAs in *N. attenuata*, accumulates in the leaf tissues after herbivory attack or upon irradiation of plants with UV light (Izaguirre et al., 2007; Kaur et al, 2010). In this study, we identified a novel spermidine monocaffeoyl transferease (SmCT) enzyme, and show that it is essential for MCS and therefore DCS biosynthesis in *N. attenuata* plants. Silencing of *SmCT* gene expression revealed a strong metabolic link between CP and DCS biosynthetic pathways in *N. attenuata*: while DCS levels were significantly reduced in *SmCT*-silenced plants, CP content strongly increased, both constitutively and after elicitation of the leaves with W+OS. Examination of *SmCT* transcript accumulation in JA-deficient and ET-insensitive plants revealed that JA regulates *SmCT* expression through the transcriptional activity of MYB8 transcription factor, while ET might be modulating *MYB8*-expression by interacting with yet unknown alternative pathway leading to *MYB8* and *SmCT* gene activation. Silencing of *SmCT* significantly reduced caterpillar performance on these plants, most probably due to high ectopic levels of CP, confirming previous report of CP as anti-herbivory defense in plants (Kaur et al., 2010) -- but hampered our intention to investigate the eco-physiological roles of DCS in plants.

3.3.1 JA-ET crosstalk in plant defense

Plants recognize and defend against biotic and abiotic stress conditions, which is often mediated by phytohormone-dependent signal transduction pathways (Katsir et al, 2008; Kazan and Manners, 2008; Bari and Jones, 2009; Pieterse et al., 2009). JA and ET are well-known plant stress-associated hormones that trigger broad range of plant defenses against biotic stress including insect herbivores and necrotrophic pathogens (Farmer et al., 2003; von Dahl et al., 2007; Paschold et al., 2008; Stepanova and Alonso; 2009). In *N. attenuata* plants, a large increase in JA concentrations 1h after W+OS elicitation (so called JA-burst) acts as an early signal to activate plant's direct (toxic secondary metabolites production i.e. nicotine and trypsin protenase inhibitors [TPIs]) and indirect (volatile production that attracts predators of herbivores) defenses against herbivores (Baldwin, 1996; Halitschke et

al., 2000; Steppuhn et al., 2004; Pluskota et al., 2007; Paschold et al., 2008). JA-burst is highly transient and the peak levels of JA normally return to their basal levels several hours after elicitation (McCloud and Baldwin, 1997; Stork et al., 2009). Interestingly, large amounts of ET (ET-burst) are emitted from W+OS-elicited plants just after or during decrease in JA content, peaking around 3h, and the amounts of ET strongly correlate with the presence of herbivore-associated elicitors (FACc) in the wounds (Von Dahl et al, 2007). In the current understanding, ET acts primarily as a modulator of JA-signaling, tailoring plant's direct and indirect defenses in response to herbivores (Von Dahl and Baldwin, 2007).

Previously, Goossens et al. (2003) demonstrated that methyl jasmonate (MeJA) alone can induce the expression of many alkaloid and phenylproponoid biosynthetic genes in tobacco BY- 2 cells. However, other reports suggest that multilayer regulation mechanisms are involved in controlling alkaloid and phenylproponoid biosynthesis at the whole plant level. For example, Kahl et al. (2000) demonstrated that ET, apart from JA, regulated alkaloid nicotine biosynthesis during herbivore attack in *N. attenuata* plants, suggesting that direct defense-related genes are co-regulated by JA and ET signals during herbivore attack. Here, we demonstrate that the *SmCT* gene (previously known as *DH29*) shows some properties of a defense gene that is regulated by crosstalk mechanisms involving JA and ET.

While the induction of *SmCT* at 3 hours after W+OS elicitation was strongly suppressed by ET insensitivity, suggesting a positive role of ethylene in regulation, its expression was somewhat promoted by the same ET-insensitivity trait at 3 and 6 h in mETR1asLOX3 plants when compared to that of asLOX3 genotype. Chemical block of ET-receptors in asLOX3 genetic background by 1-MCP also increased *SmCT* expression in these low JA producing plants (Fig. 10B), strengthening the observations made with the mETRasLOX3 genetic cross (Fig. 9B). Additionally, the increases in *SmCT* transcripts were accompanied by the corresponding increases in accumulation of DCS in MCP-1-treated leaves compared to untreated control asLOX3 leaves (Fig. 10C). Even though the exact molecular mechanism of the interaction between JA and ET remains unknown, these seemingly contradictory observations could be explained by the existence of two independent signaling pathways leading to the induction of *SmCT* gene.

It has been shown that both constitutive and ABA-induced expression of *KIN1* gene was strongly enhanced in *Arabidopsis* etr1-1 mutant (an equivalent of our

mETR1 plants), suggesting that ET can act as a suppressor of ABA signaling in vegetative plant tissues (Anderson et al. 2004). In addition, ABA is known to induce the expression of At*MYC2* gene in *Arabidopsis* (Abe et al., 1997; 2003) that can therefore lead to the hypothetical activation of the AtMYC2-dependent "JA-responsive" gene expression in the absence of JA, lending support to the following model and explanation of *SmCT* gene expression patterns in our transgenic plants (Fig. 9B).

In WT plants, JA-dependent and ET-potentiated induction of *SmCT* transcripts would reach maximal induction levels, which would be then partially reduced in mETR1 genotype due to compromised ET signaling of these plants (Fig. 9B; 3h-mETR). In asLOX3 plants, the induction of *SmCT* would be completely abolished due to the lack of primary inducer JA and, at the same time, alternative induction of At*MYC2* by ABA-dependent signaling pathway would be suppressed by the ABA antagonist; ET. Finally, in mETR1asLOX3 plants, the *SmCT* gene would be induced via JA-independent and ABA-dependent pathway, which would be now de-repressed due to ET insensitive genotype of mETR1asLOX3 plants. Even though this model fits to our observations, and could represent a more general mechanism used by plants to discriminate between wounding (associated with lower JA and ET levels) and herbivory (high JA and high ET), it is based on the strongly hypothetical role of ABA in the response, which has yet to be examined. As an essential prerequisite of the model to be operational, ABA levels usually increase together with JA and ET after elicitation of plants with W+OS, possibly as a result of local exposure of wounded tissues and drought symptoms in the wounded areas of the leaves (unpublished data).

As one possible test of this hypothesis, the plants deficient in JA and ET signaling (e.g. mETR1asLOX3 or mETR1irCOI1) should be subjected to ABA treatment and examined for their expression of some typical *N. attenuata* JA-inducible marker gene such as TPIs, expecting that the expression of these genes would be elicited via ABA-MYC2 signal transduction pathway. This response should be then absent in MYC2 mutant plants, pending the availability of *N. attenuata* plants with the down-regulated expression of *N. attenuata*'s MYC2 in JA and ET signaling-deficient background, which can be achieved, for example, by the VIGS technology already used in this paper to transiently silence the *SmCT* expression.

3.3.2 MYB8 transcription factor links JA to SmCT expression and DCS accumulation

Plant stress signals usually activate transcription factors, which then bind to the promoter sites in defense-related genes and induce or suppress their expression (Eulgem et al., 2000; Sugimoto et al., 2000; Singh et al., 2002). MYB transcription factors, a large family of plant-specific transcriptional regulators, are involved in regulation of many plant defense-related genes during exposure of plants to stress (Sugimoto et al., 2000; Chen et al., 2002; de Vos et al., 2006). Kaur et al. (2010) demonstrated that CP and DCS accumulation was dramatically reduced in irMYB8 plants, which resulted in lower defense capacity of the plants against herbivores. It suggested that MYB8 specifically regulates plant defense by activating genes involved in CP and DCS biosynthesis in *N. attenuata*. Other MYB transcription factors have already been shown to function as master regulators in very diverse phenylpropanoid pathway, leading to coordinated gene expression and accumulation of specific bioproducts. For example, the overexpression of PAP1 (AtMYB75) gene in *Arabidopsis* triggers ectopic accumulation of anthocyanins in the plants, which involves regulation of multiple anthocyanin biosynthesis-related genes by a single master regulatory transcription factor PAP1 (Teng et al., 2005; Tohge et al., 2005). Similar to PAP1, MYB8 was found to regulate multiple genes, including the rate limiting PAL expression, as well as genes downstream of PAL ensuring accumulation of a specific branch of metabolites (CP and DCS) in secondary metabolism (Kaur et al., 2010). Here, MYB8-regulated *SmCT* gene expression represents one of the important factors involved in general metabolite channeling of phenylpropanoids towards DCS biosynthesis.

3.3.3 DCS biosynthesis, derivatives and intermediates

Polyamines like spermidine offer multiple structural possibilities to form conjugates with hydroxycinnamic acids, resulting in different stereoisomers and dihydrogenated forms. In nature, DCS can occur as three possible stereoisomers (Fig. 7) due to three *N*-positions in spermidine where caffeoyl-CoA can be attached. In *N. attenuata*, a stereoisomer with retention time 3.83 min seems to be the dominant form of DCS, assuming that the stereoisomers possess comparable UV-absorbance during PDA-based detection of the metabolites by HPLC. While the amount of all three stereoisomers of DCS decreased in SmCT-silenced plants, the dihydroxy-DCS

peak areas relatively increased in *SmCT*-silenced plants compared to EV-inoculated plants, data based on selected ion monitoring (SIM) from LC-TOFMS analysis (Fig. 7). Unfortunately, the internal standards for different isomers of DCS and dihydro-DCS have not been available in this study and absolute quantification of each form of DCS could not be currently achieved. Future quantification of DCS isomers based on authentic standards should be conducted to assign biological importance of DCS structural variability in ecological and physiological function of these metabolite in plants.

The spermidine acyl transferase activity is normally required for mono-, di- and tri- substitution (Grienenberger et al., 2009; Luo et al., 2009) of spermidine with activated hydroxycinnamic acids, posing a dilemma of whether all three biosynthetic steps are mediated by the same enzyme or each of the steps requires a specific enzyme, which should be co-expressed during HCAAs biosynthesis. Two major caffeic acid conjugates with polyamines have been documented in tobacco plants, CP and DCS (Eich, 2008), while the reports of spermidine substituted with three caffeic acid moieties (tricaffeoylspermidine) in tobacco have not been, to our best knowledge, reported in the literature. While the enzyme involved in CP biosynthesis remains unknown, existence of one or two enzymes could be hypothesized in DCS biosynthesis, one or both of them being encoded by the *SmCT* gene described in this study.

HCBT, ACT and AsHHT1 are three examples of BAHD proteins that can condense amino group from polyamines and carboxyl group from hydroxycinnamoyl-CoA to form HCAAs (Yang et al., 1997; Burhenne et al., 2003; Yang et al., 2004). Recently, three novel spermidine acyl transferases (SDT, SCT and SHT) responsible for condensation of either feruloyl-CoA or sinapoyl-CoA to spermidine have been identified in *Arabidopsis* (Grienenberger et al., 2009; Luo et al., 2009). Noteworthy, these newly identified enzymes were capable of condensation of two to three molecules of feruloyl-CoA or sinapoyl-CoA to spermidine under *in vitro* reaction conditions. On the other hand, recombinant SmCT enzyme could only condense one molecule of caffeoyl-CoA to spermidine, resulting in the formation of MCS with unknown stereo-specificity (Fig. 5A, B); the defect in MCS biosynthesis then presumably leads to the low DCS content found in *SmCT*-silenced plants (Fig. 3D), even in the presence of the hypothetical enzyme for MCS to DCS conversion. However, it cannot be excluded that our recombinant fusion

protein has lost some of its natural activity to mediate the second step in DCS formation, and further experiments are needed to confirm or falsify the hypothesis of DCS biosynthesis in a two enzyme step process. The isolation of the second step enzyme would be the most straightforward falsification of this hypothesis.

3.3.4 DCS function in N. attenuata: growth or defense?

Functions of HCAAs in growth and development have been frequently proposed, based on the observations of HCAAs accumulation in the plant reproductive organs (Back et al., 2001; Facchini et al., 2002; Edreva et al., 2007). Martin-Tanguy (1985) in his early review on distribution and possible function of HCAAs in plants have reported that HCAAs, including CP and caffeoyl spermidine (CS), highly accumulate in inflorescences of tobacco plants as well as the content of HCAAs increased upon viral infection and hypersensitive lesion formation in tobacco plants. Recently, Kaur et al. (2010) demonstrated that CP distribution in *N. attenuata* follows previously reported trends of accumulation, which was further extended in this study to DCS distribution and accumulation in WT *N. attenuata* plants (Fig. 8). Again, DCS mainly accumulated in the reproductive organs of mature plants, at which stage DCS was almost absent in the leaves (Fig. 8). These results suggest that DCS could play an important role in flower and capsule development in *N. attenuata*, however this role is contradictory to the observations of completely normal development and reproductive fitness of irMYB8 plants that lack most of their CP and DCS content due to silencing of a master regulator (MYB8) in their biosynthesis (Kaur et al., 2010).

In contrast, Kaur et al. (2010) showed that CP- and DCS-deficient plants were more sensitive to herbivores, emphasizing the defensive role of HCAAs in *N. attenuata* plants. We found that *SmCT*-silenced plants were better defended against *M. sexta* caterpillars compared to EV-inoculated plants, which correlated with abnormally high levels of CP accumulated in these plants. Due to the interference of CP, methods alternative to direct silencing of DCS biosynthesis should be used to avoid compensatory effects of CP accumulation after shutting down the DCS production. This task could be, for example, resolved in the future by applying synthetic DCS to plants lacking DCS and CP; an ideal model for such study would then be the irMYB8 plants (Kaur et al., 2010).

Hydroxycinnamic acids conjugated to polyamines might also represent an

important sink of carbon and nitrogen during growth and development of plants (Facchini et al., 2002; Edreva et al., 2007). Because their accumulation is regulated by JA and ET during defense, a trade-off between growth and defense should be considered when examining the ecological roles of these compounds. Interestingly, irMYB8 plants showed slightly enhanced growth, which could be explained by decreased production of CP and DCS, and therefore allocation of more resources to vegetative growth of the plants. Unfortunately, the compensatory character of DCS and CP biosynthetic pathways did not allow us to test the role of DCS in growth and reproductive fitness of *N. attenauta* plants.

3.4 Materials and Methods

3.4.1 Plant material and growth conditions

Nicotiana attenuata Torr. Ex S. Watson (31st inbred generation) seeds, originally collected from a native population from a field site located in Utah, USA, were used for all described experiments. Transgenic plants were used as described in Chapter 2. The seeds were germinated on Gamborg's B5 media (Duchefa; http://www.duchefa.com) as previously described by Krügel et al. (2002). Seeds were maintained at 26°C /16 h, 155 µmol m^{-2} s^{-1} light: 24°C/8 h dark cycle (Percival, Perry, IA, USA) for 10 d and young seedlings were planted individually in soil in Teku plastic pots. 10 d later, early rosette plants were transferred to soil in 1 L pots and grown in the glasshouse with day/night cycle of 16 (26-28°C)/8 (22-24°C) h under supplemental light from Master Sun-T PIA Agro 400 or Master Sun-T PIA Plus 600W high-pressure sodium lamps (Philips Sun-T Agro; http://www.nam.lighting.philips.com).

3.4.2 Virus induced gene silencing (VIGS)

Vector constructs, plant growth conditions and inoculation conditions were used as described in Saedler and Baldwin (2004). Three-weeks-old *N. attenuata* plants were co-inoculated with *Agrobacterium tumefaciens* cells transformed with pBINTRA, and either pTVDH29 plasmid (carrying a fragment of *DH29*) or pTV00 plasmid (carrying an empty vector construct as control).

3.4.3 Plant treatments

A transition rosette leaf was wounded with a fabric pattern wheel and fresh wounds were immediately treated with either 20 µL water or 20 µL of 1:5 (v/v) water-diluted oral secretions (OS) from *M. sexta*; control plants remained untreated. *M. sexta* larval OS were collected after larvae were reared on *N. attenuata* WT plants until the 3rd to 5th instar; OS were collected after regurgitation through a Teflon tube connected to vacuum, and stored under argon at -20°C.

3.4.4 qRT-PCR analysis

To analyze *SmCT* and *MYB8* gene expression, total RNA was isolated from control and W+OS treated rosette leaves of WT, DH29-VIGS and EV-VIGS plants. Approximately 150 mg liquid nitrogen-ground leaf powder from each sample was extracted with Trizol reagent, following the manufacturer's protocol (Invitrogen; http://www.invitrogen.com). Crude RNA samples were treated with RQ1 RNase-free DNase (Promega; http://www.promega.com), followed by phenol/chloroform/isoamylalcohol (25:24:1) extraction and ethanol precipitation. DNA-free RNA samples were reverse-transcribed using oligo (dT_{18}) primer and Superscript II reverse transcriptase enzyme (Invitrogen; http://www.invitrogen.com), following manufacturer's recommendations. All qRT-PCR assays of were performed with Stratagene MX3005P instrument (Stratagene; http://www.stratagene.com) as recommended by the manufacturer. For normalization purpose, primers specific for the elongation factor-1α gene from tobacco (EF1-α; Acc. D63396) were used as an internal standard to adjust for variable concentrations of cDNA in the samples. Specific primers in 5'-3' direction used for SYBR Green-based analyses of *MYB8* and *SmCT* were:

MYB8-F	AACCTCAAGAAACTCAGGACATACAA
MYB8-R	GATGAATGTGTGACCAAATTTTCC
SmCT-F	AAGTTGTGTAGATTTCCTATG
SmCT-R	CGTTCAAAGTAGTGCATTAGGC.

3.4.5 Analysis and identification of caffeoyl putrescine and dicaffeoyl spermidine

CP and DCS accumulation was quantified by HPLC coupled to photodiode array (PDA) detector. Approximately 100 mg of liquid nitrogen-ground leaf powder was extracted in 1mL of 40%MeOH prepared with 0.5% acetic acid water in

FastPrep tubes containing 0.9 g of FastPrep matrix (Sili GmbH, Germany). The samples in FastPrep tubes were homogenized twice on a FastPrep homogenizer (Thermo Fisher Scientific, Germany) for 45 seconds. The homogenized samples were then centrifuged at 16000x g, 4°C for 30 minutes. The supernatants were transferred into 1.5mL Eppendorf tubes, and centrifuged as before. 400µL aliquots of the supernatants from each sample were transferred to autosampler-compatible 2mL glass vials and each sample was analyzed by an Agilent HPLC 1100 series chromatographic system (http://www.chem.agilent.com). 1µL of sample was injected onto Chromolith FastGradient RP 18-e column (1.6 µm diameter, 50x 2mm, Merck, Germany; http://www.merck-chemical.de) attached to a pre-column (Gemini NX RP18, 3µm diameter, 2x 4.6mm; http://www.phenomenex.com). A mobile phase composed of 0.1% formic acid + 0.1% ammonium water, pH 3.5 and methanol was used in a gradient mode for the separation. The mobile phase comprised of solvent A (0.1% formic acid + 0.1% ammonium water, pH 3.5) and solvent B (methanol), used in a gradient mode time/concentration for 0% B 0.5 minutes to 80% B in 6.5 minutes. The flow rate was 0.8 mL/min, and column oven was set at 50 ºC. CP, CGA and DCS that eluted at retention times 2.45 min, 2.78 min, and 3.97 min, respectively, were detected by their absorbance at 320nm.

DCS isomers and derivatives were analyzed by LC-TOFMS system (Burker; http://www.bruker.com). The samples were extracted and prepared as described in sample preparation procedure for HPLC samples and 10µL sample aliquot was injected onto Macherey und Nagel C18 Pyramid column (2x 50 mm, 1.8µm diameter; Macherey and Nagel; http://www.mn-net.com). A mobile phase composed of 0.05% formic acid in water and 0.05% MeOH in acetonitrile was used in a gradient mode for the separation. The mobile phase comprised solvent A (0.05% formic acid) and solvent B (0.05% MeOH in acetonitrile) was used in a linear gradient mode from 5% to 80%B after equilibration time of 2 min. Linear gradient was run over 7 min and kept constant for additional 3 min with flow rate 0.6 ml/min and splitting ¼. DCS isomers and derivatives, and CP were detected as positive ions. Three isomers of DCS (molecular ions M-H (+) at m/z 470.234) were detected at corresponding retention times 1.90 min, 2.05 min and 2.15 min. Di-hydroxy DCS was identified by its molecular ion M-H (+) at m/z 472.249, and tetra-hydroxy DCS was identified by its molecular ions M-H (+) at m/z 474.265. CP was identified by its molecular ion M-H (+) at m/z 251.143.

3.4.6 1-methylcyclopropane (1-MCP) treatment

To inhibit ET perception, leaves were exposed to 1-methylcyclopropane (1-MCP), a known ET receptor antagonist: 250 mg of SmartFresh (3.3% 1-MCP, AgroFresh, Rohm and Haas, Italia) was dissolved in 25 mL of alkaline solution (0.75% KOH + NaOH in 1:1 ratio) to release the active substance, 1-MCP. 500 uL of activated solution of 1-MCP was infiltrated into a small cotton bud and immediately placed inside of the tightly sealed plastic bags around the leaf. Leaves were pre-exposed overnight to 1-MCP before treating them with W+OS, essentially as described in plant treatments above. Leaves were continuously maintained in the plastic bags with 1-MCP-soaked cotton buds, which were replaced every day. Control leaves were kept in the identical bags supplied with equivalent amounts of the alkaline solvent solution. Leaved were never allowed to come in direct contact with soaked cotton buds. Whole leaves were harvested at 0, 6, 12, 20 and 60 h after W+OS treatment, tissues were snap-frozen in liquid nitrogen and kept at -80ºC until analyzed.

3.4.7 Expression and purification of recombinant protein

Full-length cDNA of *DH29* was amplified using specific restriction-site (underlined) modified primers in 5'-3' direction (DH29-F; AGTTGACTATCATATGGGTTTCCTTTGTGCCAACCTG, DH29-R; TATATATATCTCGAGATTATTCAAAATAGTAAAAGTGTT). PCR was performed with a proof-reading Phusion polymerase (Finnzymes, Finland) and PCR products were cloned into NdeI-XhoI sites of pET23a expression vector (Novagen; http://www.emdchemical.com) to produce pET23a-DH29 clone, which were verified with DNA sequencing.

Recombinant protein with introduced N-terminal 6x His-tag was expressed in BL21 (DE3) pLysS *E. coli* cells (Novagen; http://www.emdchemical.com) after 0.05mM of isopropyl-β-D-thiogalactoside (IPTG) was added to induce the accumulation of recombinant protein in bacterial cultures that reached 1.0 OD_{600} culture density. Cells were centrifuged at 2500x g for 20 minutes and the pellets were frozen at -80ºC until further use. The pellets were resuspended in lysis buffer (NaH2PO4/NaCl) containing 1mg/mL lysozyme, and incubated on ice for 30 minutes. Lysates were then sonicated and centrifuged at 10000x g, 4ºC for 30 minutes. Supernatants were collected and 6x histidine tagged DH29 fusion proteins

were adsorbed to 2 mL Hi-Trap columns charged with nickel chloride by gravity flow. Unbound proteins were first washed out with 50mM NaH2PO4/300mM NaCl buffer, and bound protein fractions were then gradually eluted with an increasing gradient of 0-0.25M imidazole. A sample from each eluted protein fraction was subjected to SDS-PAGE to identify the positive fractions containing recombinant DH29 protein. The positive protein fractions were pooled, desalted with a Zeba desalt spin columns (Pierce Biotechnoly, USA; http://www.thermo.com/pierece) and concentrated by Amicon Ultra-4 columns (Millipore; http://www.millipore.com). Purified concentrated protein fractions were snap-frozen in 100µL aliquots in liquid nitrogen and stored at -80C until used. 20µL of purified protein fraction was loaded onto SDS-PAGE to confirm protein size and purification level.

SDS-PAGE was performed using 10% polyacrylamide gel (Roth; http://www.carl-roth.de) with Tris-glycine SDS running buffer (0.25M Tris, 2M glycine and 10% SDS). Bands were visualized by bio-safe coomassie stain (Bio-rad; http://www.bio-rad.com).

3.4.7 In vitro enzyme activity

SmCT activity was determined in total 100µL volume reaction, which contained 30µM acyl donor (caffeoyl-CoA; prepared by enzymatic synthesis with recombinant 4CL protein as described in (Obel and Scheller, 2000) and 120 µM acyl acceptor (spermidine) in 0.1M Tris-HCl buffer supplemented with 10mM EDTA. The reactions were incubated at 30 ºC for 15-60 minutes before reactions were stopped by addition of 1µL 12N HCL and 15µL acetonitrile. The enzymatic reaction products were centrifuged at 16000x g for 5 minutes and analyzed by HPLC-PDA and LC-TOFMS as described above.

3.4.8 Herbivore performance

Specialist *M. sexta* herbivore performance was examined in EV-VIGS and DH29-VIGS plants as described before (Lou and Baldwin, 2006). Freshly hatched *M. sexta* neonates were placed on stem leaves of EV-VIGS and DH29-VIGS plants and neonates were allowed to feed on the plants for 4 days before first recording of the caterpillar mass. Subsequently, caterpillar mass was determined at 7, 10 and 12 days after feeding.

3.4.9 Statistical analysis

All data were analyzed with StatView software and statistical significance displayed where relevant to the measurements.

4. Concluding discussion

Defense mechanisms are generally considered to be costly for plants because the plants have to divert some of their limited resources from growth and development to local and systemic defense. Several studies reported that plant fitness has significantly reduced after pathogen infection or herbivore attack (Baldwin, 1998; Zavala and Baldwin, 2004; Walters and Boyle, 2005; Walters and Heli, 2007), proposing the existence of trade-off mechanisms between growth and defense in plants subjected to biotic stress. However, there is no conclusive evidence of which mechanisms are involved in regulation of this trade-off process.

Phytohormones are known to function in both -- regulation of plant growth and defense -- proposing phytohormones crosstalk as one possible resource allocation control mechanism in plants that are exposed to stress. To this end, jasmonic acid (JA) and ethylene (ET) play important roles in herbivore-induced plant defenses and they are also important plant growth regulators. Considering their dual function in growth and defense, we decided to explore more closely the interactions between JA and ET during herbivory stress, using the well studied ecological model plant *Nicotiana attenuata*. The main questions addressed in this study were:

(1) Does JA-ET crosstalk have adverse effect on plant growth during herbivore attack?

(2) What are the main targets of JA-ET crosstalk in plants responding to herbivory stress?

(3) Is JA-ET crosstalk involved in optimizing plant defense mechanisms against herbivores, for example at metabolic level?

JA and ET play major roles in inducing plant defenses against herbivores; however, it remains largely unknown whether (and how) JA and ET directly influence plant growth during herbivory stress. We therefore examined the effect of JA-ET on plant growth and development using transgenic *N. attenuata* plants that were deficient in their JA (asLOX3) or ET (mETR1) signaling cascades, and their genetic cross (mETR1asLOX3), after subjecting them to simulated herbivory treatments. Simulated herbivory inhibited growth of the directly-treated leaves in WT *N. attenuata* plants, while the leaf expansion in mETR1asLOX3 plants was not

affected by the treatments (Chapter 2). Our results prove a direct negative effect of both JA and ET on plant growth after herbivore attack that is shown to involve active crosstalk between JA and ET signaling pathways. These finding correlate with (and combine) the results of previous studies, in which high ET levels negatively affected leaf expansion (Bleecker et al., 1988; Hua et al., 1995; Grbic and Bleecker, 1995), and high levels of JA after mechanical wounding inhibited leaf expansion in *Arabidopsis* plants (Zhang and Turner, 2009). In the natural herbivory, both signals should be therefore considered when evaluating the impact of herbivores on plant growth.

JA-ET crosstalk not only inhibited growth at the whole plant level -- the absence of both signals led to callus-like cells development around the puncture wounds, specifically in mETR1asLOX3 leaves after both mechanical wounding and W+OS treatments. This finding suggested that JA-ET crosstalk resumes in the local growth inhibitory response at damage site; calli composed of undifferentiated plants cells develop in some plant species after tissue damage, being considered as wound healing process in these plants (Lulai and Corsini, 1998; Bucciarelli et al., 1999; Ibrahim et al., 2001; Leon et al. 2001; Paris et al., 2007). Auxin (IAA) is one of the essential factors required for cell division, cell expansion and callus development (Skoog and Tsui, 1948). We found that IAA concentrations in the callus-forming mETR1asLOX3 leaves were significantly higher after treatment with W+OS, which correlated with the development of callus-like cells around the wounds. It has long been known that ET has a positive effect on IAA function and biosynthesis (Visser et al., 1996; Vandenbussche et al., 2003; Stepanova et al., 2005; Stepanova et al., 2007; Ivanchenko et al., 2008), while JA mainly provided negative effect on IAA signaling pathway (Staswick, 2009). In mETR1asLOX3 plants, it is therefore likely that JA is one of the negative regulators of IAA biosynthesis; however, the negative effect of ET on IAA biosynthesis, when uncoupled from JA action in mETR1 plants, was rather unexpected. As one possible explanation for this negative effect of ET, the positive interaction between ET and IAA could be restricted to specific tissues -- in all studies that found a positive effect of ET on IAA, these studies used tissues with relatively high growth rates such as roots and seedlings; however, in tissues associated with lower growth rates such as fruits, ET actually showed negative effect on IAA biosynthesis (Brown, 1997).

ET not only interacted with IAA at biosynthetic and/or accumulation level,

but ET also affected the sensitivity of leaf tissues to IAA. We found that mETR1 (ET-insensitive plants) became more sensitive to exogenous IAA that was directly applied to the leaves after treatment with W+OS, which resulted in the formation of callus-like cells on the leaves. Based on our results, we propose that JA and ET inhibit plant growth by suppressing IAA biosynthesis and IAA sensitivity during herbivore attack, which therefore might allow more resources to be re-located from local healing to herbivore-induced defenses, including secondary metabolism.

Toxic secondary metabolites are part of the effective plant direct defense mechanisms against herbivores. JA and ET are known to regulate the production of induced-defense metabolites such as nicotine and TPIs in *N. attenuata* (Winz and Baldwin, 2001; Halischeke and Baldwin, 2003). In this study, we examine the influence of JA-ET crosstalk on the production of dicaffeoyl spermidine (DCS), the compound recently proposed to have a function in direct defense against herbivores in *N. attenuata* (Kaur et al., 2010). In our study, DCS biosynthesis was regulated by the novel acyl transferase gene, *SmCT*. In WT plants, *SmCT* expression and DCS accumulation both increased after simulated herbivory treatment, showing inducible character of the metabolite accumulation. The gene expression and DCS accumulation in mETR1 plants slightly decreased but *SmCT* expression and DCS accumulation were barely detected in asLOX3 plants after W+OS treatment, revealing JA as main regulator of DCS accumulation in plants. Interestingly, the gene expression and DCS accumulation in mETR1asLOX3 plants were in between the levels found in mETR1 and asLOX3 plants. This result demonstrates that JA is a positive regulator for DCS; however ET seems to have multiple accessory roles in regulating DCS.

MYB8 transcription factor was recently reported to be a major regulator of DCS biosynthesis in *N. attenuata* (Kaur et al, 2010). We found that MYB8 expression indeed correlated with *SmCT* expression in WT and asLOX3 plants, suggesting that JA regulates *SmCT* expression and DCS accumulation via MYB8-dependent mechanism. However; our results with mETR1asLOX3 plants suggested the existence of an alternative, ethylene suppressed pathway leading to *SmCT* expression and DCS accumulation. Anderson et al. (2004) showed that ET had antagonistic effect on abscisic acid (ABA) signaling. Interestingly, ABA is also known to induce the expression of AtMYC2 gene (Abe et al., 1997; 2003) that is otherwise a key transcription factor in JA-dependent signal transduction pathway

(Abe et al., 2003). Even though JA and ET are the main regulators of *SmCT* expression, it is possible that ABA might as well be involved in the regulation of this gene, most interestingly, in a MYC2-dependent manner. Therefore, it remains a possibility that DCS accumulation is actually regulated by the extended JA-ET-ABA crosstalk mechanisms. While JA has probably undertake the main regulatory function over *SmCT*, using MYB-dependent pathway, ET and ABA function as minor inducers/modulators of *SmCT* expression. Even though mETR1asLOX3 plants lacked the major inducer (JA) for *SmCT*, the minor regulatory pathway putatively operated by ABA was able to induce this gene during simulated herbivory treatment, most probably because these plants lacked the antagonistic effect of ET imposed over ABA signaling. These findings, integrated into evolutionary context, may suggest a gradual development of hormonal signaling pathways, overtaking the regulatory functions of genes when the new and more dominant signals have evolved, and the function of regulated metabolite was suited to a novel hormone-regulated function, for example defense. In this model, original ABA regulation would be replaced by a strong wound- and herbivory defense-related JA functionality, and the original regulatory circuit would be further masked by ET signaling pathway, accessory to JA regulation.

The understanding of phytohormone crosstalk regulating resource allocation between growth and defense during herbivory is an important step required for deciphering plant survival strategies in response to adverse environmental conditions. Reverse genetic approach, gene expression analysis and targeted manipulation of molecular components mediating phytohormone signaling in defense, used in this study, show that plants tried in evolution to minimize the negative impact of herbivory -- by reducing their growth and local wound-healing responses -- using JA and ET as major tools, and enhance the production of defense metabolites that are elicited by the same hormonal cues. In the signaling cascade, ET seems to amplify some JA responses as well as ET is found to be a part of the redundant pathway leading to reduced growth and local healing of the leaves.

The regulation of defense is naturally complemented and/or antagonized by the action of other hormones, activated by different stress factors like pathogens and abiotic stress. It is well known that pathogen-elicited SA signaling can induce phenylpropanoid biosynthetic pathway, similar to JA, leading to the production of phytoalexins, and mounting defense responses against invading pathogens. In this

work, we propose a novel hypothesis that ABA, a drought stress-related phytohormone, could possibly cross-activate the JA-response pathway in *N. atenuata*, which can be only uncovered when using JA- and ET-signaling deficient plants, and *SmCT* gene expression as marker of anti-hebivore defense. It is apparent that more studies are required to fully understand the complex crosstalk between plant hormones and trade-off mechanisms involved in regulation of growth and defense in plants, an important plant trait that could be used to optimize fitness of our crops, and contribute to improved human life and society at global scale in the future.

5. Summary

Plant hormones (phytohormones) are important signals that regulate plant growth, development and defense. Large number of studies in the past years has been dedicated to dissecting phytohormone signal transduction pathways and crosstalk between phytohormones in plants. The knowledge of phytohormone crosstalk should allow better understanding of how plants adapt to stress conditions and survive in the natural environment.

Jasmonic acid (JA) and ethylene (ET) play important roles in mediating plant defense against herbivores but how they affect growth and development in attacked plants is unknown. We therefore examined plant growth and development during herbivory stress in JA-deficient (silenced in *LIPOXYGENASE 3*; asLOX3), ethylene-insensitive *Nicotiana attenuata* plants (expressing a mutated dominant negative form of *ETHYLENE RESPONSE 1*; mETR1), and in their genetic cross (mETR1asLOX3). At the whole plant level, both hormones contributed to suppressing leaf growth in plants that had been wounded and had the wounds immediately treated with *Manduca sexta* oral secretions (W+OS) to simulate herbivory. In addition, large callus-like structures developed around wounds in both water- and OS-treated punctures in mETR1asLOX3 leaves but not in mETR1, asLOX3 or wild type (WT) plants. Treating asLOX3 leaves with the ethylene receptor antagonist 1-MCP before wounding resulted in local cell expansion that closely mimicked the mETR1asLOX3 phenotype, confirming that suppression of both JA and ET action is required for callus development around wounds.

We used molecular tools to dissect the unusual response of mETR1asLOX3 plants to wounding, finding that higher auxin levels in W+OS-treated mETR1asLOX3 leaves correlate with callus formation in these plants. In addition, microarray and qRT-PCR transcript profiling of OS-elicited mETR1asLOX3 leaves revealed their preferential accumulation of transcripts known to function in cell wall remodeling, suggesting that JA and ET normally act as negative regulators of these genes after wounding, possibly by counteracting IAA accumulation and/or IAA signaling. Based on our data, we propose that JA-ET crosstalk restrains local cell expansion and/or the healing process in wounded *N. attenuata* plants, allowing more resources to be allocated to induced defenses during herbivore attack.

Production of toxic secondary metabolites is one of the plant's direct defense

mechanisms against herbivores; however this process is thought to be costly for plants, and plants therefore need to wisely allocate their resources between growth and defense. As we showed in the previous paragraphs, both JA and ET are used to suppress plant growth, resulting in smaller leaves, and lack of local healing response (callus formation) during herbivore attack. We then concentrated on the role of hydroxycinnamic acid amides as potential defense metabolites that can benefit from resources released by JA-ET action, leading to induced defenses and efficient accumulation of plant defense metabolites against herbivores.

Caffeoyl putrescine (CP) and dicaffeoyl spermidine (DCS), two most abundant hydroxycinnamic acid amides in *N. attenuata* plants, are thought to function in plant growth as well as in response of plants to biotic and abiotic stress factors. Previously, an unknown function gene, *DH29*, showed low expression in response to W+OS in plants silenced in their expression of MYB8 transcription factor, a known regulator of CP and DCS accumulation in *N. attenuata*. The analysis of full length DH29 cDNA coding sequence indicated that this protein might encode a novel acyl transferase-like enzyme, classified in a large BAHD protein family in plants. The plants silenced in DH29 expression by virus-induced gene silencing (VIGS) contained strongly reduced levels of DCS, indicating that DH29 enzyme encodes a DCS synthase. However, the analysis of DH29 recombinant protein activity demonstrated that this enzyme can only mediate the transfer of a single caffeoyl moiety to spermidine, resulting in the formation of a putative DCS precursor monocaffeoyl spermidine (MCS).

The expression analysis of spermidine monocaffeoyl transferase (*SmCT*) in *N. attenuata* plants deficient in JA accumulation (asLOX3) and ET perception (mETR1), or both, indicated that *SmCT* expression is synergistically controlled by JA, ET and possibly by other hormones via MYB8 transcriptional activity. *SmCT* expression *in vivo* and enzyme activity *in vitro* suggested that SmCT and its derived end product metabolite DCS could play an important role in plant-defense against herbivores. However, interconnected character of DCS and CP metabolic pathways has currently disabled unambiguous determination of DCS function in plants, as in general, the plants deficient in DCS accumulation strongly over-accumulated CP, which then masked the effect of reduced DCS accumulation in our herbivore bioassays.

The understanding of phytohormone crosstalk regulating resource allocation

between growth and defense during herbivory is an important step required for deciphering plant survival strategies in response to environmental stress.

6. Zusammenfassung

Pflanzenhormone (Phytohormone) sind wichtige Signale, die das Pflanzenwachstum sowie deren Entwicklung und Verteidigung regulieren. Eine große Anzahl von Studien hat sich in den letzten Jahren der Analyse von Transduktionswegen und Crosstalks der Phytohormonsignale in Pflanzen gewidmet. Das Wissen über Phytohormon-Crosstalk soll zum besseren Verständnis führen, wie sich Pflanzen an Stresskonditionen anpassen und in der natürlichen Umwelt überleben.

Jasmonsäure (JA) und Ethylen (ET) spielen eine wichtige Rolle bei der Herbeiführung von Pflanzenabwehr gegen Herbivore. Dennoch ist unbekannt, wie sie das Wachstum und die Entwicklung in befallenen Pflanzen beeinflussen. Aus diesem Grund untersuchten wir das Pflanzenwachstum und die Pflanzenentwicklung, während die JA-defizitären (stillgelegt in *LIPOXYGENASE 3*; asLOX3), Ethylen unempfindlichen *Nicotiana attenuata* Pflanzen (Ausdruck einer mutierten, dominant-negativen Form von *ETHYLENE RECEPTOR 1*; mETR1) und deren genetische Kreuzungen (mETR1asLOX3) den Herbivoren ausgesetzt waren. Auf der ganzen Pflanzenebene wirken beide Hormone mit, indem sie das Blätterwachstum in den Pflanzen, die verletzt waren und deren Verletzungen sofort mit dem oralen Sekret *Manduca sexta* (W+OS) behandelt wurden, um den Befall mit Herbivoren zu simulieren, unterdrücken. Darüber hinaus entstanden um die Verletzungen herum große kallus-ähnliche Formen in beiden Wasser- und OS-behandelten Vertiefungen der mETR1asLOX3 Blätter, jedoch nicht in den mETR1, asLOX3 oder den wilden (WT) Pflanzen. Die Behandlung der asLOX3 Blätter mit dem Ethylen-Rezeptor-Antagonist 1-MCP, bevor das Verletzen lokale Zellexpansion nach sich zieht, welches genau den mETR1asLOX3 Phenotype nachahmt, bestätigt, dass die Unterdrückung beider JA- und ET-Aktionen erforderlich ist, damit sich Kalli um die Wunde bilden.

Unter Anwendung molekularer Tools konnte die ungewöhnliche Reaktion von mETR1asLOX3 Pflanzen auf die Verletzungen analysiert werden, indem herausgefunden wurde, dass ein höherer Auxin-Anteil in den W+OS-behandelten mETR1asLOX3 Blättern mit den Kallusbildungen in diesen Pflanzen korrelieren. Des Weiteren verdeutlichten Mikroarray und qRT-PCR Transkript, das die OS-hervorrufenden mETR1asLOX3 Blätter profiliert, deren bevorzugte Transkript-

Anhäufung, die dafür bekannt sind, in Zellwänden als Umwandler zu funktionieren. Hinzuweisen ist darauf, dass JA und ET normalerweise als negative Regulatoren dieser Gene nach der Verletzung auftreten, die wahrscheinlich der IAA Akkumulation und/oder der IAA Signalisierung entgegenwirken. Aufgrund unserer Daten wird vorgeschlagen, dass der JA-ET-Crosstalk die lokale Zellausdehnung und/oder den Heilprozess in den verletzten *N. attenuata* Pflanzen hemmt und erlaubt, dass mehr Ressourcen bereitgestellt werden, um Verteidigungen während herbivorärer Befälle zu bewirken.

Die Produktion von toxischen sekundären Metaboliten ist einer der direkten Verteidigungsmechanismen einer Pflanze gegen Herbivoren; dennoch wird dieser Prozess für eine Pflanze als sehr aufwendig eingeschätzt, und deshalb müssen sie ihre Ressourcen sehr weise zwischen Wachstum und Verteidigung kontingentieren. Wie in den vorangegangenen Absätzen gezeigt werden konnte, unterdrücken sowohl JA als auch ET das Pflanzenwachstum, was dazu führt, dass während herbivorärer Befälle kleinere Blätter wachsen und nicht mehr auf lokale Heilprozesse reagiert wird (Kallusbildungen). Daraufhin konzentrierten wir uns auf die Rolle des Hydroxycinnamic-Säureamides als potentielle Verteidigungsmetaboliten, die aus den Ressourcen Nutzen ziehen können, die während einer JA-ET-Aktion freigegeben werden, so dass dies zu einer eingeleiteten Verteidigung und einer effizienten Akkumulation von Verteidigungsmetaboliten gegen Herbivoren führt.

Kaffeoyl-Putrescine (CP) und Dikaffeoyl-Spermidin (DCS), zwei sehr ergiebige Hydroxyzimtsäuren-Amide in *N. attenuata* Pflanzen, werden erwogen, sowohl im Pflanzenwachstum als auch während der Reaktionen auf biotische und abiotische Stressfaktoren mitzuwirken. Bisher zeigte ein unbekanntes Funktionsgen, DH29, einen geringen Ausdruck in Erwiderung auf W+OS in Pflanzen, die in ihrer Äußerung des MYB8-Transkriptionsfaktors zum Schweigen gebracht wurden, der wiederum ein bekannter Regulator von CP und DCS-Anhäufungen in *N. attenuata* ist. Die Analyse einer vollständigen DH29 cDNA Kodierungssequenz wies darauf hin, dass dieses Protein es vermag, ein neuartiges Azyltransferase-ähnliches Enzym zu enkodieren, das in eine große BAHD-Proteinfamilie in Pflanzen eingeordnet wird. Die Pflanzen, die in DH29-Äußerungen durch Virus eingeleitete Genstilllegung (VIGS) gehemmt wurden, beinhalteten stark reduzierte Anteile an DCS, was darauf hinweist, dass DH29-Enzyme eine DCS-Synthase enkodiert. Dennoch zeigte die Analyse der DH29-rekombinanten Proteinaktivität auf, dass dieses Enzym nur den

Transfer von einzelnen Kaffeoyl-Teilen zu Spermidinen herbeiführen kann, woraus sich die Bildung des DCS Präkursors (Mono)Kaffeoyl-Spermidin (MCS) ergibt.

Die Ausdrucksanalyse der Spermidin (Mono)Kaffeoyl-Transferase (SmCT) in den *N. attenuata* Pflanzen, denen es an JA-Akkumulation (asLOX3) und ET-Perzeption (mETR1) oder beiden mangelt, wiesen darauf hin, dass der SmCT-Ausdruck durch JA, ET und wahrscheinlich weitere Hormone via MYB8 transkriptionale Aktivität synergistisch kontrolliert wird. Der SmCT-Ausdruck in vivo und die Enzym-Aktivität in vitro deuteten darauf hin, dass SmCT und dessen abgeleitetes Endprodukt Metabolit DCS eine wichtige Rolle bei der Pflanzenabwehr gehen Herbivore spielen könnte. Dennoch hat der gekoppelte Charakter des DCS- und CP-Stoffwechselwegs laufend die eindeutige Determination der DCS-Funktion in Pflanzen blockiert, da gewöhnlich die an DCS-Akkumulation mangelnden Pflanzen übertrieben CP ansammeln, welches daraufhin den Effekt der reduzierten DCS-Akkumulation in unserem Herbivoren-Biotest verbirgt.

Das Verstehen des Phytohormon-Crosstalks, welches die Ressourcenverteilung zwischen Wachstum und Verteidigung während herbivorärer Angriffe reguliert, ist ein wichtiger Schritt, um die Überlebensstrategien der Pflanzen als Antwort auf umgebungsbedingten Stress zu entschlüsseln.

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Curriculum vitae

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- Used LC/MS and HPLC to analyse phytohormones and plant secondary metabolites
- Used virus induced gene silencing (VIGS) to investigate gene function
- Used herbivory bioassays to evaluate gene function in plant defense
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Oral presentations

- **"Crosstalk between jasmonic acid and ethylene is important for tuning growth and defence in *Nicotiana attenuata"*** Utrecht Summer School on Environmental Signalling, Utrecht The Netherlands, 24-26th August 2009
- **"Identification of a novel acetyltransferase enzyme in *Nicotiana attenuata* after herbivory"** 8th IMPRS symposium Dornburg, Germany, 2nd -3rd March 2009

Poster presentations

- **"Crosstalk between jasmonic acid and ethylene is important for tuning growth and defence in *Nicotiana attenuata"*** Nawaporn Onkokesung, Ivan Galis, Hans-Peter Saluz and Ian T. Baldwin Adaptation Potential in Plants, FEBS workshop, Vienna Austria, 19th-21st March, 2009
- **"Maintaining balance between growth and defence: The dilemma in plant-herbivore interactions"** Nawaporn Onkokesung, Ivan Galis, Hans-Peter Saluz and Ian T. Baldwin, 7th IMPRS symposium Dornburg, Germany, 25th-26th Febuary 2008

Publications

- **Onkokesung, N.,** Galis, I., von Dahl, C. C., Matsuoka, K., Saluz, H.P., and Baldwin, I. T. *Jasmonic acid and ethylene modulate local response to wounding and simulated herbivory in Nicotiana attenuata leaves.* Plant Physiology *(in press)*
- **Onkokesung, N.,** Kaur, H., Saluz, H.P., Baldwin, I. T., and Galis, I., *A novel dicaffeoylsperimidine transferase is regulated by jasmonic acid and ethylene crosstalk in Nicotiana attenuata (in preparation)*

Publications (Continue)

- Galis I., **Onkokesung N**., and Baldwin, I. T. *New insights into mechanisms regulating differential accumulation of phenylpropanoid-polyamine conjugates (PPCs) in herbivore-attacked Nicotiana attenuata plants.* Plant Signaling and Behavior (in press)

Nawaporn Onkokesung

List of presentations and publications

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- **Onkokesung, N.,** Kaur, H., Saluz, H.P., Baldwin, I. T., and Galis, I., *A novel dicaffeoylsperimidine transferase is regulated by jasmonic acid and ethylene crosstalk in Nicotiana attenuata (in preparation)*
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Selbständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, daß ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

Nawaporn Onkokesung

Jena, den 07, Mai, 2010