Elucidating the roles of the hydroxyproline-rich glycopeptide systemin precursor in anti-herbivore defense and development of *Nicotiana attenuata*

Dissertation

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1. Introduction

Multicellular organisms composed of differentiated tissues require signal transduction mechanisms to coordinate and integrate metabolic functions within and between tissues. In plants, internal signaling is essential for the regulation of developmental processes like growth and reproduction, the fine-tuning of metabolic cycles and the activation and regulation of defense responses. In contrast to animals, plants lack specialized signal transduction tissues equivalent to a central nervous system for the coordination of internal processes. Nevertheless, plants are able to coordinate all of the functions mentioned above, thereby adapting to changing environmental conditions and reacting to biotic challenges like competition and herbivore- or pathogen-attack. Chemical signals having local (within a tissue or organ) or systemic (between tissues or organs) effects are the most important facilitators of signal transduction in plants.

1.1. Plant hormone signals - the "classical five and friends"

The regulation of developmental processes in plants has been classically associated with five hormones, namely auxins, gibberelins, cytokinins, abscisic acid and ethylene. The term ’hormone’, derived from the Greek word ”horman” (to set something in motion), was adopted earlier in a botanical context to describe post-flowering phenomena in orchids (Fitting, 1909). In contrast to animal hormones which are produced by endocrine glands and which affect target organs, the production of plant hormones is not limited to a certain cell type or plant stage and can affect any plant tissue.

Generally speaking, auxins, cytokinins and gibberelic acid are known to exert broad growth-promoting effects, while ethylene and abscisic acid are growth and cell division inhibitors. Cross-talk between signal transduction pathways appears to be prevalent in the regulation of many physiological processes. Auxins act as growth promotors, and are involved in cell enlargement, bud formation, flower initiation, fruit formation and root initiation (Woodward and Bartel, 2005). The ratio of auxins and cytokinins affects all major aspects of plant growth and demonstrates the required conjunction of hormone action in the regulation of growth processes (Skoog and Miller, 1957; Nordström *et al*., 2004).. Abscisic acid effects bud growth as well as seed dormancy (Nambara and Marion-Poll, 2005). Bud dormancy, induced by abscisic acid can be reversed by gibberelins, a structurally closely related class of phytohormones promoting flowering, cellular division, and seedling growth after germination (e.g. Pharis and King, 1985). The contemporary

understanding of plant hormones includes other growth regulators as well. For example, brassinolides promote cell elongation and cell division, differentiation of xylem tissues, and inhibition of leaf abscission. Jasmonates (JA's) are believed to play a role in seed germination, flower development and root growth (Creelman and Mullet, 1997). Moreover, the involvement of phytohormones in defense responses is represented by JA's, ethylene and salicylic acid (SA) (Davies (ed.) 2004). These compounds are known to induce specific chemical defense responses to biotic stressors like pathogens or herbivores (reviewed in Koorneef and Pieterse, 2008).

1.2. Plant peptide hormone signals – "newcomers" with great potential

Peptide signal molecules are well known and widely distributed throughout the animal kingdom. Their broad functional spectrum complements the signal transduction roles played by the central nervous system through the regulation of specific developmental processes. The existence of hormone-like signaling peptides in plants has been widely speculated upon. It is only just recently that the functions for plant peptides in growth and development (Casson *et al*., 2002; Wen *et al*., 2004), reproduction (Kachroo *et al*., 2002) and defense (Pearce *et al*., 2001) were shown.

1.2.1. Systemin – the past, the present, and future perspectives

Systemin, the first and most widely studied signal peptide in plants was discovered during the hunt for a systemic wound signal. Following an observation made four decades ago, that wounding tomato (*Solanum lycopersicum*) plants resulted in the induction of proteinase inhibitors (PIs) not only at the site of wounding, but also in distal parts of the plant (Green and Ryan, 1972), several kinds of signals were proposed to be involved in the transmission of a systemic wound response. The most likely candidates were pectin cell wall fragments (Bishop *et al*., 1981), abscisic acid (Pena-Cortes *et al*., 1991), methyl jasmonate (MeJa) (Farmer and Ryan, 1990), electrical potentials (Davies, 1987) and the 18 amino acid (aa) oligopeptide systemin (Pearce *et al*., 1991), all of which were believed to be capable of inducing PI production. Systemin was thought to be the most likely candidate because it elicits the accumulation of PI I and II both locally and systemically when applied to unwounded tomato plants at levels in the fmol range (Pearce *et al*., 1991). Moreover, [^{14}C] Ala-labeled systemin was found to move freely in the vascular system and was therefore postulated to be the mobile signal activating defence traits in distal undamaged parts of tomato plants (Pearce *et al*., 1991). In response to wounding, the

mature peptide is derived from the carboxy-terminal end of a larger 200 aa precursor called prosystemin. The precursor is synthesized and sequestered in vascular phloem parenchyma cells (Nárváez-Vásquez and Ryan, 2004). How the precursor molecule is proteolytically processed, and which enzymes are involved in the cleavage of systemin is still under investigation. The common processing mechanisms for signal peptides include dibasic cleavage sites, signal sequences or membrane-spanning regions. However, all of these properties seem to be missing in the prosystemin protein (Ryan and Pearce, 1998).

Systemin's role in the activation of systemic defence responses is supported by a wealth of evidence. For example, tomato plants expressing prosystemin in an anti-sense orientation to reduce systemin production accumulated less PIs after wounding compared to wild-type (WT) plants (McGurl *et al*., 1992) and were consequently more susceptible to attack by larvae of the tobacco hornworm (*Manduca sexta*) (Orozco-Cardenas *et al*., 1993). In addition, transgenic plants overexpressing the prosystemin gene constitutively produced PI I and II proteins and accumulated more PIs than WT plants in local and systemic tissue after wounding (McGurl *et al*., 1994). High endogenous prosystemin levels were further associated with up to 10-fold higher jasmonic acid (JA) levels in unwounded leaves compared to those in non-transformend plants (Stenzel *et al*., 2003), indicating systemin action upstream of the JA signal. The first model for the transmission of a systemic wound signal in tomato presumed systemin being released from its precursor at the wound site, moving through the phloem, and binding to a 160 kDa leucine-rich repeat receptor-like kinase (LRR-RLK) (SR160; Scheer and Ryan, 2002) acting as putative receptor. After binding the receptor, systemin initiates an intracellular signaling cascade including the activation of a mitogen-activated protein (MAP) kinase, phospholipase A, and the release of linolenic acid (LA) from cell membranes. LA is subsequently converted to JA via the octadecanoid pathway, and JA activates downstream defense genes (such as PI).

The early systemin model was recently reconsidered as a result of data from grafting experiments between WT tomato plants and mutants that are either deficient in JA production (*acx1*), JA insensitive (*jai1*), defective in systemin action (*spr1*), or constitutively expressing prosystemin. In summary, these grafting experiments demonstrated that systemic signaling requires JA to be produced at the site of wounding but also the ability to perceive a JA-based signal in the distal leaf. These data conform to the idea that the presence of systemin is needed but only required in the local, wounded tissue to amplify the JA-based signal. Experiments showed that a JA-derived signal is indeed the mobile signal that elicits PIs in distal, unwounded parts of the plant (Schilmiller

and Howe, 2005). The current model of systemin's mode of action in tomato is summarized in figure 1.

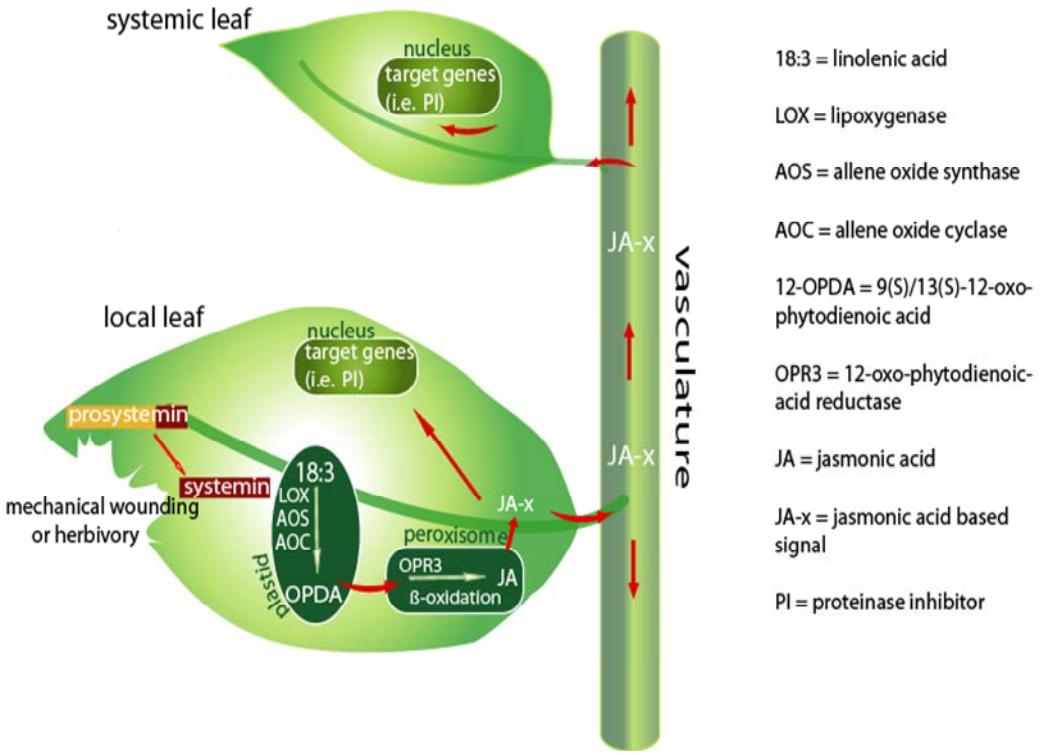

Figure 1: Simplified model of current understanding how systemin action in systemic defense signaling of tomato plants; a model after Wasternack (2006) and Schilmiller and Howe (2005).

Homologues of the proline-rich tomato systemin have so far been identified only in close relatives such as potato (*Solanum tuberosum*) (McGurl and Ryan, 1992), black nightshade (*Solanum nigrum*), and bell pepper (*Capsicum annuum*) (Constabel *et al.*, 1998). Surprisingly, systemin does not mediate PI production in the undomesticated black nightshade (Schmidt and Baldwin, 2006), and seems rather to help the plant to tolerate herbivory than to mediate direct defense (Schmidt and Baldwin, in review). Therefore, it seems that systemin function is not conserved even among closely related Solanaceous species.

1.2.2. Hydroxyproline-rich glycopeptides – a systemin subfamily

The elicitation of PIs in response to wounding is not specific for tomato *(S. lycopersicum*), but rather is conserved among many Solanaceous species like tobacco (*Nicotiana tabacum*). *N. tabacum*, for example, does not possess a proline-rich tomato systemin homologue and does not respond to treatment by tomato-systemin with increased PIs (McGurl, 1992). Searching for a functional systemin equivalent, Ryan and coworkers identified two hydroxyproline-rich peptides (TobHSI and TobHSII) in wounded *N. tabacum* plants, both of which are derived from a single 165 aa precursor (preproTobHypSys-A), and sequestered in cell walls. These peptides elicited MAP kinases when applied to tobacco cell cultures and activated the synthesis of PIs when stem-fed into young excised plants (Pearce *et al*., 1993; Pearce *et al*., 2001). Exposure of cultivated tobacco leaves to MeJA vapours, mechanical wounding, and attack by *M. sexta* larvae elicited increases in preproTobHypSys-A transcripts (Pearce *et al*., 2001). This paralleled increases in other wound-responsive genes (*PIOX*, *Nt-PI-I, TPI*) (Rocha-Granados *et al.*, 2005). Additionally, a third hydroxyproline-rich tobacco peptide (TobHSIa) was found to be encoded by a second 164 aa precursor.

More recently, when cultivated tobacco plants were transformed with preproTobHypSys-A under the control of a 35S promotor, constitutive levels of PIs increased and plants were more resistant to the polyphagous larvae of *Helicoverpa armigera* (Ren and Lu, 2006). In short, ectopic over-expression of the preproTobHypSys-A in tobacco produced results similar to those seen from the over-expression of prosystemin in tomato: plants increased their PI production and became more resistant to herbivore attack. These results support the idea that the tomato and tobacco systemins, despite their lack of phylogenetic similarity, both function in defense signaling (Ryan and Pearce, 2003). Interestingly, tomato also possesses three hydroxyproline-rich glycopeptides (TomHypSys I, II and III) which are encoded by a wound-inducible 146 aa precursor. These peptides are thought to support systemin in an amplification loop that up-regulates the octadecanoid pathway and the synthesis of jasmonates to effect the strong systemic induction of defense genes (Nárvárez-Vásquez *et al*., 2007). All hydroxyproline-rich glycopeptides are supposed to be synthesized through the peptide secretory pathway, where they are hydroxylated and glycosylated. Interestingly, HypSys signal activity may not only appear in response to wounding or herbivore attack, but also after pathogen infection as observed for the HypSys activated *defensin1* gene expression in petunia (*Petunia hybrida)* (Pearce *et al*., 2007). While three HypSys peptides isolated from *P.*

hybrida induced a gene associated with anti-pathogen defense, they are unable to induce anti-herbivore protease inhibitor or polyphenol oxidase activity (Pearce *et al*., 2007). However, similarities in biological defense signaling activities of systemins and hydroxyproline-rich glycopeptides have led to the inclusion of both in a functionally defined systemin family, with the hydroxyproline-rich glycopeptides being named HypSys peptides. The involvement of HypSys peptides in defense signaling of some cultivated members of the nightshade family (*S. lycopersicum*, *N. tabacum*, *P. hybrida*) has been demonstrated but functions of HypSys in wild relatives of the nightshade family had not been characterized up to now.

1.3. The model plant *Nicotiana attenuata*

1.3.1. Habitat and life cycle

The wild tobacco *Nicotiana attenuata*, Torr. ex Watson (synonymous with *Nicotiana torreyana* Nelson & Macbr, *Solanaceae*, genus *Nicotiana*, subgenus *Petunioides*) is a herbaceous annual occurring in newly disturbed desert habitats with populations across the southwestern USA (Goodspeed *et al*., 1954). Growing on nitrogen-rich soils, *N. attenuata* invades burned areas with high-density populations for an initial period of up to three years after a fire in sagebrush, blackbrush, and pinyon–juniper forests of the Great Basin desert. The early-successional spatial and temporal occurrence of *N. attenuata* in a post-fire environment is due to its synchronized seed germination from long-lived seedbanks promoted by smoke-derived positive cues (Baldwin *et al*., 1994) and inhibited by allelochemicals in the litter of other plant species (Preston *et al*., 2002).

N. attenuata produces white tubular flowers. Right before the flowers open, anthesis occurs and most seeds that are set in nature are thought to result from self-pollination. Yet although the diploid *N. attenuata* is largely self-compatible, it has maintained traits that can promote out-crossing. *N. attenuata* flowers look like flowers of typical moth-pollinated plants, produce more pollen than needed for self-pollination (Sime and Baldwin, 2003) and known to be visited by various pollinators.

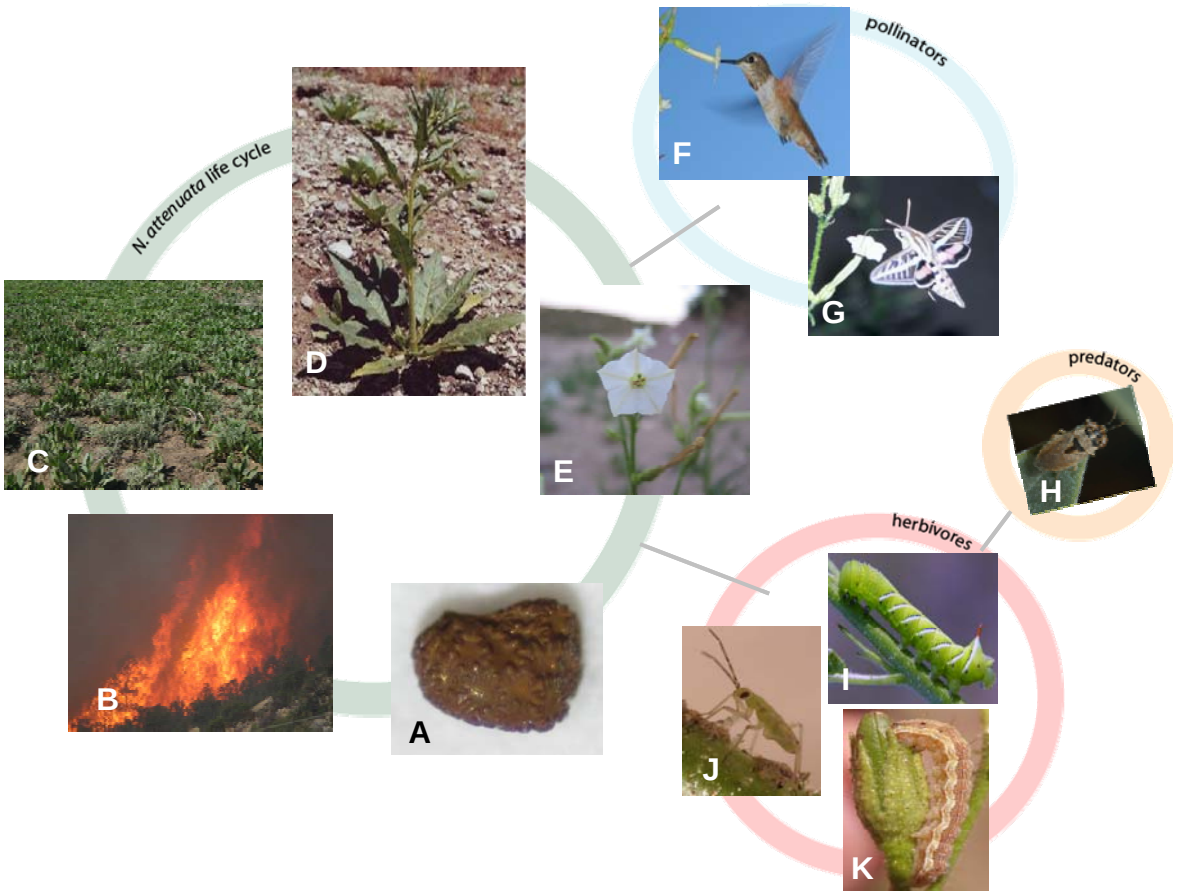

Fig. 2: *Nicotiana attenuata* life cycle: (A) *N. attenuata* seed, (B) wild-fire in the Great Basin desert, Utah, (C) N. attenuata rosette stage plants growing in monocultures on nitrogen-rich soils, (D) elongated *N. attenuata* and (E) flower of *N. attenuata* plants. Pollinators visiting *N. attenuata* flowers (F) *Selasphorus rufus* (hummingbird) and (G) *Hyles lineata* (white-lined sphinx moth). Herbivores (H) *Manduca sexta* (tobacco hornworm), (I) *Tupiocoris notatus* (suckfly) and (J) *Spodoptera exigua* (beet army worm) and predating (K) *Geocoris pallens* were observed in field-grown *N. attenuata* plants. Photographs: (A) http://www.nps.gov/plants/sos/bendcollections/images/Nicotiana%20attenuata_JPG.jpg; (B, C, F, G, H): D. Kessler, (D): B. Berger, (E): R. Halitschke, (K): A. Kessler, (I, J): A. Steppuhn.

1.3.2. Defense strategies

Because of its ”fire-chasing” behaviour, every new generation of *N. attenuata* has to cope with an unpredictable population of various herbivores from different feeding guilds, and, in turn, herbivores have to establish their own new generations on *N. attenuata* plants. The natural herbivore community mainly consists of specialized leaf-chewing larvae of the lepidopteran insect herbivores *M. sexta* and *M. quinquemaculata* (*Lepidoptera, Sphingidae*), the cell-content feeding mirid bug *Tupiocoris notatus* (*Hemiptera, Miridae*), and the flea beetle *Epitrix hirtipennis* (*Coleoptera, Chrysemelidae*)

(Kessler *et al*., 2004). Larvae of other polyphagous leaf-chewing insect herbivores *Heliothis virescens* (*Fabricius*) and *Spodoptera exigua* (*Huebner*) (*Lepidoptera, Noctuidae*) are also occasionally observed. Therefore *N. attenuata* provides an excellent system to study herbivore-resistance responses. Among these herbivores, the responses of *N. attenuata* to the Solanaceous specialist *M. sexta* are the most intensively studied. *N. attenuata* is known to tailor its wound response when fatty-acid amino-acid conjugates (FACs) present in the herbivores oral secretions (OS) and regurgitant are introduced into plant wounds during *M. sexta* attack. The metabolic reorganization after *M. sexta* attack is mediated by the octadecanoid pathway and begins with a dramatic JA burst in the damaged leaves (Schittko *et al*., 2000). This JA burst elicits secondary metabolites involved in direct defenses (Halitschke and Baldwin, 2004), such as trypsin PIs (TPI) (Zavala *et al*., 2004) and nicotine (Baldwin, 1999), and induces the release of volatile organic compounds (VOCs), which can function as an indirect defense by attracting predators of *M. sexta* larvae (Baldwin, 1999; Halitschke *et al*., 2000; Kahl *et al*., 2000; Kessler and Baldwin, 2001).

N. attenuata is not a domesticated plant and therefore functional associations between defense traits are likely to result from natural selection. Moreover, with its natural genotypic variation in defense traits among the accessions (also referred as genotypes) collected in Utah (UT) and Arizona (AZ) (Glawe *et al*., 2003), it provides a useful system to study the evolution of defense traits in general and of specific genes in particular (Wu *et al*., 2006, Steppuhn *et al*., submitted). *N. attenuata* provides us with an excellent opportunity to expand our knowledge on plant biotic interactions, but also on reproduction strategies and the evolutionary forces shaping them. This growing body of knowledge is facilitated by the use of modern methods like transcriptome- and proteome-analysis in combination with the manipulation of specific genes by *Agrobacterium*-mediated transformation.

1.4. Aim of this thesis

The present thesis focussed on trying to elucidate the role of the hydroxyproline-rich glycopeptide systemin precursor in *N. attenuata*, an well-investigated plant model system for herbivore-plant interactions.

To illuminate the role of ppHysSys in *N. attenuata*, the following subjects were investigated.

1. The occurence of tetraploidy in *Agrobacterium*-mediated transformed plants exemplified by the hydroxyproline-rich glycopeptide systemin precursor (also referred as prosystemin) in two accession of the wild tobacco, *N. attenuata* (manuscript I).
2. Whether the hydroxyproline rich systemin glycopeptide precursor (*Na*ppHypSys) of the wild tobacco, *N. attenuata*, functions in anti-herbivore defense responses as described for the ppHypSys in *N. tabacum* and the systemin precursor (Sys) in tomato, *S. lycopersicum* (manuscript II)?
3. And, if there is no overlap in a defense related role of *N. attenuata's* ppHS and *S. lycopersicum's* Sys, what function in *N. attenuata* is dependent on the hydroxyproline-rich systemin glycopeptid precursor (manuscript III)?

Manuscript I: – authors' contributions

Occurrence of tetraploidy in *Nicotiana attenuata* plants after *Agrobacterium*-mediated transformation is genotype specific but independent of polysomaty of explant tissue

Ben Bubner, Klaus Gase, Beatrice Berger, Dirk Link and Ian T. Baldwin

Plant Cell Reports 25 (7): 668-675 (2006)

This manuscript describes a genotype-specific occurrence of tetraploidy in *Nicotiana attenuata* which is caused by an *Agrobacterium*-mediated transformation. Doubling chromosome number or even the number of whole genomes by transformation, as demonstrated in this paper for the *N. attenuata* genotype Arizona, can confound functional analysis much more than those directly related to the transgene. These findings and the characterization of the hydroxyproline-rich systemin glycopeptide precursor (referred as prosystemin) silenced lines for their diploid status were prerequisites for all further experiments in this thesis.

Ben Bubner, who was responsible for the planning and analysis of the experimental work, was advised by Ian T. Baldwin. Klaus Gase provided all vectors for the investigated transgenic plants and wrote the first draft on the vector construction of the Materials and Methods. For the experimental part of the work, Ben Bubner investigated the influence of the transformation process and inbred status of the plants on ploidy levels. He first analyzed the ploidy status of transgenic plants containing different constructs. I analyzed the ploidy levels of transformed plants of two different genotypes (Arizona and Utah) but with the same vector (pRESC5SYS2) and at the same post-transformational state. Hence I contributed the most complete data set on transformed plants available at this time to confirm the genotype-specific occurrence of tetraploidy by *Agrobacterium*-mediated transformation. Ben Bubner and Dirk Link generated the data on ploidy levels in different somatic tissue of *N. attenuata* Arizona and Utah seedlings. The first draft of the manuscript was written by Ben Bubner and revised after suggestions from Ian T. Baldwin and the other co-authors.

Manuscript II: – authors' contributions

The hydroxyproline-rich glycopeptide systemin precursor *Na*preproHypSys does not play a central role in *Nicotiana attenuata´*s anti-herbivore defense responses

Beatrice Berger and Ian T. Baldwin

Plant, Cell and Environment 30: 1450-1465 (2007)

The impact of the hydroxyproline-rich glycopeptide systemin precursor (*Na*ppHS) on jasmonic-acid-mediated anti-herbivore defenses of *Nicotiana attenuata* was elucidated in glasshouse and field experiments for this manuscript. Our results reveal that the precursor in *N. attenuata's* herbivore defense response does not play a central role. I was responsible for the planning, realization and analysis of the glasshouse- and field-experiments and advised by Ian T. Baldwin. I wrote the first draft of the manuscript, which was in turn optimized by Ian T. Baldwin.

Manuscript III: – authors' contributions

Silencing the hydroxyproline-rich glycopeptide systemin precursor in two accessions of *Nicotiana attenuata* alters flower morphology and rates of self-pollination

Beatrice Berger and Ian T. Baldwin

Reviewed in Plant Physiology

Following up on the results in manuscript II – namely, that *Na*ppHS does not play a role in anti-herbivore defense as previously expected -- this study aimed to elucidate an alternative function for *Na*ppHS. We found evidence for the involvement of *Na*ppHS in developmental processes in *N. attenuata*. I observed the described phenotypic alterations in *Na*ppHS-silenced *N. attenuata* flowers for both ecotypes. Further experiments were planned, conducted, and analyzed by me under the supervision of Ian T. Baldwin. I wrote an outline and a first draft of the manuscript, which in turn will be optimized by Ian T. Baldwin

Manuscript I

Occurrence of tetraploidy in *Nicotiana attenuata* plants after *Agrobacterium*-mediated transformation is genotype specific but independent of polysomaty of explant tissue

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Abstract

Genotypes of *Nicotiana attenuata* collected from Utah and Arizona were transformed with 17 different vectors (14 unpublished vectors based on 3 new backbone vectors) using an *Agrobacterium*-mediated procedure to functionally analyze genes important for plant-insect interactions. None of 51 T1 to T3 transgenic Utah lines analyzed by flow cytometry were tetraploid, as opposed to 18 of 33 transgenic Arizona lines (55%). Analysis of T0 regenerants transformed with the same vector carrying an inverted repeat (IR) *N. attenuata* prosystemin construct confirmed the genotype dependency of tetraploidization: none of 23 transgenic Utah lines were tetraploid but 31 (72%) of 43 transgenic Arizonas were tetraploid. We tested the hypothesis that differences in polysomaty of the explant tissues accounted for genotype dependency of tetraploid formation by measuring polysomaty levels in different seedling tissues. Hypocotyls, cotyledons and roots of Utah and Arizona genotypes contained similar percentages of 4C nuclei (61 and 60; 7 and 5; and 58 and 61%, respectively). Since we used hypocotyls as explant sources and the nonoccurrence of tetraploid Utah transformants does not correspond to the high percentage of 4C nuclei in Utah hypocotyls, we can rule out a direct relationship between tetraploid formation and polysomaty level. We hypothesize that the difference between the Utah and Arizona genotypes results from the failure of polyploid Utah callus to regenerate into fully competent plants. We propose that future work on post-transformation polyploidy concentrate on the processes that occur during callus formation and plant regeneration from callus.

Keywords

Flow cytometry, Polyploidy, Polysomaty, Transformation, Transgenic plants

Introduction

Agrobacterium-mediated transformation has revolutionized the analysis of gene function in the plant sciences. Lines transformed with endogenous and heterologous genes in sense or antisense/inverted repeat (IR) orientations are frequently used to alter the expression of particular genes and thereby understand their function. Transformation success of plants regenerating from callus is usually determined by antibiotic screens, PCR, and Southern analysis and confirms the function of the resistance marker, the presence of the transgene, and the number of transgene copies. However, functional analyses can be confounded when genetic changes, such as the doubling of chromosomes or even whole genomes that are much larger than those directly related to the transgene, occur in the transformation process. Tetraploidization of plants after regeneration from calli with or without transformation was demonstrated more than 2 decades ago (Jacobson 1981, Imai et al. 1993, Lavia et al. 1994), but the analysis of ploidy levels has not yet been routinely integrated into post-transformation screening programs. More recently, flow cytometry, which allows plant transformants to be reliably and rapidly analyzed (Goldman et al. 2004, Ellul et al. 2003, Sigareva et al. 2004, Ducreux et al. 2005) has demonstrated that transformation can double the chromosome number of the transformed plants. In diploid tomato, 24.5 - 80% of transformants were tetraploid (depending on cultivar and method; Ellul et al. 2003) and up to 92% of originally triploid bermuda grass *Cynodon dactylon* x *transvaalensis* cv. TifEagle transformants were found to be hexaploid (Goldman et al. 2004).

The production of tetraploids by the transformation system for *Nicotiana attenuata* Torrey ex. Watson (Krügel et al. 2002), a native diploid tobacco used for the analysis of ecologically important traits (Baldwin 2001), is analyzed in this study. Two genotypes of *N. attenuata*, both from field collections -- one from Utah (Baldwin et al. 1994) and one from Arizona (Glawe et al. 2003) -- are routinely transformed in our laboratory to study the interaction between *N. attenuata* and its herbivores. We transformed the Utah and Arizona genotypes with the following *N. attenuata* genes in antisense or IR orientations: RUBISCO activase, prosystemin, WRKY3 transcription factor, lipoxygenase 3, putrescine-*N*-methyl-transferase, and trypsin proteinase inhibitor. In addition, we constitutively over-expressed the *N. attenuata* trypsin proteinase inhibitor, the prosystemin gene, and a mutated ethylene receptor 1 of *Arabidopsis thaliana* (Table 1). Transgenic lines had previously been subject to the routine post-transformation analysis of antibiotic screens and Southern

hybridizations. Here we add flow cytometry to the analysis to determine the tetraploidy of the Utah and Arizona genotypes and test a mechanism that could account for the transformation-induced tetraploidization.

Table 1 Vectors used for transformation

Vector	Type	Gene function	Accession number	Reference for cloning	Resistance in vector
pNATLOX1	AS	*N. attenuata* lipoxygenase *NaLOX3*	AY254349	Halitschke and Baldwin (2003)	Nourseothricin
pNATPI1	AS	*N. attenuata* trypsin proteinase inhibitor partial sequence	AY184823	Zavala et al. (2004)	Nourseothricin
pRESC2PIA2	CE	*N. attenuata* trypsin proteinase inhibitor, full length	AF542547	Zavala et al. (2004)	Hygromycin
pNATNC	EV	-	-	-	Nourseothricin
pRESC2NC	EV	-	-	-	Hygromycin
pRESC2RCA	AS	*N. attenuata* RUBISCO activase	BU494545	Völckel and Baldwin (2003)	Hygromycin
pRESC2SYS1	AS	*N. attenuata* pro-systemin	AY456270	Unpublished	Hygromycin
pRESC2SYS2	CE				
pRESC2TFN	AS	*N. attenuata* transcription factor WRKY3	AY456271	Unpublished	Hygromycin
pRESC5LOX	IR	*N. attenuata* lipoxygenase *NaLOX3*	AY254349	Halitschke and Baldwin (2003)	Hygromycin
pRESC5PMT	IR	*N. attenuata* putrescine-*N*-methyl transferase	AF280402	Winz and Baldwin (2001)	Hygromycin
pRESC5SYS2	IR	*N. attenuata* pro-systemin	AY456270	Unpublished	Hygromycin
pRESC2ETR1	CE	*Arabidopsis thaliana* functional restricted. ethylene receptor 1, *etr1-1*	AC020665	Chang et al. (1993)	Hygromycin
pSOL3LOX	IR	*N. attenuata* lipoxygenase *NaLOX2*	AY254348	Halitschke and Baldwin (2003)	Hygromycin
pSOL3PIA	IR	*N. attenuata* trypsin proteinase inhibitor partial sequence	AY184823	Zavala et al. (2004)	Hygromycin
pSOL3NC	EV	-	-	-	Hygromycin
pSOL4PIA	IR	*N. attenuata* trypsin proteinase inhibitor partial sequence	AY184823	Zavala et. al. (2004)	Nourseothricin

Construction of the first three vectors published (see references in Methods section); construction of remaining vectors is described in Supplementary Material S1.
AS: antisense expression, IR: inverted repeat, CE: constitutive expression, EV: empty vector

Materials and Methods

Plant Material

The Utah genotype was collected in 1992 from plants growing at the DI ranch, Santa Clara, southwestern Utah, USA (Baldwin et al. 1994). These seeds were used to establish an inbreeding line from which seeds of plants in the 7th, 11th, 12th, 14th or 15th generation have been used for transformation. For an inbreeding-free explant source, seeds were collected from one plant at the same site in 2004. The Arizona genotype was collected in 1996 from a 20-plant population near Flagstaff, Arizona, USA (Glawe et al. 2003). Plants grown from these seeds were selfed in 2001 and their bulk-collected seeds (1st generation) were used for transformation. From this 1st generation an inbred line to the 7th generation was established. Seed germination and rearing of plants was performed as described in Krügel et al. (2002).

Plasmid construction

A summary of the vectors used and the genes of interest carried by these vectors is provided in Table 1. The construction of pNATLOX1 (11.2 kb) was described in Krügel et al. (2002); details for the construction of pNATPI1 (9.0 kb) and pRESC2PIA2 (11.1 kb) can be found in the Supplementary Material of Zavala et al. (2004). The construction of all other vectors is described in Supplementary Material S1. Maps of the backbone vectors pRESC501, pSOL3RCA, and pSOL4RCA as the basis for all described vectors are given in Supplementary Material S2, S3 and S4.

Generation of transgenic plants

The transformation procedure is described in detail in Krügel et al. (2002). Briefly, hypocotyls from 8-day-old seedlings germinated on Gamborg´s B5 medium were cut with a scalpel in two to three 3 mm long pieces after the tip of the scalpel was dipped into a suspension containing the vector-harboring *Agrobacterium tumefaciens* (strain LBA 4404, Life technologies-Gibco BRL). On different phytagel-based media, the explants and resulting calli/plants went through five stages: co-cultivation (3 days), callus growth (14-21 days), shoot regeneration (14-21 days), shoot maturation (14-21 days) and rooting (21 days). After rooting, the plants were transferred to soil in Magenta boxes (77mm x 77mm x 77mm) and finally planted in 2-l pots in the greenhouse for breeding.

Characterization of transformants

For each T0 plant, 60 T1 seeds are germinated on plates with germination medium containing 35 mg/L hygromycin as described in Krügel et al. (2002) for seedling selection. If 75% of the seedlings survived after 7 days, the line was considered to be a putative transformant. For nourseothricin resistant T0 plants, half a cotyledon of 60 T1 seedlings was transferred to plates with callus-inducing medium containing 250 mg/L nourseothricin as described in Krügel et al. (2002) for callus selection. If 75% of the cotyledon halves showed callus growth within 7 days, the line was considered a putative transformant.

Flow cytometry

Nuclei were extracted and stained using the Partec Cystain UV-precise P kit (containing the dye 4´,6´-diamidino-2-phenylindole, DAPI) and 30 µm mesh filters (Partec, Münster, Germany) according to the manufacturer's instructions. Samples were mascerated together with *Brassica oleracea* cv. Rosella, *Hordeum vulgare* cv. Sultan, or *Secale cereale* cv. Petkus Spring as internal standards. Measurements were taken on the flow cytometer CCA-II (Partec, Münster, Germany) with UV excitation by a mercury arc lamp. The gain was generally set to 435 but was occasionally fine-tuned between 433 and 440. To measure transformants, plants were grown to the elongation stage and leaves from the upper part of the elongated shoot were used. For those lines identified in Table 2, flow cytometry was performed on cotyledons of 8-day-old seedlings.

Chromosome counting

Five-day-old seedlings that had been germinated as described in Krügel et al. (2002) were incubated for 2 h in 2 mM 8-hydroxychinolin at room temperature. After being fixed in alcohol/acetic acid (3:1: v:v), seedlings were macerated in 1 N HCl at 60 ºC for 4 min. After separation from the seedling, root tips were stained with carmine acetic acid, squeezed between a microscope slide and cover slip, and immediately observed under a microscope with 100x10 magnification and oil immersion. Pictures were taken with an Axioskop2 with digital camera and Axiovision 3.0 software for image acquisition (Carl Zeiss, Oberkochen, Germany).

Results and Discussion

Distribution of tetraploid transformants

Ploidy levels were determined by dividing the average fluorescence of 2C nuclei of the sample by the average fluorescence of 2C nuclei of the standard, yielding the ratio R. For measurements with the standard *Brassica oleracea* cv. Rosella, a sample with an R of 3.32 ± 5% CV was considered diploid and a plant with R=6.64 ± 5% CV tetraploid. For *Hordeum vulgare* cv. Sultan the ratios were 0.655 ± 5% and 1.31 ± 5% CV, and for *Secale cereale* cv. Petkus Spring, the ratios were 0.408 ± 5% CV and 0.816 ± 5% CV. A small number of samples were measured without a standard; as all samples were measured with a similar gain, the location of the 2C peak in the histogram of a diploid plant could be readily determined. When the 2C peak was absent and the 4C peak was very prominent, a plant was considered tetraploid. The flow cytometric ploidy determinations were confirmed by counting metaphase chromosomes for pRESC2RCA-transformed Arizona lines A02-415 (2*n*=24) and A02-416 (2*n*=48, Fig. 1), the former being diploid by flow cytometry and the latter tetraploid (Table 2).

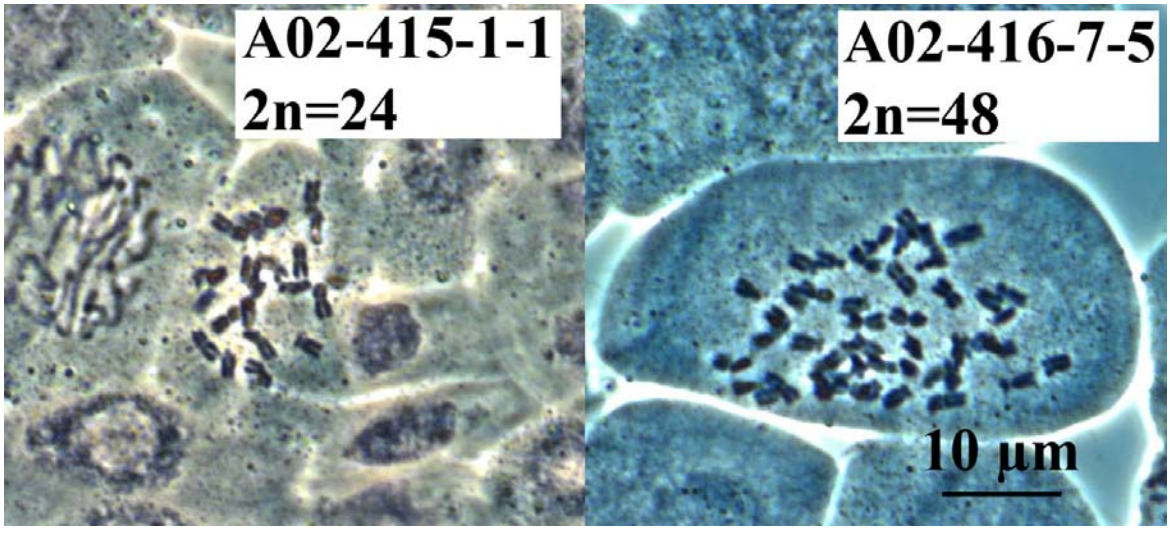

Figure 1. Metaphase plates in root tips of transgenic *N. attenuata* of the Arizona genotype at 1000x magnification. T3 seeds of lines growing in T2 were used. The T2 lines analyzed are given in the figure and described in Table 2.

A majority of the ploidy determinations of the transgenic lines presented in Table 2 were conducted after hygromycin resistance screens and Southern blot analysis had established that the lines had been transformed.

That none of the 51 transgenic Utah lines were found to be tetraploid was not influenced by the vectors used for the transformation or by the generation of inbred line used for transformation. Even when the Utah genotype was subjected to two consecutive transformations (pSOL4PIA transformed into A03-108-3, which had been transformed with pSOL3LOX), none of the regenerated lines were tetraploid.

The situation was quite different for transgenic Arizona lines (Table 2): there more than half of the lines were tetraploid. It was not possible to distinguish tetraploid from diploid plants by visually examining external morphological features.

Table 2 Ploidy levels of transgenic plants

Construct name	Transformant	Generation of transformants	Inbred generation of starting material	Standard	Ploidy
Utah genotype transformants					
pNATLOX	A300-1-1	T2	6x	NST	2x
pNATNC	A03-412-4-1[a]	T2	15x	HV	2x
pNATPI1	A315-1-5-1	T3	7x	BO	2x
	A339-1-1-1	T3	7x	BO	2x
pRESC2ETR1	A03-328-8-1	T2	14x	NST	2x
	A03-408-7-1	T2	14x	NST	2x
pRESC2NC	A03-09-3-1	T2	14x	BO	2x
pRESC2RCA	A991-2-1-1	T3	13x	BO	2x
	A02-19-4-7-1	T3	13x	BO	2x
	A02-31-9-3-1	T3	13x	BO	2x
	A02-177-4-2-1	T3	11x	BO	2x
pRESC2SYS1	A137-7-x-1	T3	11x	BO	2x
	A148-3-x-1	T3	11x	BO	2x
	A160-5-x-1	T3	11x	BO	2x
pRESC2SYS2	A430-14-x-1	T3	14x	BO	2x
pRESC2TFN	A02-489-x-x-1	T3	14x	NST	2x
	A02-491-x-x-1	T3	14x	NST	2x
	A784-x-x-1	T3	12x	NST	2x
	A793-x-x-1	T3	12x	NST	2x

Table 2 continued

Construct name	Transformant	Generation of transformants	Inbred generation of starting material	Standard	Ploidy
pRESC5LOX	A03-499-1	T1	15x	NST	2x
	A03-499-3-1	T2	15x	HV	2x
	A03-507-6	T1	15x	NST	2x
	A03-507-6-1	T2	15x	SC	2x
	A03-514-2	T1	15x	NST	2x
	A03-515-3	T1	15x	NST	2x
	A03-533-1	T1	15x	NST	2x
	A03-534-5	T1	15x	NST	2x
	A03-534-5-1	T2	15x	SC	2x
	A03-542-4	T1	15x	NST	2x
	A03-542-5-1	T2	15x	SC	2x
	A03-562-2-1	T2	15x	HV	2x
pRESC5PMT	A03-108-3-1	T2	14x	BO	2x
	A03-108-3-1-1[a]	T3	14x	HV	2x
	A03-145-1-1-1[a]	T3	14x	HV	2x
pSOL3LOX	A04-52-2-1	T2	14x	HV	2x
	A04-57-1-1	T2	14x	SC	2x
	A04-59-2-1	T2	14x	HV	2x
	A04-65-10-1	T2	14x	SC	2x
	A04-67-4-1	T2	14x	HV	2x
pSOL3NC	A04-266-1	T1	14x	HV	2x
pSOL3PIA	A04-141-4	T1	14x	BO	2x
	A04-142-4	T1	14x	BO	2x
	A04-143-5	T1	14x	BO	2x
	A04-160-1	T1	14x	BO	2x
	A04-169-4	T1	14x	BO	2x
	A04-186-1	T1	14x	BO	2x
pSOL4PIA in A03-108-3	A04-103-2	T1	1x	BO	2x
	A04-105-2	T1	1x	BO	2x
	A04-106-2	T1	1x	BO	2x
	A04-107-2	T1	1x	BO	2x
	A04-111-2	T1	1x	BO	2x

Table 2 continued

Construct name	Transformant	Generation of transformants	Inbred generation of starting material	Standard	Ploidy
	A04-215-1	T1	1x	HV	2x
	A04-216-1	T1	1x	HV	2x
	A04-226-1	T1	1x	HV	2x
	A04-227-1	T1	1x	HV	2x
Arizona genotype transformants					
pRESC2NC	A03 367-2	T1	1x	HV	4x
	A03 389-7	T1	1x	HV	4x
	A04-364-1[a]	T1	1x	HV	2x
pRESCPIA2	A966-x-x-1	T3	1x	BO	2x
	A981-x-x-1[b]	T3	1x	BO	2x
	A989-x-x-1	T3	1x	BO	4x
	A995-x-x-1	T3	1x	BO	4x
pRESC2RCA	A02-306-12-1-1	T3	1x	BO	4x
	A02-348-8-1-1	T3	1x	BO	4x
	A02-363-1-2-1	T3	1x	BO	4x
	A02-414-2-3-1	T3	1x	BO	4x
	A02-415-1-1-1	T3	1x	BO	2x
	A02-416-7-5-1	T3	1x	BO	4x
	A02-418-5-1-1	T3	1x	BO	2x
	A02-424-14-5-1	T3	1x	BO	4x
	A02-434-16-1-1	T3	1x	BO	2x
	A02-443-4-1-1[c]	T3	1x	BO	2x
	A02-458-8-1-1	T3	1x	BO	4x
	A02-484-3-2-1	T3	1x	BO	4x
	A02-485-2-2-1	T3	1x	BO	2x
	A02-504-4-1-1	T3	1x	BO	2x
pRESC5PMT	A04-108-6	T1	1x	BO	4x
	A04-108-6-1[a]	T2	1x	HV	4x
	A04-139-1	T1	1x	BO	4x
	A04-140-3	T1	1x	BO	4x
	A04-144-3	T1	1x	BO	2x
	A04-145-1	T1	1x	HV	4x

Table 2 continued

Construct name	Transformant	Generation of transformants	Inbred generation of starting material	Standard	Ploidy
	A04-157-3	T1	1x	BO	2x
	A04-161-3	T1	1x	BO	2x
	A04-189-1	T1	1x	HV	2x
	A04-190-1	T1	1x	HV	2x
	A04-196	T0	1x	BO	4x
	A04-220	T0	1x	BO	4x
	A04-163-4	T1	1x	HV	2x

All transformants were confirmed by their resistance to hygromycin or nourseothricin. The digits beyond the line number are the numbers for a specific individual in a certain generation after T0. Example: A03-328-8-1 is a T2 offspring of transformation event A03-328 (T0). An x means that no number was assigned to the individual that gave rise to the next generation. Abbreviations for internal standards: NST no internal standard, BO *Brassica oleracea* cv. Rosella, *Hordeum vulgare* cv. Sultan, SC *Secale cereale* cv. Petkus Spring. [a]Measured with one cotyledon of an 8 day-old plant. [b]This diploid line has been used for the experiments described in Zavala et al. (2004). All conclusions drawn in this work are due to the experimental conditions described there and not to tetraploidization. [c]Not transformed according to hygromycin-resistance screen.

The occurrence of tetraploid transformants did not appear to influence the transgenes inserted into the vectors. For example, for the more than 10 independently transformed lines that were analyzed after being transformed with vectors pRESC2RCA and pRESC5PMT, the tetraploidization rates were 57 and 50% (Table 3).

Table 3 Summary of tetraploidy occurrence from Table 2

Genotype	Vector	*N*	Tetraploid number	Tetraploid frequency (%)
Utah	All	51	0	0
Arizona	All	33	18	55
	pRESC2NC	3	2	67
	pRESC2PIA2	4	2	50
	pRESC2RCA	14	8	57
	pRESC5PMT	12	6	50

To rule out the prosaic explanation for transformed tetraploids, namely, that they resulted from naturally occurring Arizona tetraploids among the seeds used for the transformation, we examined the ploidy levels of 22 individual seedlings from the seed source used for the transformation; all were found all to be diploid (Table 4).

Table 4 Occurrence of tetraploidy in T0 transformants of Utah and Arizona genotypes of *N. attenuata* containing the vector pRESC5SYS2 (*N. attenuata* prosystemin gene in an inverted repeat orientiation) and for untransformed seedlings of the Arizona genotype

Genotype	*N*	Tetraploid number	Tetraploid (%)
T0 Utah	23	0	0
T0 Arizona	43	31	72
Wild type Arizona	22	0	0

Hordeum vulgare cv. Sultan served as internal standard.

Since all of the Utah lines listed in Table 2 passed the antibiotic screen and were therefore fertile, the lack of tetraploids among the T1-T3 generation Utah may have resulted from the discarding of infertile tetraploid T0 plants during the post-transformation breeding process. To test this hypothesis, we analyzed 23 T0 Utah and 43 T0 Arizona lines that had been transformed with the vector pRESC5SYS2 before the hygromycin resistance test was carried out (Table 4). As we found no T0 Utah lines that were tetraploid, we conclude that our transformation system does not produce tetraploid transformants of the Utah genotype. This experiment also highlighted the efficiency of using flow cytometric analysis before the antibiotic resistance screen for Arizona plants: only 12 of 43 transformants were diploid.

Compared to Arizona explant sources, sources of the Utah genotype are from a higher inbreeding generation (6x to 15x) (all 1x, Table 2). To exclude the possibility that the nonoccurrence of tetraploidy for Utah is due to inbreeding, we transformed Utah and Arizona explant sources with vector pRESC2RCA. For both genotypes low and high inbreeding generations were used (Table 5).

Table 5 Comparison of tetraploidy occurrence after transformation with vector pRESC2RCA for different stages of inbreeding of the explant source

Genotype	Inbreeding generation of explant source	*N*	Tetraploid (%)
Utah	0x	1	0
	17x	19	0
Arizona	1x	11	27
	7x	24	62

Utah 0x are seeds from plant DI04/18 collected in 2004 from the same location where the founder seeds for the Utah inbred line have been collected. *Brassica oleracea* cv. Rosella was used as internal standard.

Unfortunately only one transformant resulted from the transformation of wild-type Utah material, probably due to the nontransformability of offspring of this specific plant DI04/18. Nevertheless, Table 5 shows that the general statement, "no tetraploid Utah transformants occur," remains true; in contrast, tetraploid plants occurred for both Arizona inbreeding stages. The low percentage of tetraploid of Arizona 1x is probably due to the low *n* and highlights the high variability of percentages which forbids any statistical analysis beyond a statement about whether tetraploidy occurs. Despite this limitation, we can conclude that inbreeding up to the 7th generation played no role in the absence of tetraploidy for the Arizona genotype.

Polysomaty in seedlings and tetraploidization

Polysomaty occurs when nuclei with a higher ploidy level than that found in particular tissues are found and results from endoreduplication of the chromosomal DNA without mitosis and cell division (Joubès and Chevalier 2000). Polysomaty was described half a century ago (Bradley 1954; Swift 1950) but only recently has it been shown to be widespread among herbaceous plants (Barow and Meister 2003) or correlated with the frequency of polyploidy regenerants. When *Lycopersicon esculentum* cv. Moneymaker callus was regenerated (without transformation), 58% of the regenerants were polyploid when hypocotyls were used as explants, 12% were polyploids when cotyledon were used as explants, and only 1.5% were polyploids when leaf explants were used. This correlated well with the percentages of nuclei which were diploid in hypocotyls, cotyledons, and leaves (22, 60, and 93%, respectively) (Bulk et al. 1990). A similar correlation was observed by Sigareva et al. (2004) who both transformed and regenerated three different genotypes of *Lycopersicon esculentum*. Regenerants from hypocotyl explants were 25, 36,

and 27% diploid, while regenerants from leaves were 85, 82, 100% diploid (the latter with *n*=4). Hence, the tetraploidization observed in the Arizona lines may result from transformed tetraploid cells in the explant tissues (Bulk et al. 1990; Ellul et al. 2003), and the differences in the degree of polysomaty in the explants of Utah and Arizona genotypes may account for the differences in tetraploid formation.

To test this hypothesis, we measured the percentage of 4C nuclei in hypocotyls, cotyledons, and roots of 8 day-old Utah and Arizona seedlings (Fig. 2; Table 6). The cotyledons of *N. attenuata* harbored 5 and 7% 4C nuclei in Utah and Arizona, respectively, which contrasts with the observations in solanaceaous plants that cotyledons are highly polysomatic (Barow and Meister 2003; Ellul et al. 2003; Sigareva et al. 2004). On the other hand, the high percentage of 4C cells in the roots (58 and 61%; Table 6) is similar to that reported from the roots of *Lycopersicon pimpinellifolium* (Barow and Meister 2003). More importantly, the explant tissues used for the transformation, namely the hypocotyls, had similar frequencies of 4C cells (60 and 61%; Table 6) in the Arizona and Utah genotypes. A direct relationship between tetraploidization rate in transformants and polysomaty of explant tissue should have yielded a flow cytometric histogram in the Utah hypocotyls similar to the upper left one measured from the Utah cotyledons (Fig. 2). As there was no polysomaty difference between the two genotypes, we can reject a direct relationship between the occurrence of tetraploidization in transformants of a genotype and the polysomaty level in its tissues.

However, our experiments and the results from Ellul et al. (2003) demonstrate that explant genotypes can influence the occurrence of tetraploid transformants. Ellul et al. (2003) reported that 80% of tetraploids resulted from one genotype and 30, 36, 28, and 43% from four others and that these frequencies were only weakly related to the polysomaty of explant tissue. However, Ellul et al. (2003) also showed that small changes in the transformation procedure led to changes in the percentage of tetraploids. Our own experiences (27% for Arizona 1x, Table 5) indicate that tetraploidy frequency varies hugely and to an extent that impairs statististical correlation when genotypes with low and high post-transformation tetraploidy are compared. The advantage of the experimental system introduced here is clear: One genotype with no post-transformation tetraploidy and another with a tetraploidy occurrence that can be detected even among a low number of transformants demonstrates that there is genotypic component in the occurrence of tetraploidy.

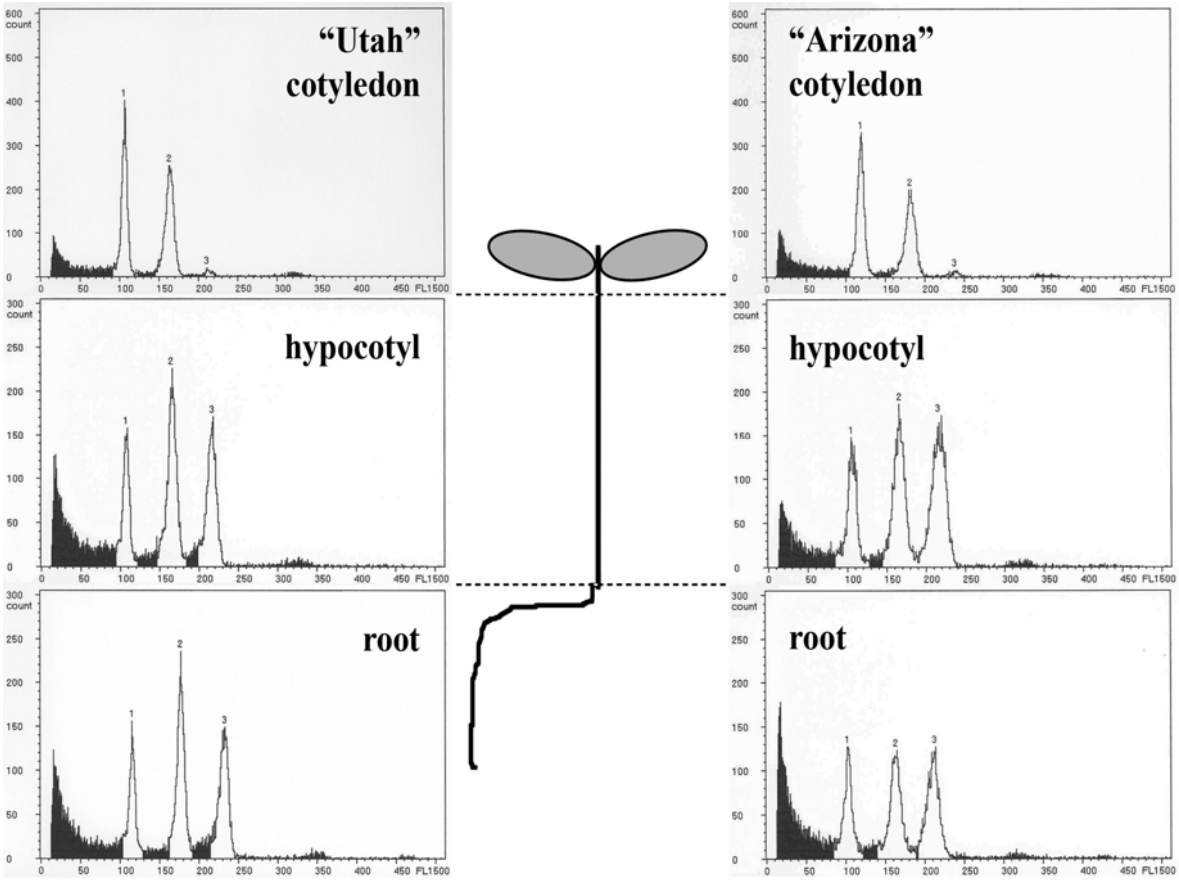

Fig. 2 Histograms of *N. attenuata* seedling tissue: the highlighted area under each peak is the integral of the number of nuclei counted. In order to obtain sufficient nuclei for the analysis, parts of several seedlings were pooled for each measurement (10 hypocotyls, 10 roots, 3 cotyledons). Peaks are numbered: 1, 2C peak of *N. attenuata*; 2, 2C peak of standard *Hordeum vulgare* cv. Sultan; 3, 4C peak of *N. attenuata*.

Table 6 Percentage (± SD of three replicates) of 4C nuclei in different parts of 8-day-old *N. attenuata* seedlings (numbers refer to seedling parts pooled for the analysis).

Genotype	Hypocotyls (10)	Cotyledons (3)	Root (10)
Utah	61% ± 2[a]	7% ± 2	58% ± 4
Arizona	60% ± 4	5% ± 2	61% ± 5

No nuclei with a higher C level were observed.

[a] Measurements as seen in Fig. 2 were repeated three times.

Conclusion

The occurrence of tetraploidy during transformation highlights the need to include ploidy tests into post-transformation-screening programs. Our observations suggest that tetraploid plants cannot always be distinguished by morphology, but that flow cytometry provides a rapid and robust determination of ploidy. When genotypes that produce a high percentage of tetraploids are to be transformed, a ploidy screen of T0 plants by flow cytometry can substantially reduce the post-transformation workload.

While in many transformation systems with callus-mediated regeneration, tetraploidy has been observed with different frequencies for different genotypes, the *N. attenuata* transformation system is unusual in having one genotype with no tetraploid transformants. As the clear-cut distinction between occurrence and nonoccurrence is easy to observe without statistical analysis, we were able to rule out three trivial causes: namely, that Arizona explant sources are tetraploid, that inbreeding is the single reason for no Utah tetraploids, and that the transgene triggers tetraploidy. As a fourth possible cause we were able to refute the hypothesis that tetraploidy occurrence resulted from differences in the polysomaty of explant tissue. Since the excluded causes are not directly related to callus-mediated transformation, future research on post-transformation tetraploidy should concentrate on the processes that take place during callus formation and subsequent plant regeneration.

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Supplementary Material

S1: Plasmid construction

pNATLOX1, pNATPI1, pNATNC

The construction of pNATLOX1 (11.2 kb) and pNATPI1 (9.0 kb) was described elsewhere (Krügel *et al.* 2002 and Zavala *et al.* 2004, Supplementary Material). pNATNC (8.7 kb; empty vector control) was obtained by digesting pNATGUS3 (10.8 kb; Krügel *et al.* 2002) with *Bst*EII and *Nco*I, blunting the overhangs with T4 DNA-Polymerase and re-circularization of the 8.7 kb fragment with T4 DNA ligase.

pRESC200, pRESC2PIA2, pRESC2NC, PRESC2RCA, pRESC2SYS1, pRESC2SYS2, pRESC2TFN

The construction of pRESC2PIA2 and pRESC20 was described in the Supplementary Material of Zavala *et al.* (2004). Plant transformation vector pRESC200 (11.8 kb) consists of the 9.8 kb *Bst*EII-*Nco*I fragment of pRESC20 and the *gusA* containing 2.1 kb *Bst*EII-*Nco*I fragment of pCAMBIA-1301 (GenBank AF234297). The 9.7 kb *Xho*I-*Bst*EII-fragment of pRESC200 led after T4 DNA polymerase treatment and recircularization to plasmid pRESC2NC (9.7 kb; empty vector control) and also served as a vector to clone the following *N. attenuata* gene PCR fragments digested with the same enzymes: RUBISCO activase (*rca*, antisense; BU494545), primers RCA1-36 (5'- GCGGCGGGTCACCGGAC AAACTTCTGAACTCTTTCG-3') and RCA2-35 (5'- GCGGCGCTCGAGGACTTCAG CAACACGACAATTAG-3'), template previously cloned *rca* cDNA; prosystemin (*sys*, antisense; AY456270) primers SYS1-36 (5'-GCGGCGGGTCACCGAGAGTTCTGTTT CTCATCTACC-3') and SYS2-32 (5'-GCGGCGCTCGAGACACACTGTTGGATACCTC C -3'), template previously cloned *sys* cDNA; prosystemin (*sys*, full length sense expression; AY456270) primers SYS3-33 (5'-GCGGCGGGTCACCTTAATAGGACTG AAGAGGAC -3') and SYS4-33 (5'-GCGGCGCTCGAGATGAGAGCTCTCTTTCT CATC-3'), template previously cloned *sys* cDNA; transcription factor NaWRKY3 (*wrky3*, antisense; AY456271) primers TFN4-23 (5'-CCATAAACATGTCATCTCGAGGC-3') and TFN5-34 (5'-GCGGCGGGTCACCCTCAGAGCAATGGTGGGAACC-3'), template previously cloned *wrky3* cDNA; yielding plasmids pRESC2RCA (10.0 kb), pRESC2SYS1 (9.9 kb), pRESC2SYS2 (10.2 kb) and pRESC2TFN (10.7 kb).

pRESC501, pRESC5LOX, pRESC5PMT, pRESC5SYS2

cDNA of the *Nicotiana benthamiana* phytoene desaturase gene (*pds*; AJ571700) resident on a plasmid kindly provided by Prof. D. Baulcombe, JIC Norwich, United Kingdom, was PCR amplified using primer pairs PDS13-40 (5'-GCGGCGAAGCTTGAGCTCAGGCAC TCAACTTTATAAACCC-3'), PDS14-40 (5'-GCGGCGGAATTCCTCGAGTCAGCTCG ATCTTTTTTATTCG-3') and PDS15-40 (5'-GCGGCGAAGCTTCTGCAGAGGCACTC AACTTTATAAACCC-3'), PDS16-40 (5'- GCGGCGGAATTCCCATGGTCAGCTCGA TCTTTTTTATTCG-3'). After digestion with *Eco*RI and *Hin*dIII both 0.2 kb PCR fragments were cloned in pUC18 (L08752) cut with the same enzymes, yielding pUCPDS4 (2.9 kb) and pUCPDS5 (2.9 kb), respectively. After treatment with *Pst*I, T4 DNA Polymerase and *Eco*RI the 4.5 kb fragment of pHYB34 (Gase *et al*. 1998), served as cloning vector for the 0.2 kb *'pds'* fragment obtained by treating pUCPDS4 with *Hin*dIII, T4 DNA Polymerase and *Eco*RI, resulting in pUCPDS6 (4.2 kb). pUCPDS7 (4.5 kb) was created by fusing the 2.4 kb *Aat*II-*Hin*dIII vector fragment of pUCPDS5 to the 2.1 kb *'pds'* containing fragment of pUCPDS6. The 9.8 kb *Bst*EII-*Nco*I fragment of pRESC200 was blunt ended with T4 DNA Polymerase and circularized. The resulting plasmid pRESC201 (9.8 kb), cut with *Nco*I and *Xho*I, served as cloning vector for the 1.8 kb *Nco*I-*Xho*I *'pds'* inverted repeat fragment from pUCPDS7, yielding pRESC500 (11.5 kb). Intron 3 of the *Flaveria trinervia* pyruvate, orthophosphate dikinase gene (X79095) resident on a plasmid kindly provided by Dr. P. Westhoff, Düsseldorf, Germany, was PCR amplified with primers PDK1-29 (5'-GCGGCGGAGCTCCTTGGTAAGGAAATAAT-3') and PDK2-30 (5'-GCGGCGCTGCAGTCCCAACTGTAATCAATC-3'). After digestion with *Pst*I and *Sac*I the obtained 0.8 kb PCR fragment was used to replace the 1.4 kb *Pst*I - *Sac*I spacer between both inverted *'pds'* copies on pRESC500. Due to *'pds'* inverted repeat structure the resulting plant transformation vector pRESC501 (10.9 kb) allowed efficient silencing of the *pds* gene in *N. attenuata*. For the construction of silencing vectors for other target genes the 0.2 kb *Nco*I-*Pst*I and the 0.2 kb *Sac*I-*Xho*I *'pds'* fragments of pRESC501 were subsequently replaced by two identical, divergently oriented PCR fragments (0.2-0.9 kp) of the target gene, flanked by the appropriate cloning sites (*Nco*I-*Pst*I and *Sac*I-*Xho*I).

Target genes relevant for this paper were the *Nicotiana attenuata* genes for lipoxygenase 3 [*lox3*, AY254349; PCR with primer pairs LOX5-30 (5'-GCGGCGCCATGGACAACGT CTTGGGTAAGG-3'), LOX6-30 (5'-GCGGCGCTGCAGGCAGTGTTGAAGGATTGG-3') and LOX8-31 (5'-GCGGCGGAGCTCGGCAGTGTTGAAGGATTGG-3'), LOX7-30 (5'-GCGGCGC TCGAGACAACGTCTTGGGTAAGG-3'); template previously cloned

lox3 cDNA; resulting silencing vector pRESC5LOX (11.4 kb)], putrescine N-methyltransferase 1 [*pmt1*, AF280402; PCR with primer pairs PMT7-31 (5'-GCGGCG CCATGGAGCCCTT AAAGACTTGACG-3'), PMT8-33 (5'-GCGGCGCTGCAGTAC CAACACAAATGGCT CTAC-3') and PMT10-33 (5'-GCGGCGGAGCTCTACCAAC ACAAATGGCTCTAC-3'), PMT9-33 (5'-GCGGCGCTCGAGCGAGCCCTTAAAGA CTTGACG-3'); template previously cloned *pmt1* cDNA; resulting silencing vector pRESC5PMT (12.4 kb)] and prosystemin [*sys*, AY456270; PCR with primer pairs SYS11-32 (5'-GCGGCGCTGCAG CTCATCTACCTTATTCTTGC-3'), SYS12-34 (5'-GCGGC GCCATGGCAGTGTTTTC AGAGGTTTTCTC-3') and SYS9-29 (5'-GCGGCGGAGCT CATCTACCTTATTCTTGC-3'), SYS10-34 (5'-GCGGCGCTCGAGCAGTGTTTTCAG AGGTTTTCTC-3'); template previously cloned *sys* cDNA; resulting silencing vector pRESC5SYS2 (10.9 kb)].

pRESC2ETR1

The chromosomal region of the *Arabidopsis thaliana* ethylene-response protein gene *etr-1* (AC020665, positions 33116-40381, mutation 36538 C to A), resident on a plasmid kindly provided by Prof. E. M. Meyerowitz, California Institute of Technology, Pasadena, California, USA, was cut with *Xho*I, blunt ended with T4 DNA-Polymerase and digested with *Nco*I. The obtained 3.7 kb fragment was ligated to the 9.7 kb vector fragment obtained by treating pRESC201 with *Xho*I, T4 DNA polymerase and *Nco*I, yielding plasmid pRESC2ETR1 (13.4 kb, *etr-1* sense expression).

pSOL3RCA, pSOL3LOX, pSOL3PIA, pSOL3NC

Plasmid pRESC200 was cut partially with *Aat*II and completely with *Hin*dIII. The resulting 10.0 kb fragment was circularized, yielding pRESC000. The 0.9 kb *Afl*III-*Ahd*I-fragment of pUC19 (L09137) was T4 DNA polymerase treated and ligated to pRESC000 linearized with *Bst*1107I (orientation of fragments: *Ahd*I end borders on T-DNA). The 6.3 kb *Bst*EII-*Eco*RI-fragment of the resulting plasmid pSOL1(10.9 kb) was fused to the 3.8 kb *Bst*EII-*Eco*RI-fragment of pRESC5RCA, giving pSOL3RCA (10.1 kb), a plant gene silencing vector with the same target gene inverted repeat structure as pRESC501, but without rescuing functionality. A number of target gene silencing vectors were derived from pSOL3RCA analogous to the procedure described above for pRESC501. Target genes for these constructs were the *Nicotiana attenuata* genes for lipoxygenase 2 [*lox2*, AY254348; PCR with primer pairs LOX9-33 (5'-GCGGCGCCATGGCCATATATC

TCAGGGTCTAGC-3'), LOX10-33 (5'-GCGGCGCTGCAGCCCTTATCCTAGAAGAT GCAG-3') and LOX12-32 (5'-GCGGCGGAGCTCCCTTATCCTAGAAGATGCAG-3'), LOX11-33 (5'-GCGGCGCTCGAGCCATATATCTCAGGGTCTAGC-3'); template previously cloned *lox2* cDNA; resulting silencing vector pSOL3LOX (10.6 kb)] and proteinase inhibitor [*pi*, AY184823; PCR with primer pairs PIA2-32 (5'-GCGGCGCCAT GGCTTACAACCCTTCGTGCCTG-3'), PIA14-32 (5'-GCGGCGCTGCAGTACTTTAG TGATGATGGAAC-3') and PIA15-33 (5'-GCGGCGGAGCTCGTACTTTAGTGATGAT GGAAC-3'), PIA13-32 (5'-GCGGCGCTCGAGCTTACAACCCTTCGTGCCTG-3'); template previously cloned *pi* cDNA; resulting silencing vector pSOL3PIA (9.9 kb)]. The 8.8 kb *Xho*I-*Bst*EII-fragment of pSOL3RCA formed after T4 DNA polymerase treatment and recircularization plasmid pSOL3NC (9.7 kb; empty vector control).

pSOL4PIA

The 8.4 kb *Xba*I-*Kpn*I-fragment of pRESC7 (Zavala *et al*., 2004, Supplementary Material) was ligated to the 0.9 kb *Xba*I-*Kpn*I-fragment of pUCNAT3 (Zavala *et al*., 2004, Supplementary Material), yielding plant transformation vector pRESC100 (9.3 kb) with the *sat-1* streptothricin-acetyl-transferase gene (X15995) as plant selectable marker. The 8.1 kb *Xba*I-*Eco*RI -fragment of pSOL3RCA served as cloning vector for the 1.5 kb *Xba*I-*Eco*RI-fragment containing *sat-1* of pRESC100. The 8.3 kb *Nco*I-*Xho*I-fragment of the resulting plasmid pSOL4RCA (9.6 kb) and the 1.1 kb-*Nco*I-*Xho*I-fragment of pSOL3PIA containing the *'pi'* inverted repeat were joined, yielding plasmid pSOL4PIA (9.4 kb).

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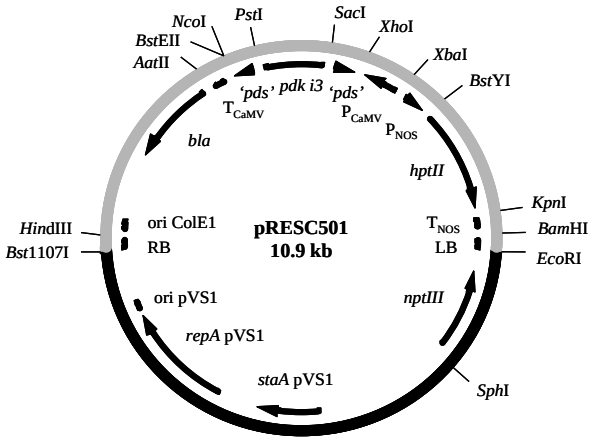
S2



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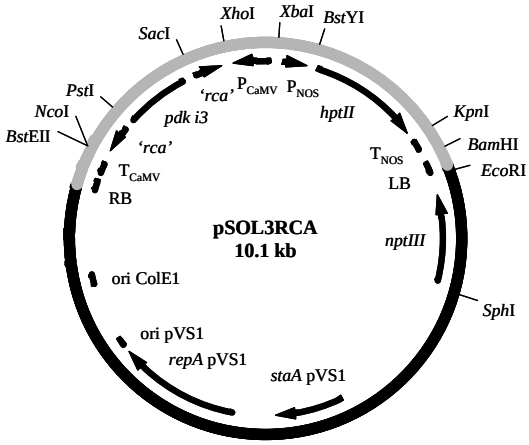
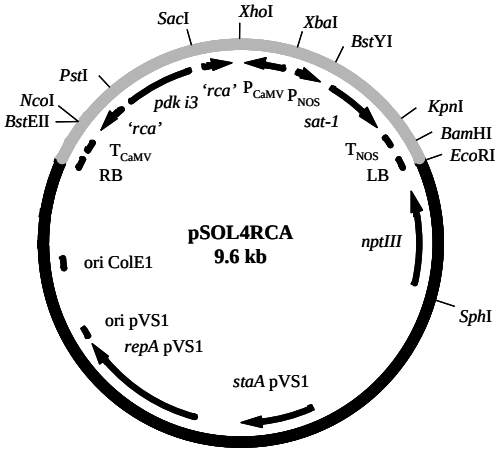


S4



Suppl. Fig. S2, S3, S4: Plasmids used as backbone for the construction of inverted repeat gene silencing vectors are shown in Supplementary Material **S2** (pRESC501), **S3** (pSOL3RCA) and **S4** (pSOL4RCA). The genes of interest can be easily exchanged as *Nco*I-*Pst*I and *Sac*I-*Xho*I fragments.

Functional elements on T-DNA (grey): *'pds'* , *pds* gene fragment; *'rca'* , *rca* gene fragment; *pdk i3*, intron 3 of *Flaveria trinervia pdk* gene for pyruvate, orthophosphate dikinase; LB/RB, left/right border of T-DNA; P_{NOS}/T_{NOS}, promoter/terminator of nopaline synthase gene; *hptII*, hygromycin phosphotransferase gene from pCAMBIA-1301 (AF234297); *sat-1*, streptothricin-acetyl-transferase gene (X15995); P_{CaMV}/T_{CaMV}, 35S promoter/terminator of cauliflower mosaic virus; *bla*, beta-lactamasease gene from pUC19; ori ColE, pUC19 origin of replication. Functional elements outside T-DNA (black): ori pVS1, *repA* pVS1, *staA* pVS1, origin of replication, replication protein gene, partitioning protein gene from plasmid pVS1; *nptIII*, aminoglycoside phosphotransferase of type III from *Streptococcus faecalis*. Displayed sites mark the borders of functional elements.

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Cover caption: *Manduca* larvae feeding on a *Nicotiana attenuata* plant growing in southwestern Utah, USA. Berger and Baldwin (pp. 1450-1464) studied the role of a hydroxyproline-rich glycopeptide prosystemin precursor in *N. attenuata's* anti-herbivore defense. *M. sexta* performance was not affected by silencing or over-expressing the prosystemin precursor, and prosystemin does not seem to be a strong regulator of anti-herbivore defenses in this species (Photograph by Danny Kessler).

Manuscript II

The hydroxyproline-rich glycopeptide systemin precursor *Na*preproHypSys does not play a central role in *Nicotiana attenuata´s* anti-herbivore defense responses

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Abstract

To determine whether the *Nicotiana tabacum* preproHypSys homologue in *N. attenuata* (*Na*preproHypSys) mediates anti-herbivore responses, we silenced (IR*sys*) and ectopically over-expressed (OV*sys*) *Na*preproHypSys in *N. attenuata*. Neither herbivore simulation nor methyl jasmonate (MeJA) application increased transcripts in wild-type (WT) or transformed lines. Compared to WT plants, OV*sys* plants had marginally higher constitutive levels but normally induced levels of trypsin proteinase inhibitors (TPIs) and nicotine; IR*sys* plants did not differ from WT plants. Herbivory-associated signaling [salicylic-acid-induced protein kinase (SIPK) activity, jasmonic acid (JA), jasmonic acid-isoleucine (JA-Ile/Leu), and ethylene production or perception] did not differ strongly among the lines, but JA, JA-Ile/Leu, and ethylene were marginally higher in OV*sys* plants. *Manduca sexta* larval performance did not differ among the lines, but feeding induced levels of TPI and nicotine in OV*sys* plants and decreased them in IR*sys* plants relative to WT. The secondary metabolite profiles of plants transplanted into *N. attenuata's* native habitat in the Great Basin Desert (Utah, USA) mirrored those of glasshouse-grown plants, and compared to WT plants, OV*sys* plants suffered marginally less damage from grasshoppers, mirids, and flea beetles but did not differ in their ability to attract *Geocoris* predators. We conclude that *Na*preproHypSys does not play a central role in anti-herbivore defense signaling in this native tobacco.

Keywords

Nicotiana attenuata, direct defenses, herbivore resistance, hydroxyproline-rich glycopeptide systemin precursor, over-expression, RNAi, silencing

Introduction

Plants have evolved an array of defenses to thwart attack from herbivores. Defense responses are activated not only in the attacked tissues but also in distal, unwounded parts of the plant. These systemic defense responses have been thoroughly studied in tomato (*Solanum lycopersicum*), and an 18-aa-peptide has been christened 'systemin' for its role in activating systemic responses (Pearce *et al*., 1991). The mature systemin peptide is processed from a larger precursor, prosystemin, which is synthesized and processed in vascular phloem parenchyma cells (Narvaez-Vasquez & Ryan, 2004). When applied to unwounded tomato plants at very low levels (fmol/plant), systemin elicits the accumulation of proteinase inhibitor (PI) I and II, which act as anti-nutritive defense compounds. [^{14}C] Ala-labeled systemin was found to move freely in the vascular system and, hence, was thought to be the mobile signal activating defense traits in distal undamaged parts (Pearce *et al.*, 1991; Nárváez-Vasquez *et al*., 1995). Consistent with this view, tomato plants expressing prosystemin in an anti-sense orientation to silence transcript accumulation had less PIs after wounding compared to wild-type (WT) plants (McGurl *et al*., 1992) and were dramatically more susceptible to attack by *Manduca sexta* larvae (Orozco-Cardenas, McGurl & Ryan, 1993). Young tomato plants constitutively expressing prosystemin were found to accumulate 10-fold higher levels of the phytohormone jasmonic acid (JA) in unwounded leaves compared to non-transformed tomato plants (Stenzel *et al*., 2003). JA in combination with ethylene is thought to mediate the accumulation of PIs (Farmer, Johnson & Ryan, 1992; O'Donnell *et al*., 1996). These and other findings supported a model that has systemin released from its precursor at the wound site, moving through the phloem and binding to a putative cell surface receptor (SR160; Scheer & Ryan, 2002). When bound to the receptor, systemin initiates an intracellular signaling cascade including the activation of a mitogen-activated protein (MAP) kinase, a phospholipase A, and the release of linolenic acid (LA) from the membranes. LA is then converted to JA via the octadecanoid pathway, and JA leads to the activation of defense genes (such as PI).

This model was recently revised as a result of data from grafting experiments between WT tomato plants and mutants that are either deficient in JA production (*acx1*), or are JA insensitive (*jai1*) or defective in systemin action (*spr1*), as well as from transgenic tomato plants that constitutively express prosystemin. These grafting experiments demonstrated that systemic signaling requires JA not only to be produced at the site of wounding but also to have the ability to perceive a JA-based signal in the distal leaf. The

presence of systemin is only required in the local, wounded tissue, where it is needed to amplify a JA-based signal. The experiments showed that a JA-based signal is indeed the mobile signal that elicits PIs in distal, unwounded parts of the plant (Schilmiller & Howe, 2005).

Homologues of tomato systemin have only been identified in close relatives such as potato (*Solanum tuberosum*) (McGurl & Ryan, 1992), black nightshade (*Solanum nigrum*), and bell pepper (*Capsicum annuum*) (Constabel, Yip & Ryan, 1998; Schmidt & Baldwin, 2006). Cultivated tobacco *(Nicotiana tabacum*), another *Solanacea*, does not possess a tomato systemin homologue and does not respond to treatment of tomato systemin with increased PIs. However, Ryan and coworkers identified two hydroxyproline-rich peptides (TobHSI and TobHSII) in wounded tobacco plants, both of which are derived from a single 165 AA precursor (preproTobHypSys-A). These peptides elicited MAP kinases when applied to tobacco cell cultures, and activated the synthesis of PIs when stem-fed into young excised plants (Pearce, Johnson & Ryan, 1993; Pearce *et al*., 2001). Exposure of cultivated tobacco leaves to methyl-jasmonate (MeJA) vapors, wounding, and attack by *M. sexta* larvae elicited increases in preproTobHypSys-A transcripts (Pearce *et al.*, 2001), which paralleled increases in other wound-responsive genes (*PIOX*, *Nt-PI-I, TPI*) (Rocha-Granados *et al.*, 2005). More recently, when cultivated tobacco plants were transformed with preproTobHypSys-A under the control of a 35S promoter, constitutive levels of PIs increased and plants were more resistant to the polyphagous larvae of *Helicoverpa armigera* (Ren & Lu, 2006). In short, ectopic over-expression of the preproTobHypSys-A in tobacco produced results similar to those seen in tomato. The plants increased their PI production and became more resistant to herbivore attack. These results support the idea that the tomato and tobacco systemins, despite their lack of phylogenetic similarity, both function in defense signaling (Ryan & Pearce, 2003). However, unlike the work with tomato, the work with cultivated tobacco did not include the analysis of plants silenced in their expression of preproTobHypSys-A. In tomato, silencing the endogenous systemin gene impaired JA signaling (Stenzel *et al*., 2003), PI accumulation, and herbivore resistance (Orozco-Cardenas, McGurl & Ryan, 1993).

To better understand the role that the hydroxyproline-rich glycopeptide precursor plays in mediating anti-herbivore defenses, we manipulated the expression of the preproTobHypSys-A homolog in a native diploid tobacco species, *Nicotiana attenuata*, whose anti-herbivore defenses against the specialist herbivore *M. sexta* have been thoroughly studied (Baldwin, 2001; Kessler & Baldwin, 2002). This native plant

recognizes attack from *M. sexta* larvae when fatty-acid amino-acid conjugates (FACs) present in the oral secretions (OS) and regurgitants (of the larvae are introduced into wounds during feeding. FACs elicit a JA burst, which, in turn, elicits secondary metabolites involved in direct defenses (Halitschke & Baldwin, 2004), such as trypsin PIs (Zavala *et al*., 2004) and nicotine (Baldwin, 1999), and releases volatile organic compounds (VOCs), which function as an indirect defense by attracting predators of *M. sexta* larvae (Kessler & Baldwin, 2001).

We isolated a full-length *Na*preproHypSys from a cDNA library of *M. sexta*-attacked *N. attenuata* leaves using primers based on the pioneering work from Ryan's group on the preproTobHypSys of cultivated tobacco (Pearce *et al.*, 2001), and subsequently silenced and ectopically overexpressed the gene by expressing a 178 bp fragment as an inverted repeat construct (IR*sys*) and the full-length gene under control of a 35S promoter (OV*sys*), respectively. To understand if *N. attenuata's* preproHypSys mediates defense responses, we measured the expression patterns of *Na*preproHypSys in response to elicitation treatments known to activate traits via the octadecanoid pathway, namely, wounding and the application of *M. sexta* OS (McCloud & Baldwin, 1997; Schittko, Preston & Baldwin, 2000) and MeJA (Baldwin, 1999; Halitschke *et al*., 2000; Kahl *et al*., 2000). JA and ethylene, which are known to mediate the accumulation of PIs, were analyzed, as was an salicylic acid-induced protein kinase (SIPK), which is rapidly activated after the application of OS to wounds and thought to activate defense-related genes (Wu *et al*., 2007). TPIs (Glawe *et al*., 2003; Zavala *et al.*, 2004) and nicotine (Baldwin & Ohnmeiss, 1993), the plant's important defense metabolites, were measured to monitor the elicitation of downstream defenses. We measured the performance of *M. sexta* larvae feeding on plants with altered *Na*preproHypSys transcripts and analyzed the TPI and nicotine contents of those plants. Finally, we planted *Na*preproHypSys-silenced and over-expressing transformants in *N. attenuata's* native habitat in the Great Basin Desert, and evaluated the degree to which the native herbivore community attacked these plants in comparison to paired WT plants. In short, we have provided the most thorough characterization to date of a hydroxyproline-rich glycopeptide systemin precursor's role in mediating herbivore defense responses in any plant.

Materials and Methods

Plant material and growing conditions

N. attenuata Torr. Ex. Wats. (synonymous with *Nicotiana torreyana* Nelson and Macbr.) seeds originally collected in Santa Clara, UT, USA, and inbred for 14 or 15 generations were used to conduct experiments and to generate transformed lines. For glasshouse experiments, seeds were germinated as described in Krügel *et al*. (2002). Plants were grown at 26-28 °C under 16 h of light supplemented by Philips Sun-T Agro 400-600W Na lights. For field experiments, the seeds of WT from the same generation that was used to generate the transformed lines were germinated on agar plates. The plates were kept at 25 °C/16 h (200µm s^{-1} m PAR) and 20°C/8 h darkness. After 10 d, the seedlings were transferred to Jiffy 703 pots (4.45 cm x 4.45 cm; AlwaysGrows, Newark, OH, USA) that had been soaked in borax solution (0.4mg, 45mL^{-1} water). The seedlings were fertilized with an iron solution (stock solution: 2.78 g $FeSO_4$ $7H_2O$ and 3.93 g Titriplex in 1L H_2O, diluted 100-fold) after 7 d. After 3-4 weeks, plants of similar sizes were transferred to a field plot in the Great Basin Desert, UT, USA, and were watered daily. The plants were planted in WT-IR*sys* (14 pairs) or WT-OV*sys* (20 pairs) combinations with a distance of 50 cm between plants of each pair. Releases of the transformed plants were conducted under APHIS notification 06-003-08n.

Cloning and sequencing of the *Na*preproHypSys

A DNA fragment synthesized by PCR using the following primers, 1F (5'-GCGGCGCTCGAGACACACTGTTGGATACCTCC-3') and 2R (5'-GGTNACCCACTAGTGATTATG-3'), was cloned into a pCR© 2.1 TOPO vector (Invitrogen, Carlsbad, CA, USA) and sequenced. To obtain positive clones, cDNA library screening was performed as described by Halitschke & Baldwin (2003). Positive clones with similarity to known *N. tabacum* preproHypSys sequences were fully sequenced (AY456270).

Generation of OV*sys* and IR*sys* lines

The *Agrobacterium tumefaciens* strain LBA 4404 mediated all transformation processes. The plasmid pRESC501 was used as a backbone. Lines with silenced transcripts were generated using the vector pRESC5SYS2 (10.9 kb), and lines with the full-length sense expression were generated using the transformation vector pRESC2sys2 (10.2 kb) as

described in Bubner *et al*. (2006). All transformation vectors contain the hygromycin resistance gene *hptII* as a selectable marker. Progeny of homozygous lines were selected by segregation analysis in the T1 generation and by hygromycin (HYG) screening in the T2 generation as described by Lou & Baldwin (2006).

DNA extraction and Southern blot analysis

To determine the number of *Na*preproHypSys genes in *N. attenuata*, genomic DNA was extracted from leaves using the CTAB method (Doyle & Doyle, 1987), and 10 μg of DNA was digested either with EcoRI, EcoRV, BamH1 or Hind III, separated on a 1% agarose gel, blotted onto nylon membranes and UV-crosslinked onto this membrane. The probe for detecting the *Na*preproHypSys gene was prepared by PCR using the primers NaSYSF (5'-GCTTAATAGGACTGAAGAGGACGC-3') and NaSYSR (5'-GAGCAAGGAAGTATCCAACA-3') and cDNA as a template. To determine the number of transformation vector insertions in the transformed lines, isolated genomic DNA was hybridized with a PCR fragment of the *hptII* gene, the selection marker used on the transformation vector (pRESC). The PCR product for both probes was eluted from the gel using Geneclean Kit (BIO 101, Vista, CA, USA), labeled with ^{32}P using a random prime labeling kit (RediPrime II, Amersham-Pharmacia, Little Chalfont, UK) and purified on G50 columns (Amersham-Pharmacia). After overnight hybridization, blots were washed one time with 2xSSPE at 62 °C and three times with 2xSSPE / 2% sodium dodecyl sulphate (SDS), and were analyzed with a phosphorimager (model FLA-3000; Fuji Photo Film Co., Tokyo, Japan).

RT-qPCR and Northern blot analysis

To quantify *Na*preproHypSys transcripts, total RNA was extracted using TRI Reagent (Sigma, St. Louis, MO, USA) according to the protocol of Chomczynski & Sacchi, (1987), with minor modifications for polysaccharide-rich plant tissue to minimize the co-precipitation of impurities. An additional salt buffer (1.2 M $NaCl_2$, 0.8 M Na citrate) was added to the isopropanol precipitation step.

For real-time quantification of the *Na*preproHypSys transcripts, cDNA was prepared from 200 ng total RNA with MultiScribe^{TM} reverse transcriptase (Applied Biosystems, Foster City, CA, USA). The primers and probes specific for *Na*preproHypSys mRNA expression detection by qPCR were as follows: NaSYS forward primer: 5'-CGCACTGATGAGAAAACCTCTG-3', NaSYS reverse primer: 5'-CAAGTGGA

AAACATTAATCAGCTGTT -3', ECI forward primer: 5'-AGAAACTGCAGGGTACTG TTGG-3', ECI reverse primer: 5'-CAAGGAGGTATAACTGGTGCCC-3', FAM labeled ProSYS probe: 5'-ACACTGAGCTCGTGATTGCAACATTTGC- 3', VIC labeled ECI probe: 5'- CGTCAAAATTCTCCACTTGTTTCAACTGT-3'. The assays using a double dye-labeled probe were performed on an ABI PRISM® 7700 Sequence Detection System (qPCRTM Core Kit, Eurogentec, Brussels, Belgium). The expression of the *Na*preproHypSys gene was normalized to the expression of the endogenous control gene ECI (sulfite reductase), which is not regulated under our experimental conditions as described in Bubner & Baldwin (2004) and Wu *et al.* (2007).

For Northern blotting, 10 µg of RNA from each sample was denatured in loading buffer at 65 ^{o}C, separated on a 1.2 % formaldehyde-agarose gel, blotted onto a nylon membrane and UV-crosslinked to the membrane. Equal loading was ascertained by ethidium bromide staining. The probe to detect the *Na*preproHypSys expression was prepared by PCR using the following primers: NaSYSF (5'- GCTTAATAGGACTGAAG AGGACGC-3') and NaSYSR (5'-CGAGCAAGGAAGTATCCAACA-3').

Transformation and sequencing of *Na*preproHypSys clones

*Na*preproHypSys cDNA was amplified with AccuPrime *Taq* polymerase (Invitrogen) using the following primers: forward: 5'- ATG AGA G(CT) TCT (GC) TTT CTC ATC-3' and reverse: 5'- TTA ATA GGA (GC)TG AAG AGG ACG-3'. PCR consisted of an initial denaturation at 94 °C for 1 min, and 33 cycles at 94 °C for 15 s, 55 °C for 40 s and 72 °C for 2 min, followed by final elongation at 72 °C for 6 min on a thermal cycler. PCR products were separated on 1 % agarose gels, excised and gel-purified using the Geneclean Kit (BIO 101) according to the manufacturer's instructions. Purified cDNA was cloned into pCR T7/CT TOPO vector and electrotransformed into *E. coli* TOP10 (Invitrogen) cells, and 192 clones were sequenced.

MAPK assay

SIPK activity is transiently elicited by OS treatment of puncture wounds (attaining maximum activity 10 and 15 min after elicitation) (Wu *et al*., 2007). Second fully expanded rosette-stage leaves from four individual plants each from IR*sys* line 1 and OV*sys* line 1, and from WT plants were treated with wounding (W) and OS, and harvested after 10 min. To determine whether the spread of OS-elicited MAPKinase activity into unwounded portions of an OS-elicited leaf is influenced by *Na*preproHypSys, the leaf was

divided into four parts from the leaf apex to the basis. The leaf was elicited in the basal portion of the leaf and samples from four different regions of the leaf were harvested after 5 min. Equal amounts of tissue from four replicates were pooled for protein extraction. Total protein was extracted with extraction buffer [100 mM Hepes, pH 7.5, 5 mM ethylene diamine tetraacetic acid (EDTA), 5 mM EGTA, 10 mM dithiothreitol (DTT), 10 mM Na_3VO_4, 10 mM NaF, 50 mM - glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 5 µg mL^{-1} antipain, 5 µg mL^{-1} aprotinin, 5 µg mL^{-1} leupeptin, and 10% glycerol]. After centrifugation at 18,000*g* for 30 min, the supernatant was transferred to a new tube and the concentration of protein extracts determined using the Bio-Rad protein assay (Munich, Germany) with BSA as a standard. The in-gel kinase activity assay was conducted as described by Zhang & Klessig, (1997). In detail, 10 µg of protein was separated on a 10 % SDS-polyacrylamide gel containing 0.25 g mL^{-1} myelin basic protein (MBP) as a substrate for the kinases. After electrophoresis, the SDS was washed from the gel with washing buffer (25 mM Tris, pH 7.5, 0.5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 0.5 mg mL^{-1} BSA, and 0.1% [v/v] Triton X-100) three times each for 30 min at room temperature. To renature the kinases, the gel was incubated in 25 mM Tris, pH 7.5, 1 mM DTT, 0.1 mM Na_3VO_4 and 5 mM NaF at 4 °C overnight with three buffer exchanges, followed by incubation at room temperature in 30 mL of reaction buffer (25 mM Tris, pH 7.5, 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT and 0.1 mM Na_3VO_4) with 200 nM ATP plus 50 µCi of ^{32}P-ATP (3000 Ci $mmol^{-1}$) for 60 min. To stop the reaction, the gel was transferred to 5% (w/v) trichloroacetic acid and 1% (w/v) NaPPi. The unincorporated ^{32}P-ATP was removed by washing the gel in the same solution for at least 6 h with hourly changes of the solution. The gel was dried on Whatman 3MM paper and analyzed on a phosphorimager (model FLA-3000; Fuji Photo Film Co., Tokyo, Japan). Pre-stained size markers (Bio-Rad) were used to estimate the sizes of the kinase.

Phytohormone analysis

The second fully expanded leaf of rosette-stage plants was OS elicited as follows: we wounded the leaves with a pattern wheel by rolling it three times on each half of the leaf lamina and immediately applied 20 µL (1:1 v/v diluted) OS from *M. sexta* larvae to the puncture wounds. Approximately 150 mg of leaf material from the same nodal leaf positions was harvested and immediately frozen 30, 45, 60, and 90 min after elicitation. To detect differences in the levels of JA or JA-Ile/Leu among the genotypes, control samples from unelicited plants were taken at the respective time points. The frozen tissue was

homogenized and extracted in FastPrep tubes containing 0.9 g of FastPrep Matrix (BIO 101), and 1 mL ethyl acetate containing methanolic 200 ng mL^{-1} [$^{13}C_2$] JA and 200 ng mL^{-1} and Paracoumaric acid as the internal standard. The FastPrep tubes were shaken two times at 6.0 m s^{-1} for 45 s. Samples were centrifuged at 13,000 rpm for 20 min at 4 °C, and the supernatant was collected. The extraction step was repeated with 1 mL ethyl acetate. Both supernatants were combined and evaporated in a SpeedVac concentrator (Eppendorf, Hamburg, Germany). The dried samples were dissolved in 500 µL MeOH (70 %) and centrifuged for 10 min at 13,000 rpm at 4 °C. Analysis of the samples was performed using a Varian 1200L Triple-Quadrupole-LC-MS (Varian, Palo Alto, CA, USA) as described by Wang *et al*. (2007).

Ethylene quantification and perception

To quantify the ethylene emission, the first fully expanded leaves of WT, IR*sys* or OV*sys* rosette-stage plants were elicited with OS, immediately excised from the plant and placed in a 250 mL glass cuvette. The cuvette was sealed, and the headspace was accumulated for 5 h. The ethylene accumulation was measured non-destructively (non-invasively in real-time) with a photoacoustic spectrometer (INVIVO, Adelzhausen, Germany). The light source consisted of a line-tunable CO_2-laser, and a resonant photoacoustic cell served as the detection unit. To fine-tune the ethylene detection based on the 'fingerprint' spectrum of ethylene in the infrared spectral region, the photoacoustic signals on the CO_2-laser lines 10p14 and 10p16 were measured. The detection unit consisted of two acoustic cells. One cell was filled with ethylene of a known concentration (516 ppb) and used to calibrate and continuously adjust the laser line. The second cell, which served as the sampling cell, was calibrated with the calibration gas (516 ppb) every time before starting the measurements. To remove ambient hydrocarbons, the air was cleaned by organic oxidation at 540 °C using a platinum catalyst (Sylatech, Walzbachtal, Germany) and then channelled to the sampling unit. For single measurements, the cuvettes were flushed with a 130-140 mL min^{-1} gas flow. The air coming out of the cuvettes was pumped through a cooling trap to remove CO_2 and H_2O.

To determine if the ability of transgenic lines to perceive ethylene is altered, we conducted a triple response assay as follows: 80 mL of GB5 with or without 1-aminocyclopropane-1-carboxylic acid (ACC) at 40 µg per 80 mL^{-1} was poured onto 12 cm square plates. Half of the agar was cut and transferred to sterile empty plates, and seeds were placed on the edge of the agar to germinate. The plates were positioned vertically in a

plant growth chamber (for growth conditions see Krügel *et al.* 2002). Three days later, when the radical had emerged from the seed coat, the light was switched off. Ten days after germination, root and hypocotyl lengths were measured. Each treatment consisted of four plates with 15 seeds in each plate.

Direct defense compounds

The first and the second fully expanded leaves of rosette-stage plants were elicited with OS as described previously. Three days after elicitation, the second fully expanded leaf was harvested to quantify responses in elicited tissues, and the unwounded source-sink leaf was harvested to measure systemic responses. Total protein was extracted as described by Jongsma *et al*. (1994), and TPI activity was quantified using the radial diffusion assay protocol described by van Dam *et al*. (2001). The nicotine content in the unwounded source-sink leaf was analyzed by high performance liquid chromatography (HPLC) as described in Keinänen, Oldham & Baldwin (2001) with the modification that approximately 100 mg frozen tissue was homogenized in 1 mL extraction buffer utilizing the FastPrep® extractions system (Savant Instruments, Holbrook, NY, USA).

We deduced the sequence of possible mature peptides from *Na*preproHypSys by aligning *Na*preproHypSys and the *Nicotiana tabacum* preproTobHypSys-A and –B, and, acquiring synthetic peptides from the corresponding aa sequence. To test whether the synthetic *Na*preproHypSys peptides are able to elicit TPI activity, as described for the tomato systemin, we supplemented both *N. attenuata* peptides (AnaSpec, San Jose, CA, USA) to excised WT leaves at different concentrations (100 fmol, 2.5 pmol, 2.5 nmol, and 20 nmol) and 1.3 µmol MeJA as described by Schmidt & Baldwin (2006) and measured the TPI activity after 72 h.

Herbivore and predation experiments

Freshly hatched neonates of *M. sexta* eggs (North Carolina State University, Raleigh, NC, USA) were placed on each of the 15 individual plants of each genotype and allowed to feed for nine days. Larval mass gain was measured on days 3, 5, 7, and 9. After 9 d, caterpillars were removed and samples for TPI and nicotine analysis were taken. To measure the susceptibility of plants to *N. attenuata*'s native herbivore community, we estimated the feeding damage resulting from attack by flea beetles, mirids, and grasshoppers three times starting 16 d after the size-matched WT-IR*sys* and WT-OV*sys* pairs were transplanted into the field plantation. The percentage of characteristic damage

caused by flea beetles and mirids or of leaf area removed by grasshoppers was estimated relative to total leaf area. Damage was expressed as the percentage of canopy damage per plant after dividing the total percentage of damage by the total number of leaves. Damage was estimated every second day, and data shown in Fig. 7 are representative for all observations. In addition, leaves sampled for TPI and nicotine analysis were harvested from plants 20 d after being transplanted to the field plot.

We conducted an egg predation assay (Kessler & Baldwin, 2001): five *M. sexta* eggs were glued, using a cellulose glue known to have no effects on VOC production, on the second stem leaf of ten replicate WT-IR*sys* and WT-OV*sys* plant pairs, which had not been previously attacked by *M. sexta*. Predation rates were measured four times at an interval of 48 h after the *M. sexta* eggs were attached. Because OS elicitation mimics the release of VOCs that normally occurs after larval attack and the VOCs attract *Geocoris pallens* predators, which prey on *M. sexta* eggs and larvae, the first stem leaves were elicited with *M. sexta* OS, and the number of eggs predated 48 h after elicitation was determined. We complemented the field study by measuring JA and JA-Ile/Leucine levels in unelicited and OS-elicited leaves 60 min after elicitation.

Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) followed by Bonferroni post hoc test or paired *t*-tests with Stat View, version 5.0 (SAS, Cary, NC, USA).

Results

Isolation and characterization of *Na*preproHypSys cDNA

We identified and cloned a 489 bp tobacco preproHypSys homolog from a *N. attenuata* cDNA library (Supplementary Fig. S1A). Sequence comparisons revealed an 87.3 % identity to preproHypSys A (AY033148) and an 84.8% identity to preproHypSys B (AY033149) from *N. tabacum* on the nucleotide level, and no significant similarity to any tomato systemin. The amino acid sequence was deduced from the ORF of the above-mentioned *Na*preproHypSys, and was compared to the two *N. tabacum* analogues by a sequence alignment (Supplementary Fig. S1B). We found an 87.3% identity of preproTobHypSys-A and an 86.1% identity of preproTobHypSys-B to the aa sequence of *Na*preproHypSys. The homologue regions in *N. attenuata* for the mature peptides identified in *N. tabacum* revealed an 88.9% (preproTobHypSys-A) and an 83.3%

(preproTobHypSys-B) sequence identity for peptide 1, and a 94.4% (preproTobHypSys-A) sequence identity for peptide 2.

To determine the copy number of *Na*preproHypSys in *N. attenuata,* we amplified a partial sequence of *Na*preproHypSys and used it as a probe in Southern blot hybridizations. We found at least two copies of the gene (Supplementary Fig. S2a), but Northern blot analysis using the same gene-specific probe showed only one expressed precursor. In order to enhance our ability to detect transcripts of a possible second *Na*preproHypSys gene, we designed primers based on the two known *N. tabacum* and one *N. attenuata* preproHypSys sequences, amplified a PCR product, and cloned it into *E. coli*. We sequenced 192 clones but found only one expressed *Na*preproHypSys sequence. From the Northern blot results and from the sequence analysis, we conclude either that only one gene is expressed or that the two sequences code for an identical *Na*preproHypSys mRNA.

Transgenic manipulation of the *Na*preproHypSys gene

Transgenic plants either expressing *Na*preproHypSys as an inverted repeat (IR*sys*) construct to silence expression or under the control of a 35S promoter (OV*sys*) to over-express the gene were generated by *Agrobacterium tumefaciens*-mediated transformation (Krügel *et al.*, 2002). RT-qPCR results revealed that levels of *Na*preproHypSys transcripts were ≤ 1% of WT levels in both IR*sys* lines (Fig. 1, inset; ANOVA: $F_{5/24}$=63.987, P < 0.001).

To ensure that we had successfully silenced the mRNA encoded by the loci we had identified on the Southern blot, we used the same *Na*preproHypSys-specific probe for Northern as well as for Southern blot analyses. We did not detect any transcripts in the Northern blot analysis using this probe, in unwounded or OS-treated leaves of either IR*sys* line, while *Na*preproHypSys transcripts were clearly detectable in WT leaves (Supplementary Fig. S2C). *Na*preproHypSys transcripts detected by RT-qPCR were at least two-fold higher in both OV*sys* lines than in WT plants (Fig. 1, inset). All lines were in T2 generation, homozygous for a single insertion of the transgene as determined by Southern blot analysis (Supplementary Fig. S2B) (except for OV*sys* line 2, which harbors two insertions) and diploid as determined by flow cytometry (Bubner *et al.*, 2006).

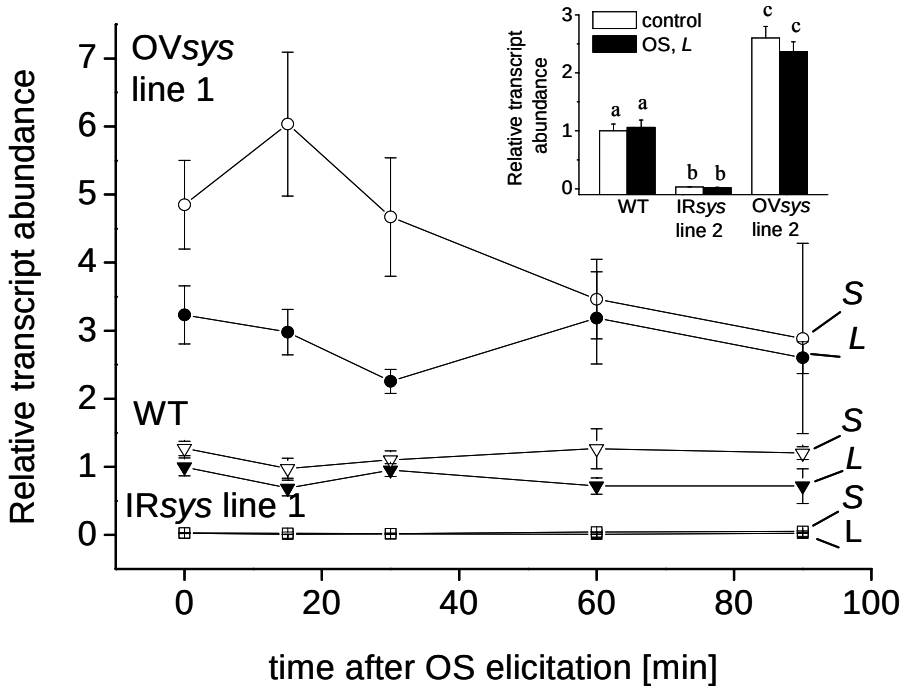

Figure 1. Kinetic of *Na*preproHypSys mRNA accumulation in *Nicotiana attenuata* after elicitation with wounding and *Manduca sexta* oral secretions (OS). Mean ± SE *Na*preproHypSys mRNA abundance in locally OS-elicited (*L*) and systemically unelicited (*S*) leaves of rosette-stage plants. Five plants per wild-type (WT), inverted repeat (IR*sys*) and over-expression (OV*sys*) lines 1 and at indicated timepoint were sampled. **Inset**: mean ± SE *Na*preproHypSys mRNA abundance in locally OS-elicited leaves of rosette-stage plants. WT, IR*sys* line 2, and OV*sys* line 2 plants 30 min after OS elicitation. Different letters in the inset indicate significant differences between genotypes and are also representative of differences among WT (triangles), IR*sys* line 1 (squares), and OV*sys* line 1 (circles) as determined by ANOVA (Bonferroni post-hoc tests, $P < 0.05$).

Endogenous *Na*preproHypSys mRNA pools are not increased by OS and MeJA elicitation

We quantified *Na*preproHypSys transcripts by RT-qPCR in different plant parts and found them expressed only in above-ground tissues (Supplementary Fig. S3). To determine how *Na*preproHypSys mRNA accumulates in response to elicitors known to trigger defense traits, we treated WT plants with either OS or MeJA and harvested their tissues at different times after elicitation. *Na*preproHypSys transcripts were not regulated in response to OS in wounded or unwounded systemic tissues within 90 min of elicitation (Fig. 1), nor was any difference observable when we compared the *Na*preproHypSys mRNA abundance of lanolin and MeJA-containing lanolin-elicited WT plants within 24 h of elicitation (Supplementary Fig. S4; ANOVA: $F_{9/29}$=3.276, P > 0.309).

Early OS-elicited responses

SIPK activity

SIPK activity was recently reported to mediate OS-elicited defense responses in *N. attenuata* (Wu *et al*., 2007). To determine if *Na*preproHypSys functions upstream of SIPK, we measured SIPK activity with an in-gel activity assay using MBP as a substrate. No differences in SIPK activity in OS- or in W-elicited leaves were found among WT, IR*sys* line 1, or OV*sys* line 1 plants 10 minutes after elicitation (Fig. 2A). To determine if *Na*preproHypSys alters the rapid spread of elicited SIPK activity into unwounded parts of an elicited leaf, we measured the spatial distribution of SIPK activity 5 min after OS elicitation in elicited and unelicited parts of leaves from WT, OV*sys,* and IR*sys* plants; levels of SIPK did not differ from those in WT plants (Fig. 2B).

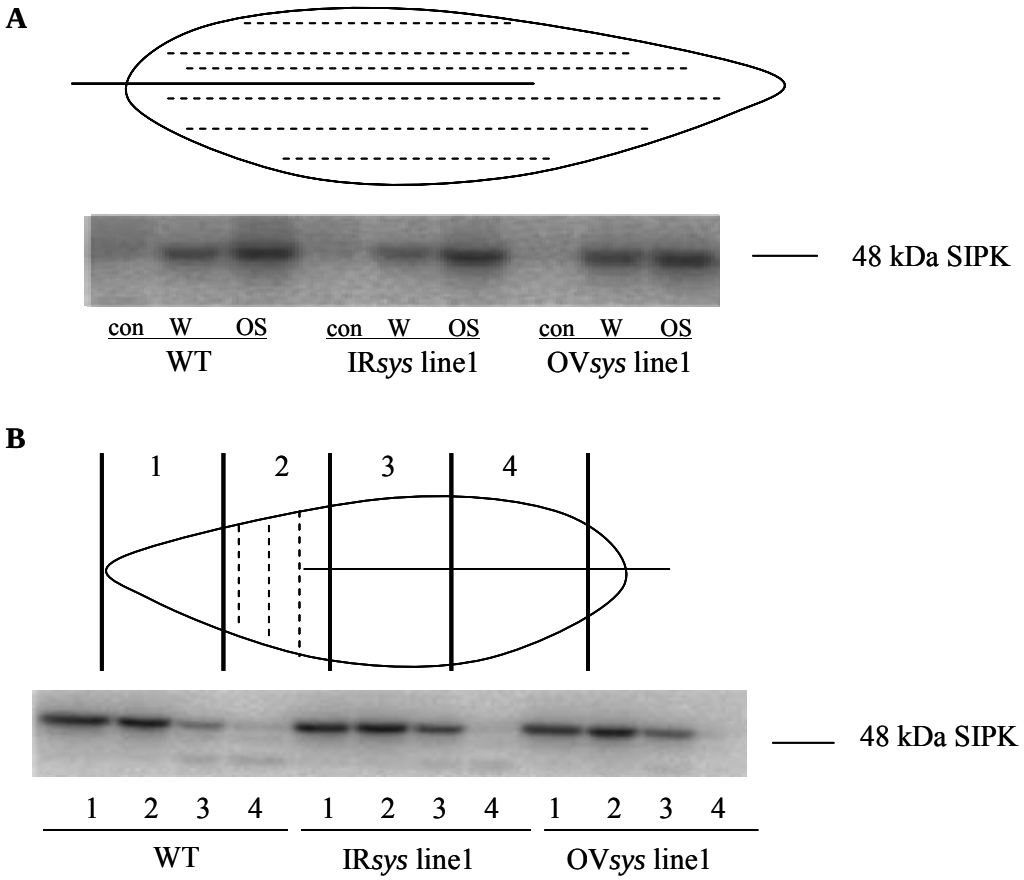

Figure 2. Salicylic acid-induced protein kinase (SIPK) activity in WT, IR*sys*, and OV*sys* plants (**A**) after elucidation with wounding (W) or oral secretions (OS) from *Manduca sexta*.

Leaves of the same phyllotaxic position from four replicate unelicited or elicited rosette-stage plants were harvested 10 min after elicitation. (**B**) Spatial distribution of OS-elicited SIPK activity within leaves of WT, IR*sys*, and OV*sys* plants. A leaf was elicited in region 2 (region with dotted lines), and the leaf was harvested in four sections after 5 min. Kinase activity was analyzed by an in-gel kinase assay using myelin basic protein (MBP) as the substrate.

JA and JA-Ile/Leucine

The main components of *N. attenuata's* direct defenses, TPI and nicotine, are mediated by JA (Halitschke & Baldwin, 2003) and JA-Ile/Leu (Kang *et al*., 2006). To understand the impact of *Na*preproHypSys on the elicitation of JA, we quantified JA in OS-elicited leaves that had been harvested at different times after elicitation.

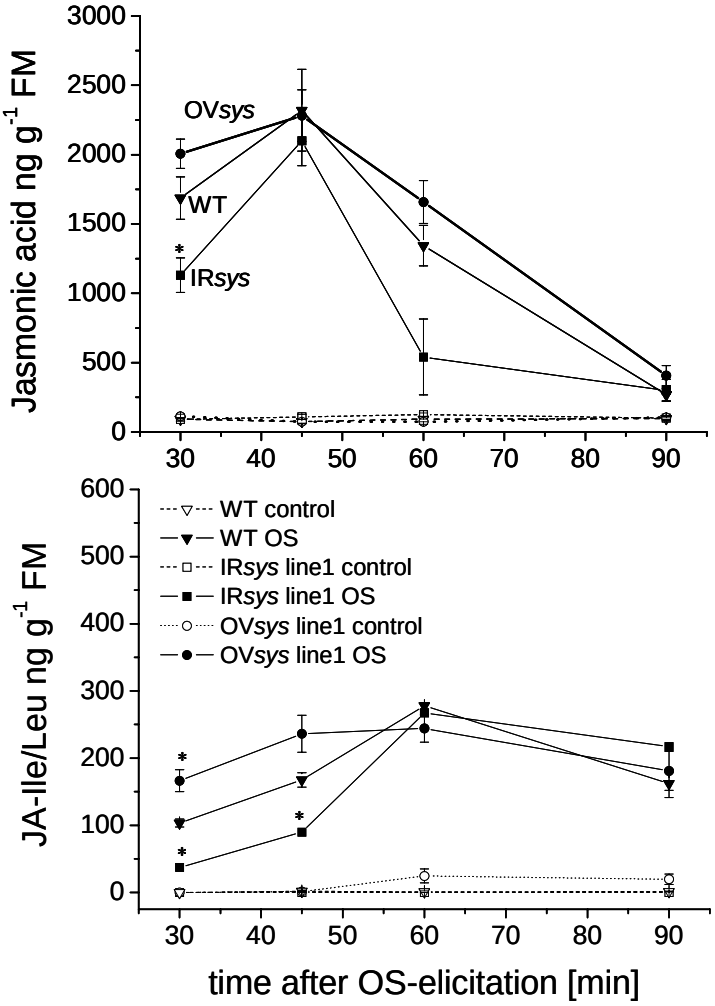

Figure 3. Jasmonic acid (JA) (**top**) and jasmonic acid-isoleucine/leucine (JA-Ile/Leu) (**bottom**) contents in OS-elicited (OS, filled symbols) and unelicited (control, empty symbols) leaves of WT (triangles), IR*sys* line 1 (squares), and OV*sys* line 1 (circles) plants over a 90 min time-course. Values are mean ± SE of four to five replicate plants per harvest. Asterisks represent significant differences between WT and IR*sys* or OV*sys* plants at respective time points as determined by ANOVA (Bonferroni post-hoc tests: * $P < 0.0167$).

After 30 minutes, JA levels were higher in OV*sys* plants compared to WT and IR*sys* plants , while JA levels in IR*sys* plants were lower than those in WT and OV*sys* plants [Fig. 3 (top); ANOVA: $F_{2/13} = 11.71$, $P < 0.0102$]. In contrast, we found no significant differences in JA values among any genotype at the time of maximum JA

levels. The JA burst declined more slowly in the OV*sys* compared to WT and IR*sys* plants [Fig. 3(top)]. The OS-elicited JA is known to be rapidly conjugated with isoleucine or leucine to form JA-Ile/Leu, conjugates which our LC-MS-MS procedure can not differentiate (Wang *et al*., 2007). Recently, JA-Ile was shown to mediate defenses against *M. sexta* in *N. attenuata* (Kang *et al*., 2006). To test if *Na*preproHypSys influences this conjugation step, we also measured JA-Ile/Leu levels in the same samples used for JA analysis. The higher level of JA in OV*sys* plants after 30 min is correlated with higher levels of JA-Ile/Leu after 30 and 45 min compared to WT plants, whereas IR*sys* plants showed lower JA-Ile/Leu values after 30 min [Fig. 3 (bottom); t = 30 min; ANOVA: $F_{2/10} = 112.241$, $P < 0.013$]. We conclude that *Na*preproHypSys does not directly influence the conjugation of JA with Ile/Leu, and its impact on the biosynthesis of JA seems to be transient.

Ethylene

The application of OS has been shown to lead to a rapid, five-fold increase in the release of ethylene in *N. attenuata* (von Dahl *et al.,* 2007), which in turn suppresses the accumulation of putrescine methyl transferase (*pmt*) transcripts and, therefore, the accumulation of nicotine (Kahl *et al.*, 2000, Winz & Baldwin, 2001). To test if the OS-elicited ethylene accumulation is regulated by *Na*preproHypSys, we measured the accumulation of ethylene for 5 h in OS-elicited leaves. Ethylene accumulation did not differ among WT and IR*sys* line 1 (Fig. 4C, inset left) and line 2 (Fig. 4C, inset right) leaves, but leaves of the OV*sys* line 1 accumulated 20 % more ethylene within 5 h (Fig. 4D, inset; ANOVA: $F_{1/19} = 7.021$, $P = 0.0163$). To test whether the plants' ethylene perception was influenced by *Na*preproHypSys, we conducted triple response assays.

No differences in hypocotyl or root growth among genotypes were found when seedlings were grown on agar plates containing 20 µg ACC. Although the hypocotyls and roots grew more on plates without ACC than on plates containing ACC, we did not see differences in growth among the WT, the two IR*sys* lines, or the two OV*sys* lines on the plates lacking ACC (Supplementary Table S1).

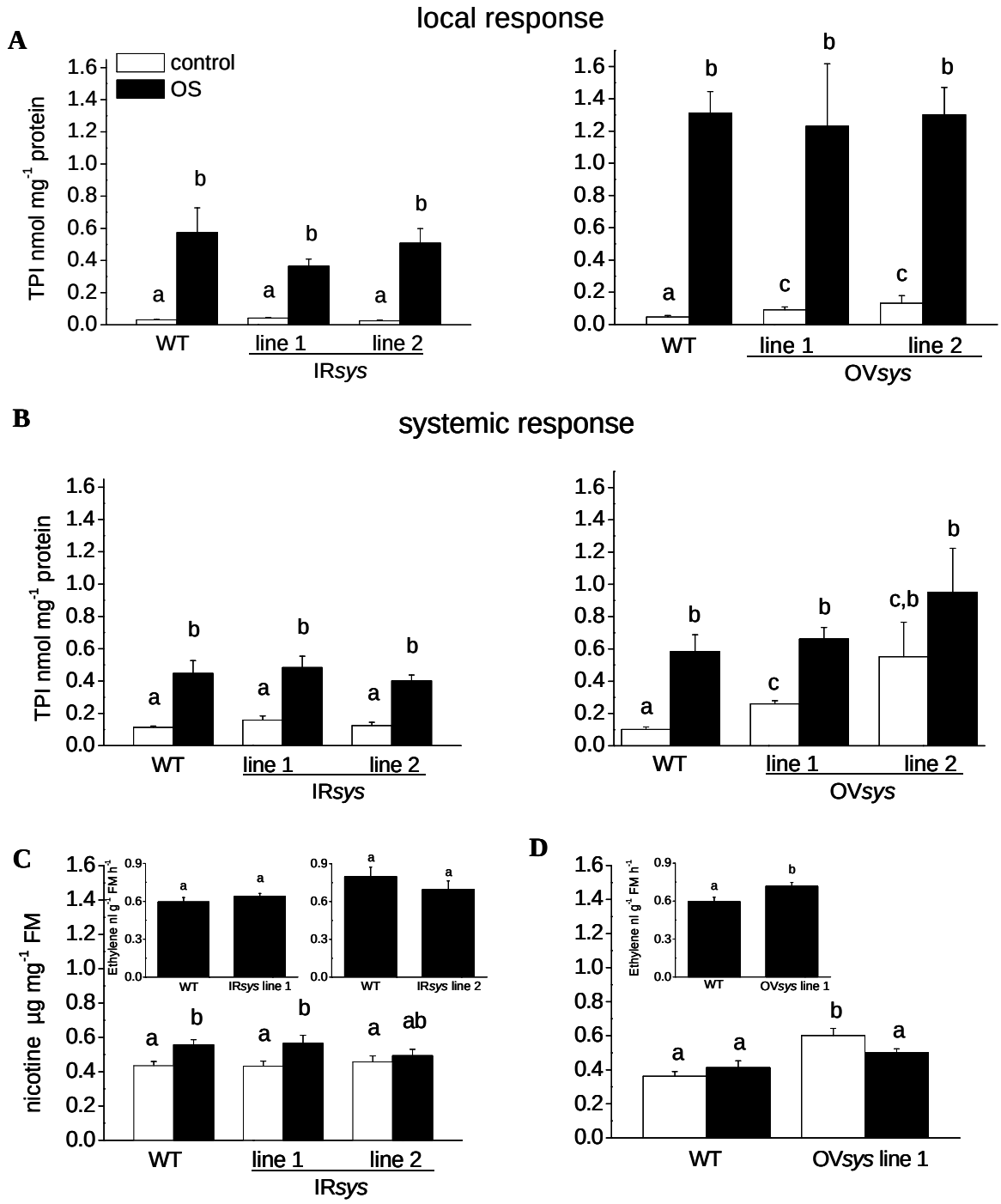

Figure 4. Defense metabolite accumulation in oral secretion (OS)-elicited (black bars) and unelicited (white bars) WT, IR*sys*, and OV*sys* plants. Trypsin-proteinase inhibitor (TPI) accumulation in WT and IR*sys* lines 1 and 2, and in WT, and OV*sys* lines 1 and 2, three days after OS elicitation in locally elicited (**A**) and unelicited systemic (**B**) leaves. Nicotine accumulation in leaves of control and OS-elicited WT, IR*sys* lines 1 and 2 (**C**) and WT and OV*sys* line 1 plants (**D**). Values are mean ± SE of four to five replicate plants. **Insets**: Ethylene emission of OS-elicited leaves of WT, IR*sys* lines 1 and 2, and OV*sys* line 1 plants. Leaves of the same phyllotaxic position were OS-elicited and cut off the respective plants and were allowed to accumulate ethylene in a 250 mL cuvette for 5 h. Values are the mean ± SE of nine to ten replicates. WT values vary

among panels due to between-experiment variation in constitutive levels. Different letters indicate significant differences as determined by ANOVA (Bonferroni post-hoc tests, $P < 0.05$).

Late OS-elicited responses

Direct defense metabolites

To test whether levels of *Na*preproHypSys related to levels of TPI in wounded or in distal, unwounded leaves, TPI values were analyzed before and after OS elicitation. Although constitutive levels of TPI were higher in OV*sys* than in WT plants, in both local (Fig. 4A; ANOVA: $F_{2/14} = 7.946$, $P < 0.0258$) and systemic leaves (Fig. 4B; ANOVA: $F_{2/14} = 38.910$, $P < 0.0002$), their ability to accumulate TPI after OS elicitation did not increase. Furthermore, we did not detect differences when we compared unelicited WT to IR*sys* plants, specifically their ability to increase their TPI values in response to OS, in either local or systemic tissues (Fig. 4A, B). When excised leaves were supplied with synthetic *Na*preproHypSys peptides through their xylem stream in concentrations from fmol to µmol, TPI activity did not differ from that in leaves supplied with water. Leaves supplied with 1.3 µmol MeJA accumulated two- to three-fold more TPIs compared to the water controls (data not shown; ANOVA; $P < 0.05$).

Nicotine, the neurotoxic alkaloid that is produced in the roots of *N. attenuata* and accumulates in above-ground tissue in response to wounding, was the second major defense compound that we examined for its dependence on *Na*preproHypSys. Silencing *Na*preproHypSys did not significantly decrease nicotine values, either in unelicited or in elicited IR*sys* plants (Fig. 4C). A positive correlation between *Na*preproHypSys and nicotine levels was detected for unelicited OV*sys* plants (Fig. 4D; ANOVA: $F_{1/6} = 0.834$, $P < 0.05$), whereas the OS treatment suppressed levels of nicotine to those observed in elicited WT plants (Fig. 4D).

Herbivory

M. sexta performance

We measured the weight gain of freshly hatched *M. sexta* larvae that fed on OV*sys*, IR*sys*, and WT plants. To gauge the effectiveness of *Na*preproHypSys as a mediator of defense responses against *M. sexta*, we included JA-deficient *NaLOX3*-silenced plants that are known to be highly vulnerable to these insects (Halitschke & Baldwin, 2003).

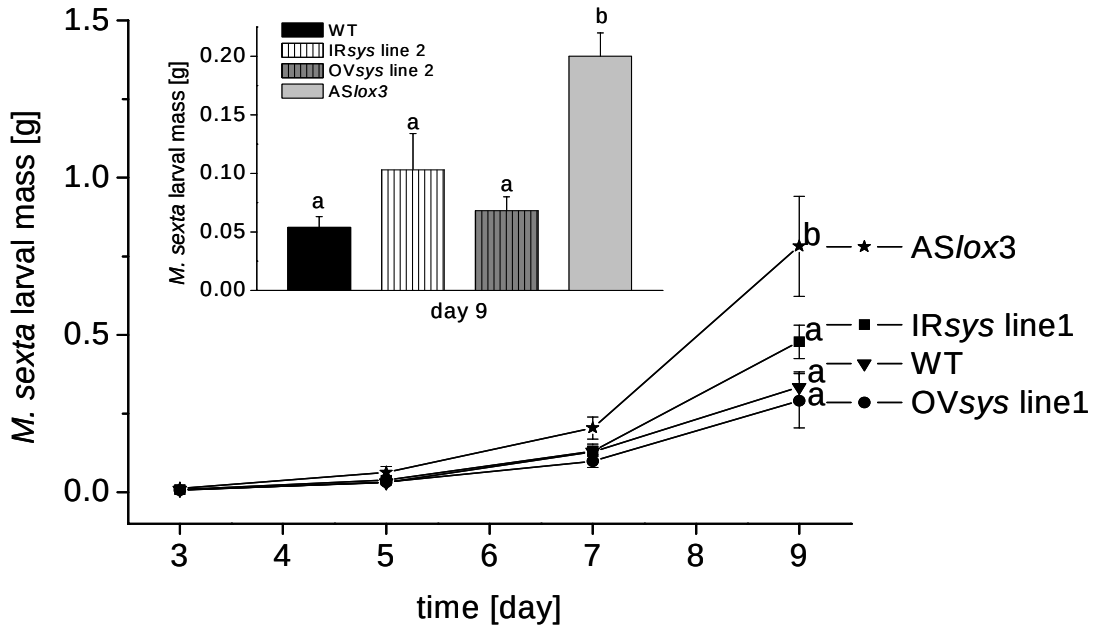

Figure 5. *Manduca sexta* performance under glasshouse conditions on plants with altered *Na*preproHypSys levels and AS*lox3* plants for comparison. Mean ± SE mass of 15 *M. sexta* larvae feeding on WT (filled triangles), IR*sys* line 1 (filled squares), OV*sys* line 1 (filled circles), and AS*lox3* (filled stars) plants for 3, 5, 7, and 9 d. Inset: Mean ± SE *M. sexta* larval mass reared on WT (black bar), IR*sys* line 2 (white striped bar), OV*sys* line 2 (gray striped bar), and AS*lox3* (light gray bar) plants after feeding for 9 d. Different letters indicate significant differences as determined by ANOVA (Bonferroni post-hoc test, $P < 0.05$) as shown for day 9.

The larvae that fed on the *NaLOX3*-silenced plants attained masses that were 2.9-fold larger than those that fed on WT plants after 9 d (Fig. 5; ANOVA: $F_{3/41} = 16.15$, $P < 0.001$). Caterpillars that fed on *Na*preproHypSys-silenced plants performed marginally, but not significantly, better than those that fed on WT plants, and the performance of larvae that fed on OVsys lines was not distinguishable from those that fed on WT plants (Fig. 5). Measures of *M. sexta* larval performance were repeated twice.

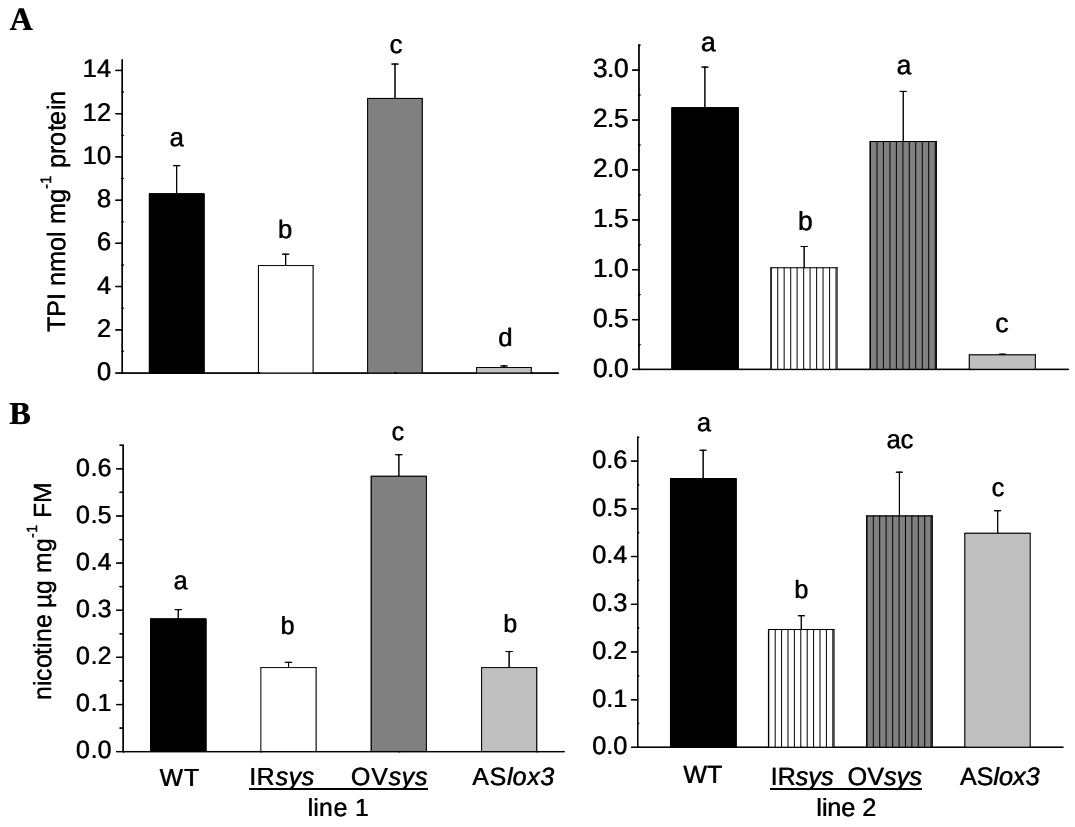

Figure 6. Defense metabolites in WT, IR*sys* and OV*sys* plants attacked by *M. sexta* for 9 d. Shown are the mean ± SE (n=10) of (**A**): TPI and (**B**): nicotine content of leaves of WT (black bars), IR*sys* line 1 (white bar), OV*sys* line 1 (gray bar) and AS*lox3* (light gray bars) plants and of WT, IR*sys* line 2 (white striped bar), OV*sys* line 2 (gray striped bar), and AS*lox3* plants after nine days of *M. sexta* larvae attack. Different letters indicate significant differences as determined by ANOVA (Bonferroni *post-hoc* test, $P < 0.05$).

Although after 9 d the larvae that fed on WT plants did not differ from those that fed on *Na*preproHypSys transformed plants, the levels of TPI (Fig. 6A) and nicotine (Fig. 6B) differed in those plants. Silenced plants had nicotine and TPI levels that were 45 % and 50 % lower than those of WT plants; plants over-expressing *Na*preproHypSys accumulated 100% more nicotine in OV*sys* line 1 than did WT plants, but no differences were found for OV*sys* line 2, TPI levels in line 1 were 50% higher, and there were no differences in line 2 compared to WT plants.

Susceptibility to the native herbivore community

To determine if *Na*preproHypSys mediates anti-herbivore defenses under natural conditions, we grew transgenic plants, either silenced or sense-expressed, and paired them with WT plants at a field site in *N. attenuata's* natural habitat, the Great Basin Desert. We measured the damage caused by the three species of herbivores that were responsible for the majority of damage at the time of the experiment, namely, flea beetles (*Epitrix hirtipennis*), mirids (*Tupicorius notatus*), and grasshoppers (*Trimerotropis spp.*).

We observed 47% (paired *t*-test: P = 0.0324) less flea beetle damage, 28% (paired *t*-test: P= 0.0149) less mirid damage, and 80% (paired *t*-test: P = 0.0664) less grasshopper damage in plants over-expressing *Na*preproHypSys compared to the paired WT plants. Herbivore damage to IR*sys* plants did not differ from the damage to their WT paired partners (Fig. 7). As a comparison, 20 AS*lox3* plants, which were planted into an adjacent field plot at the same time received three-fold (ANOVA; P < 0.0001) more damage from flea beetles compared to their paired WT plants (Paschold, Halitschke & Baldwin, unpublished data). To analyse TPI and nicotine levels, leaf samples were taken from these plants 20 d after they were planted into field plots. The levels of TPI activity and nicotine in IR*sys* plants did not differ from levels in their WT paired plants (Fig. 8A, B). In addition, among the paired genotypes we found no differences in plants' ability to accumulate JA and JA-Ile/Leu within 60 min after OS elicitation (Fig. 8C, D).

To examine if the lower damage rates on OV*sys* plants correlated with more predators of the herbivores on those plants, we conducted an egg predation assay: no differences were found in predation rates among the genotypes before (data not shown) or after OS elicitation (Fig. 8E). These results demonstrate that indirect defenses mediated by VOCs are not likely activated in OV*sys* plants, and we conclude that the lower damage rates are likely a result of the higher nicotine concentrations in these plants.

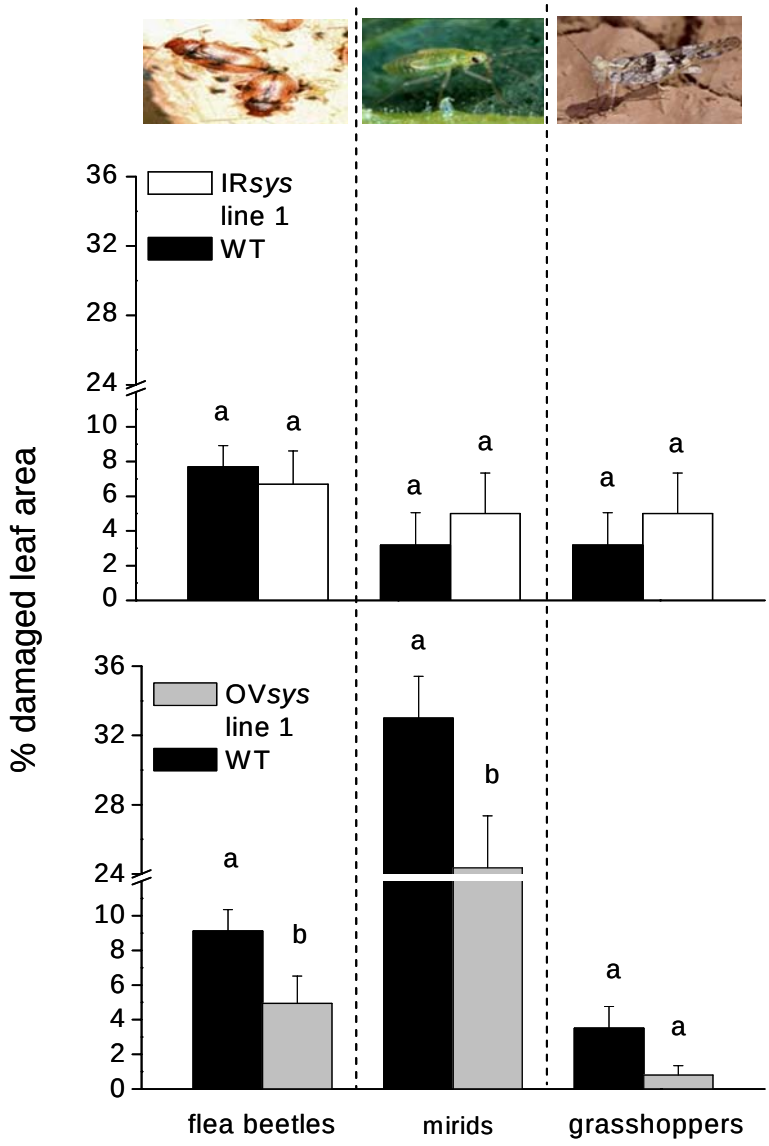

Figure 7. Herbivore damage on WT, IR*sys*, and OV*sys* plants grown in their native habitat in the Great Basin Desert. Mean ± SE leaf area damaged (% of canopy) by flea beetles (*Epitrix hirtipennis*), mirids (*Tupicorius notatus*), and grasshoppers (*Trimerotropis spp.*) in field-grown WT (black bars) and IR*sys* line 1 (white bars) plant pairs, and WT and OV*sys* line 1 (gray bars) plant pairs. Different letters indicate significant differences between genotypes as determined by paired *t*-test, $P < 0.05$ (n=10-12).

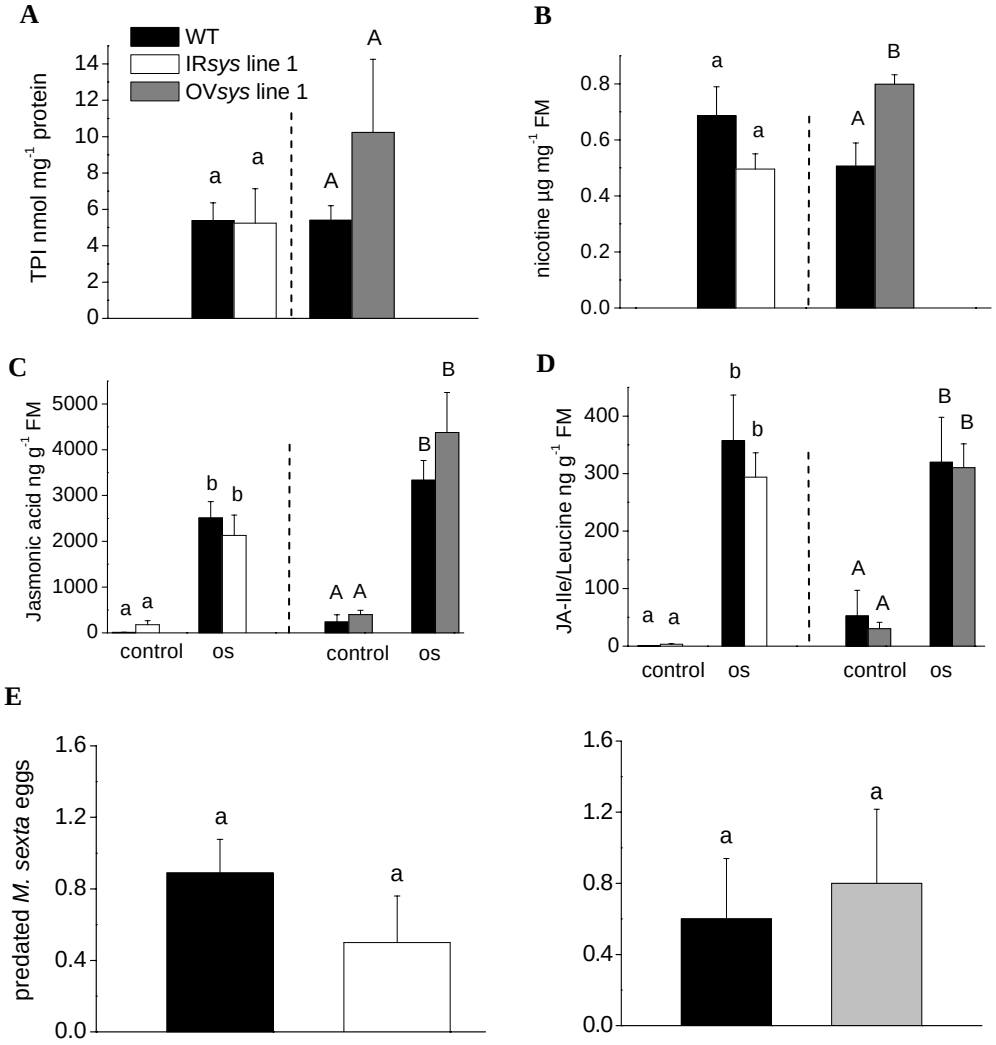

Figure 8. Defense-related compounds in field-grown plants with different *Na*preproHypSys mRNA levels. Mean ± SE (n=5-7) trypsin proteinase inhibitor (TPI) (**A**) and nicotine (**B**) content of plants 20 d after being transplanted to the field and exposed to the natural herbivore community. Mean ± SE JA (**C**) and JA-Ile/Leu (**D**) accumulation 60 min after OS elicitation (OS) of field-grown WT (black bars), IR*sys* (white bars), and OV*sys* (gray bars) plants. (**E**) *Manduca sexta* egg predation assay. Mean ± SE (n=10-12) number of predated *M. sexta* eggs 48 h after five eggs had been experimentally glued to the first stem leaf of WT (black bars), IR*sys* (white bar), and OV*sys* (gray bar) plants. At the same time plants had been OS-elicited to induce volatile emissions and to attract predators. *Geocoris pallens* was responsible for all egg predations, as determined by its signature feeding damage to the eggs. Different letters indicate significant differences between genotypes as determined by paired *t*-test $P < 0.05$.

Discussion

To test the hypothesis that the tomato and tobacco systemins, despite their lack of phylogenetic similarity, both function in defense signaling (Ryan & Pearce, 2003; Ren & Lu 2006), we silenced and ectopically expressed *Na*preproHypSys by expressing either a *Na*preproHypSys fragment in an inverted repeat orientation or the full-length sequence under the control of a 35S promoter to examine its role in mediating anti-herbivore defense responses in *N. attenuata,* a native diploid tobacco whose defenses have been particularly well studied. We compared the performance of transformed plants with that of WT plants by measuring the following responses, known to be elicited by *M. sexta* attack and OS elicitation: SIPK activity, JA and JA-Ile/Leu levels, ethylene emission, TPI activity, and nicotine accumulation. In addition, we compared the plants' resistance to herbivore attack in glasshouse experiments as well as in the Great Basin Desert (UT, USA), where they were exposed to the plant's native herbivore community.

Silencing the expression of an endogenous gene provides a more rigorous test of the gene's function than does ectopically expressing the gene in all plant tissues, and the results of the analysis of the *Na*preproHypSys -silenced plants were clear: *Na*preproHypSys does not play a role in mediating *N. attenuata*'s defense responses. IR*sys* plants did not differ from WT plants in any of the defense responses that we measured in either the glasshouse or the field (TPI, nicotine, JA, JA-Ile/Leu, SIPK activity, resistance to *M. sexta* larvae, grasshoppers, mirids, or flea beetles, or their ability to attract predators after OS elicitation). These results are consistent with results from systemin-silenced *S. nigrum* (Schmidt & Baldwin, 2006) but stand in marked contrast to those from studies with systemin-silenced tomato plants, which have shown that silencing systemin impairs PI-mediated herbivore resistance (Orozco-Cardenas *et al.*, 1993). It is always possible that another, yet-to-be identified *Na*preproHypSys in *N. attenuata* plays a central role in defense signaling, but our qPCR and Northern blot analyses clearly demonstrated that we had silenced the *N. tabacum* preproHypSys homolog in *N. attenuata*. Silencing the tobacco preproHypSys in cultivated tobacco will be an interesting experiment to conduct because ectopic over-expression in *N. attenuata* produced phenotypic changes similar to those reported from the ectopic over-expression of preproHypSys/ prosystemin in cultivated tobacco (Ren & Lu, 2006) and tomato (Orozco-Cardenas *et al*., 1993).

Ectopic over-expression of prosystemin under the control of a 35S promoter resulted in PI accumulation in tomato in the absence of wounding (Orozco-Cardenas *et al.*,

1993) and increased constitutive levels in *N. tabacum* by eight-fold (Ren & Lu, 2006). Smaller (1.5- to 3-fold; Fig. 4A, B) increases in constitutive TPIs levels in OV*sys N. attenuata* lines were found. Nicotine is one of the most important defense metabolites for herbivore resistance in *Nicotiana* species (Halitschke & Baldwin, 2003; Steppuhn *et al*., 2004), but this metabolite was not analyzed in *N. tabacum* plants over-expressing preproTobHypSys-A (Ren & Lu, 2006). Like levels of TPIs, constitutive nicotine levels increased slightly in OV*sys* compared to WT plants (Fig. 4A, B). However, neither TPIs nor nicotine increased their levels after OS elicitation (Fig. 4A, B), but levels of both tended to be higher after 9 d of continuous attack by *M. sexta* larvae in glasshouse experiments (Fig. 6A, B) or in plants attacked by the natural herbivore community (Fig. 8A, B). While cultivated tobacco plants over-expressing preproTobHypSys-A are more resistant to *H. armiguera* larvae (Ren & Lu, 2006) in glasshouse experiments, *N. attenuata* lines over-expressing *Na*preproHypSys were not more resistant to *M. sexta* larvae (Fig. 5). However, they did suffer less damage from members of three different feeding guilds, namely flea beetles, mirids, and grasshoppers (Fig. 7). The lower amount of damage done to plants over-expressing the *Na*preproHypSys gene might be explained by the higher nicotine levels in those plants compared to their WT counterparts (Fig. 8B). To exclude the possibility that the lower herbivore damage rates on OV*sys* plants resulted from a greater ability to attract *Geocoris* predators, we conducted egg predation assays. These revealed that predation rates from *Geocoris* predators did not differ among genotypes (Fig. 8E), which in turn suggests that OS-elicited emissions of volatile organic compounds in *Na*preproHypSys-transformed plants are indistinguishable from those in WT plants. These results indicate a correlation between continuous attack by herbivores, ectopic *Na*preproHypSys expression, and plants' ability to accumulate TPI and nicotine.

The activation of defense responses (i.e. PIs) in unwounded, distal parts of tomato plants is thought to be mediated by JA or JA-associated signals. In tomato, systemin is causally linked to the elicitation of JA or JA-associated signals in wounded tissues. Stenzel *et al*. (2003) reported a more rapid and dramatic increase of JA in tomato plants ectopically expressing the prosystemin gene than in WT tomato plants, and observed that wound-elicited JA levels in prosystemin-silenced plants were significantly impaired, reaching only 50% of the JA levels found in WT plants. In contrast, in *N. attenuata*, where the OS-elicited JA burst peaks between 45 and 60 min after elicitation, only modest differences were found after the first 30 min among WT, IR*sys,* and OV*sys* plants, and no significant differences in the peak JA amounts were found [Fig. 3 (top)]. The same pattern was

evident in the OS-elicited dynamics of JA-Ile/Leu [Fig. 3 (bottom)]. We found no significant differences 60 min after OS elicitation among the genotypes in field-grown plants, (Fig. 8C, D). We conclude that *Na*preproHypSys is clearly not playing a central role in regulating JA-signaling, though it may fine-tune some aspects of JA-signaling in *N. attenuata*.

While over-expression was associated with higher constitutive levels of TPIs in OV*sys* lines (Fig. 4A, B), over-expression had no influence on the OS-induced levels of TPI activity (Fig. 4A, B). Whereas stem-feeding tomato systemin peptide to young tomato plants through their excised petioles increased PI levels within 24 h, stem-feeding 100 fmol to 20 µmol of the two synthetic *N. attenuata* HypSys peptides to excised *N. attenuata* leaves through their petioles did not influence TPI activity. Treating plants with 1.3 µmol MeJA, however, does have an effect. Even though the lack of response to the systemin peptide may have resulted from the use of synthetic peptides, which do not contain the correct decorations at the two hydroxyl proline residues, undecorated tobacco systemins were found to actively elicit pH changes in *N. tabacum* cell cultures; such changes are thought to indicate defense-related functions (Pearce *et al.*, 2001). To reconcile these results with those from the continuous feeding experiment, which show that *Na*preproHypSys over-expression influences the accumulation of secondary metabolites (Fig. 6, 8), we propose that *Na*preproHypSys expression influences defense production by influencing resource allocation rather than directly influencing defense signaling, as it clearly does in tomato.

One of the early steps involved in defense signaling is the activation of MAPKs. Treating tomato and tobacco cell cultures with tomato or either of the two tobacco hydroxyproline-rich peptides, TobHypSys I and II, rapidly activates a 48 kDa MAPK (Pearce *et al.*, 2001; Stratmann & Ryan, 1997). In *N. attenuata*, OS elicitation has recently been shown to enhance the wound activation of a 48 kDa SIPK (Wu *et al*., 2007). We were able to confirm that compared to the W treatment, OS treatment activated higher levels of SIPK, but OS-elicited WT, IR*sys*, and OV*sys* plants all responded similarly. Moreover, we found no differences in how SIPK activity was distributed in an OS-elicited leaf among the different genotypes (Fig. 2A, B). We conclude that *Na*preproHypSys does not regulate OS-elicited SIPK activity in *N. attenuata*. In addition to rapidly activating SIPK, *M. sexta* attack and OS elicitation also result in a rapid ethylene burst in *N. attenuata* which suppresses nicotine production (Kahl *et al.*, 2000; von Dahl *et al.,* 2007). Treating tomato plants and cell cultures with tomato systemin also elicits a rapid ethylene burst (Felix &

Boller, 1995; O'Donnell *et al*., 1998). OS elicitation of OV*sys N. attenuata* increased levels of ethylene emission by a modest 20% above those in WT plants (Fig. 4D inset), while the OS-elicited ethylene releases from IR*sys* plants did not differ from those from WT plants (Fig. 4C insets). We conclude that in *N. attenuata* the production of ethylene is independent of *Na*preproHypSys, and that the increase observed in OV*sys* plants is likely another consequence of ectopic expression.

The tissue-specific constitutive accumulation of *Na*preproHypSys mRNA we observed in *N. attenuata* -- namely, in above-ground tissues but not in roots -- is consistent with the patterns reported from *Solanum lycopersicum* (McGurl *et al*., 1992) and *S. nigrum* (Schmidt & Baldwin, 2006). Although the constitutive accumulation patterns do not vary among these solanaceous taxa, the transcriptional responses to elicitation differ dramatically. We found no significant increase in *Na*preproHypSys transcripts in response to OS elicitation within 90 minutes (Fig. 1), and no increase within 24 h after MeJA elicitation (Supplementary Fig. S4). In contrast, prosystemin transcripts increase dramatically 3-4 h after wounding in *S. lycopersicum* (McGurl *et al.,* 1992), as well as NtppHS in *Nicotiana tabacum* (Pearce *et al.*, 2001; Rocha-Granados *et al.*, 2005). Interestingly, in *S. nigrum,* whose prosystemin sequence shares an 81% aa identity to *S. lycopersicum*, transcript levels decrease rapidly after OS treatment (Schmidt & Baldwin, 2006), which suggests that even systemin's response to herbivore attack and wounding is highly species specific among closely related solanaceous taxa.

We found a correlation between *Na*preproHypSys and defense traits in *N. attenuata*, but compared to the susceptibility of *N. attenuata* plants impaired in their JA signaling, such as plants silenced in *NaLOX3* (Halitscke and Baldwin, 2003; Kessler, Halitschke & Baldwin, 2004), the resistance of plants silenced in *Na*preproHypSys was largely indistinguishable from that of WT plants (Figs. 2, 4, 5, 7,& 8). We propose that the small effects on herbivore resistance result indirectly from a yet-to-be discovered function of *Na*preproHypSys and are not a direct influence of *Na*preproHypSys. It will be an exciting challenge to understand why *N. attenuata* expresses a preproHypSys gene in its above-ground tissues.

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Supplementary Material

A

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taccaattag caagtttcaa gaaactgttc gtctcatacc ttctgcctgt
ctcatacgtg tggaacctga tcagttcatc gtcttttaat cacgatcttg   B
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gttgtaactt agcgtggtcg tttggttttc agataataag ttaaacgtga
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tttgatgtat taacaattac taatgtgtaa tctaagatgg tgtgtgtttt
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B

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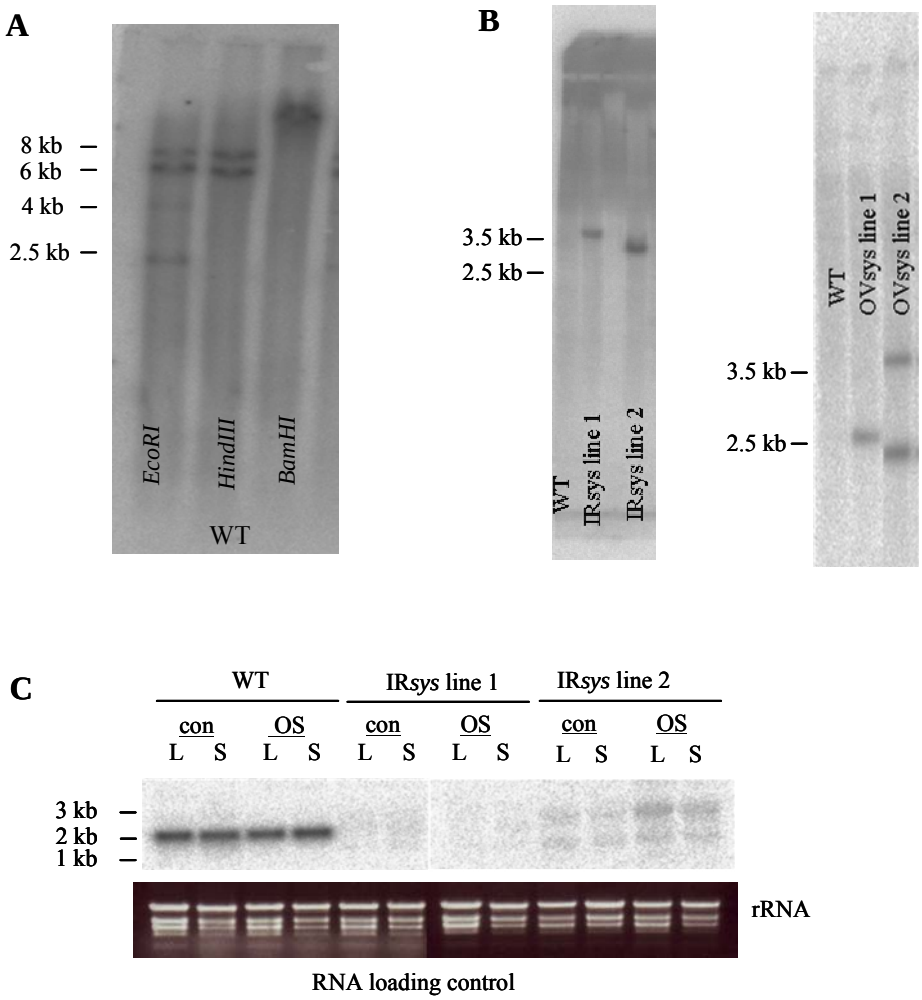
NapreproHypSys   :  MRALFLIYLILAPFRAEARTLQENDEGLNVGSGYGRGAHLSPPSPASSPPSKEVSNSVSP
NtpreproHypSys-A :  MRVLFLIYLILSPFGAEARTLLENHEGLNVGSGYGRGANLPPPSPASSPPSKEVSNSVSP
NtpreproHypSys-B :  MRALFLIYLILSPFRAVSRTLLENHEGLNVGSGYGRGTNLPPPSPASSPPSKEVSNSVSP
                                                    <=   mature peptide 1   =>

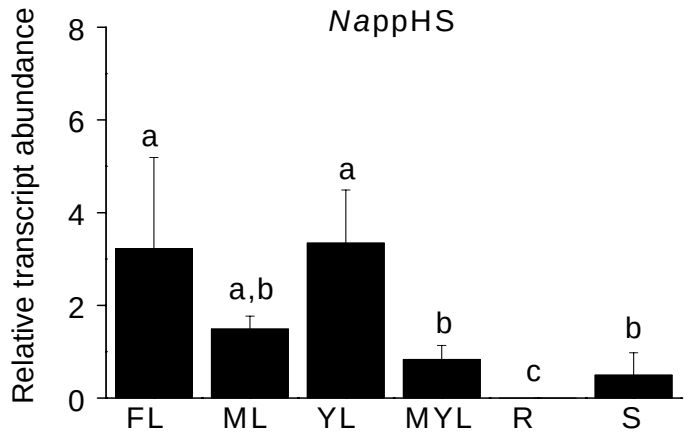
                    61--------x---------x---------x---------x---------x------120
NapreproHypSys   :  TRTDEKTSENTELVIATFAQVENINQLFSFPTSSVNYYQLASFKKLFVSYLLPVSYVWNL
NtpreproHypSys-A :  TRTDEKTSENTELVMTTIAQGENINQLFSFPTSADNYYQLASFKKLFISYLLPVSYVWNL
NtpreproHypSys-B :  TRTDEKTSENTELVMTTIAQGENSNQLFPFLTSSDN-YQLASFKKLSISYLLPVSYVWKL

                    121-------x---------x---------x---------x---------x------180
NapreproHypSys   :  ISSSSFNHDLVDIFDTKFDEKYWNHKPLSPPSPKPADGQRPLQSY.
NtpreproHypSys-A :  IGSSSFDHDLVDIFDSKSDERYWNRKPLSPPSPKPADGQRPLHSY.
NtpreproHypSys-B :  ISSSSFNHDLVDIFDTSSDEKYWNRKPLSPPSPKPADGHRPLQSY.
                                        <=   mature peptide 2   =>
```

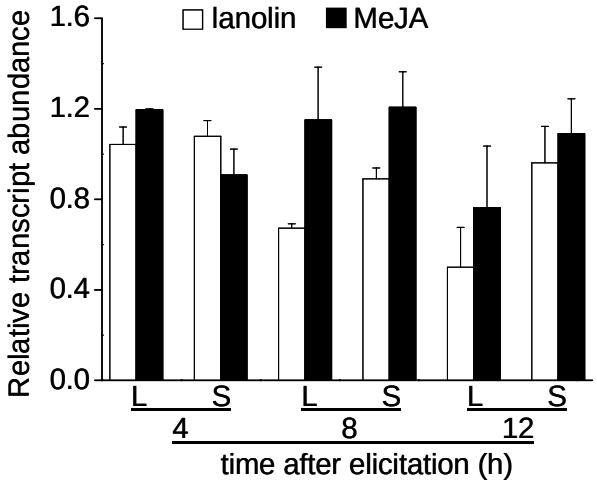
Suppl. Fig. 1: (A) cDNA sequence of *N. attenuata Na*preproHypSys (AY456270) gene. Two primers (underlined letters) and the probe (underlined italic letters) for real-time quantitative PCR, and the region used for inverted repeat transformation (box A), for Northern and Southern blotting (box B) and the peptide coding regions (bold letters) are shown. (**B**) Shown are the published aa sequences of preproTobHypSys-A and -B aligned with the deduced aa sequence of *Na*preproHypSys. The mature peptides of the preproTobHypSys´s are marked by frames, the homolog regions in all three polypeptides are gray, and the homolog mature peptide regions are in bold face.



Suppl. Fig. 2: (**A**) Southern blot analysis of *N. attenuata Na*preproHypSys gene. Genomic DNA of plants from of *N. attenuata* WT was digested with EcoRI, Hind III, and BamHI, blotted onto a nylon membrane, and hybridized with gene-specific probes. (**B**) Number of transgene insertions of IR*sys* and OV*sys* lines. Southern hybridization of genomic DNA extracted from WT and IR*sys* lines 1 (A-04-366-10) and 2 (A-04-464-1) and OV*sys* lines 1 (A-03-430-14) and 2 (A-03-475-15) plants. 10 µg of genomic DNA was digested with EcoRV. The digest was separated on an agarose gel, blotted onto nylon membranes, and hybridized with a ^{32}P-labelled *hptII*-specific probe. DNA marker size (kb) is indicated. (**C**) Endogenous expression of the *Na*preproHypSys gene in WT and IR*sys* lines. Total RNA (10 µg) of OS-elicited (L) and systemic unelicited (S) leaves of *N. attenuata* plants harvested 30 min after elicitation. Control samples were harvested of untreated plants (con). RNA was blotted onto a nylon membrane and hybridized with a *Na*preproHypSys-specific probe.



Suppl. Fig. 3: Tissue-specific *Na*preproHypSys mRNA accumulation in *Nicotiana attenuata*.
Relative transcript abundance (mean ± SE, n=4) in flowers (F), mature stem leaves (ML), young leaves (YL), midribs of YL (MYL), roots (R), and stems (S) of unelicited flowering *N. attenuata* WT plants. Different letters indicate significant differences as determined by ANOVA (Bonferroni post-hoc test, $P < 0.05$).



Suppl. Fig. 4: Endogenous expression of *Na*preproHypSys in elicited (L) and unelicited (S) leaves after rosette-stage plants were elicited with pure lanolin or lanolin (white bars) containing MeJA (black bars). Shown are mean ± SE of four to five replicate plants at each harvest. Transcript accumulations were normalized to those of an endogenous control gene (ECI) and statistically analyzed by ANOVA (Bonferroni post hoc test).

	GB 5 media hypocotyl [mm]	root [mm]	**GB 5 media +20 µg ACC** hypocotyl [mm]	root [mm]
WT	25.321± 0.961	7.923± 0.875	5.240± 0.238	1.926± 0.099
IR*sys* line1	22.321± 1.121	9.000± 1.001	3.259± 0.193	1.446± 0.101
WT	24.769± 1.005	11.923± 0.903	4.352± 0.269	1.889± 0.090
OV*sys* line1	20.680± 1.787	10.192± 1.027	3.074± 0.348	1.759± 0.142

Suppl. Table 1: Triple response assays. Root and hypocotyl lengths of WT, IR*sys* and OV*sys* seedlings growing in the presence or absence of ACC (5 µM) ten days after germination. Shown are mean ± SE of 15 (statistically analyzed by ANOVA (Bonferroni post hoc test)) seedlings per genotype.

Manuscript III

Silencing the hydroxyproline-rich glycopeptide systemin precursor in two accessions of *Nicotiana attenuata* alters flower morphology and rates of self-pollination

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Abstract

Systemins and their hydroxyproline-rich glycopeptide systemin (ppHS) subfamily members are known to mediate anti-herbivore defenses in some solanaceous taxa but not others; functions other than in defense remain largely unexplored. *Nicotiana attenuata's* ppHS precursor is known not to function in herbivore defense. *NappHS* transcripts are very abundant in flowers, particularly in pistils and when two *N. attenuata* accessions from Utah and Arizona were transformed to silence *NappHS* by RNAi (IRsys), seed capsule production and seed number per capsule were reduced in both accessions. These reductions in reproductive performance could not be attributed to impaired pollen or ovule viability because hand-pollinated IRsys plants of all lines of both accessions restored seed production per capsule to levels found in wild-type (WT) plants. Rather, changes in flower morphology that decreased the efficiency of self-pollination are likely responsible: IRsys plants of both accessions have flowers with pistils that protrude beyond their anthers. Because these changes in flower morphology are reminiscent of *COI1* (*CORONATINE-INSENSITIVE 1*)-silenced *N. attenuata* plants, we measured jasmonates (JA) and JA-biosynthetic transcripts in different floral developmental stages, and found levels of JA-isoleucine/leucine(JA-Ile/Leu) and threonine deaminase (TD) transcripts, which are abundant in WT pistils, to be significantly reduced in IRsys buds and flowers. TD supplies isoleucine for JA-Ile biosynthesis and we propose that ppHS mediates jasmonate-signaling during flower development and thereby changes flower morphology. These results suggest that the function of *ppHS* family members in *N. attenuata* may have diversified to modulate flower morphology and thereby out-crossing rates in response to herbivore attack.

Introduction

Signaling peptides that activate plant defensive genes are defined as members of the systemin family (Pearce and Ryan, 2003); until now, however, no common mode of action has been uncovered. While the systemin peptide in tomato (*Solanum lycopersicum*) amplifies a jasmonic acid (JA)-based signal to activate the anti-digestive trypsin proteinase inhibitor (TPI) and other defensive genes (Schilmiller and Howe, 2005), its homolog in black nightshade (*Solanum nigrum*) is not involved in TPI's production (Schmidt and Baldwin, 2006), but, rather, appears to help the plant tolerate rather than resist herbivory (Schmidt and Baldwin, in review). Hydroxyproline-rich glycopeptides (HypSys) purified from cultivated tobacco (*Nicotiana tabacum*) are members of a systemin subfamily; recently they were reported to act as defense signals in cultivated tobacco. The 164-amino acid precursor that encodes the two tobacco peptides has no sequence homology with the 200 aa systemin tomato precursor. The two mature HypSys peptides from tobacco share a weak amino acid homology to the tomato systemin. Moreover, the -PPS- motif found in tomato systemin, is modified to –OOS- and the hydroxyproline residues are posttranslationally modified with pentose glycosylations (Pearce et al., 2001). The hydroxylation of the proline residues, the carbohydrate decorations and a leader sequence indicate that tobacco HypSys peptides are synthesized through the secretory system, unlike tomato systemin which has none of these features (Pearce et al., 2001, McGurl et al., 1992). These modifications increase the technical challenges of characterizing and synthesizing the HypSys peptides and likely add another level of specificity to their potential function.

HypSys peptides isolated from attacked tobacco leaves were shown to elicit the tobacco trypsin inhibitor (TTI), a paralog of the tomato protease inhibitor II, when applied to the cut petioles of tobacco leaves (Pearce et al., 2001). HypSys peptides involved in anti-herbivore defense signaling were, however, also found in tomato (Pearce and Ryan, 2003) and may regulate systemic wound signaling as well as systemin in tomato (Narávez-Vásquez et al., 2007). Whereas HypSys peptides in cultivated tobacco and tomato were found to mediate anti-herbivore defense signaling, HypSys peptides purified from petunia (*Petunia hybrida*) did not. Although they were found to activate *defensin1*, a gene associated with anti-pathogen defense, they were not able to induce anti-herbivore protease inhibitor or polyphenol oxidase activity (Pearce et al., 2007). This variability in function

within the systemin gene family as well as within the HypSys systemin subfamily argues against a commonly conserved function.

Nicotiana attenuata, a wild tobacco, is native to the southwestern deserts of the USA, with populations across Utah, Arizona, and California. The plant is self-compatible, and is known to exhibit adaptive morphological and chemical phenotypic plasticity in response to biotic and abiotic factors, including the jasmonate-mediated production of defense metabolites such as nicotine or TPIs after herbivore attack (Baldwin, 2001). Two morphologically similar *N. attenuata* accessions, Arizona (AZ) and Utah (UT), differ in their responses to insect herbivore attack. AZ accession plants do not produce TPIs due to a nonsense mutation in the *pi* gene (Glawe et al. 2003; Wu et al. 2006). AZ plants' reproductive performance is higher than that of UT, as evidenced by an increase in the number of seed capsules when AZ plants are grown in competition with UT plants. The loss in fitness has been shown to be directly attributable to production of TPIs (Glawe et al., 2003; Zavala et al. 2004).

N. attenuata contains a homologue of the hydroxyproline-rich systemin glycopeptide precursor found in *N. tabacum*. But *N. attenuata* plants of the UT accession that were silenced for their ability to express the HypSys precursor gene (*NappHS*) by RNAi did not differ from wild-type (WT) plants in their ability to produce TPIs or other JA-mediated anti-herbivore defenses (Berger and Baldwin, 2007). This work ruled out a central role for *NappHS* in the JA-mediated anti-herbivore defense responses of *N. attenuata* UT. The hydroxyproline-rich systemin glycopeptide genes may evolve rapidly, perhaps even attaining new functions, as suggested by the findings in *N. tabacum* and *P. hybrida*. Hence it is possible that while the UT accession does not rely on ppHS for its anti-herbivore defenses, other *N. attenuata* accessions might. To test this hypothesis, we transformed the AZ accession with an RNAi construct to silence *NappHS* transcript accumulation and evaluated defense-related attributes. Again we found no evidence that *NappHS* functions in the JA-mediated anti-herbivore defense responses, as we had found for plants of the UT accession. Hence the question remains: does ppHS function as a signal peptide in *N. attenuata* and if so what functions does it mediate? *NappHS* transcripts are particularly abundant in flowers (Berger and Baldwin, 2007) and we turned our attention to functions that this peptide might be playing in reproductive organs.

Results

PpHS does not mediate anti-herbivore defense responses in AZ plants

We expanded our previous analysis of *NappHS's* role in mediating *N. attenuata's* anti-herbivore responses to plants of the AZ accession. We detected no change in *NappHS* transcripts in locally OS-elicited or systemic leaves, AZ wild-type (AZ-WT) plants (Suppl. Fig. 1A). We generated two independently transformed lines each harboring a single insertion of the transformation vector (Suppl. Fig. 1B) and confirmed the reduction in ppHS transcripts by real-time qPCR analysis (Suppl. Fig. 1C) and that they were diploid by flow cytometry. IRsys lines contained only 1.2 % of the ppHS transcripts of AZ-WT plants. Interestingly, the transformed plants, with low endogenous ppHS levels, contained the same amount of nicotine as non-transformed plants (Suppl. Fig. 1D, ANOVA, $F_{2,9}$= 0.414, P > 0.45), and they were not impaired in the wound-induced increase in nicotine accumulation (Suppl. Fig. 1D, ANOVA, $F_{2,12}$= 1.925, P > 0.0812). Similar observations were found in ppHS-silenced plants of the UT accession.

Growth and flowering

To examine whether the *NappHS* gene influences the growth performance of *N. attenuata*, we measured stalk lengths of AZ and UT accessions and their respective silenced lines and found no significant difference between WT and IRsys lines (AZ. Suppl. Fig. 2A, left panel ANOVA, Bonferroni post hoc test, $F_{2,26}$= 2.585, *P's* > 0.05; UT; Suppl. Fig. 2A, right panel ANOVA, Bonferroni post hoc test $F_{2,27}$= 0.047, *P's* > 0.05). Only plants from AZ IRsys line 2 differed in their stalk length from AZ-WT plants on day four (ANOVA, Bonferroni post hoc test, $F_{2,26}$= 4.424, *P* = 0.0133) and six (ANOVA, Bonferroni post hoc test, $F_{2,26}$ = 5.375, *P* =0.0033).

AZ IRsys and UT IRsys lines did not differ significantly in the timing of the appearance of the first flower, compared to their respective WT (AZ, Suppl. Fig. 2A, arrows; ANOVA, Bonferroni post hoc test, $F_{2,27}$= 5.424, *P's* > 0.05, UT, Fig. 2A, arrows, ANOVA, Bonferroni post hoc test, $F_{2,27}$= 3.580, *P's* > 0.05) plants. In addition, the number of flowers per plant in AZ WT and IRsys lines was the same (Suppl. Fig. 2B, left panel ANOVA, Bonferroni post hoc test, $F_{2,26}$= 0.118, *P's* > 0.05); this was also true for the UT accession (Suppl. Fig. 2B, right panel ANOVA, Bonferroni post hoc test, $F_{2,27}$= 3.022, *P's* > 0.05). Reductions in above-ground biomass were correlated with low endogenous *NappHS* levels in AZ (Suppl. Fig. 2C, left panel ANOVA, Bonferroni post hoc test, $F_{2,45}$=

10.037, *P's* ≤ 0.0061) and in UT IRsys line 2 (Suppl. Fig. 2C, right panel ANOVA, Bonferroni post hoc test, $F_{2,37}$= 23.658, *P* < 0.0001), but not in UT IRsys line 1.

Silencing *Na*ppHS reduces the number of seed capsules produced in both UT and AZ accessions

A plant's reproductive success and therefore its fitness are strongly correlated with its seed capsule production. To determine whether silencing the *NappHS* gene has fitness consequences for *N. attenuata,* we counted the number of seed capsules per plant. We found conspicuously lower numbers of seed capsules in *NappHS*-silenced lines of both accessions compared to their respective WT: AZ (Fig. 1A top, left panel, ANOVA, Bonferroni post hoc test $F_{2,52}$= 92.379, *P's* < 0.0001) and UT (Fig. 1A top, right panel, ANOVA, Bonferroni post hoc test $F_{2,39}$= 16.403, *P's*≤ 0.0009). Consistent with the results from Glawe et al. (2003), the number of seed capsules in AZ plants was higher than in UT plants. Since a plant's fitness is largely determined by the product of the number of seed capsules times the number of seeds per capsule, we counted the number of seeds per capsule in the first four to five ripened capsules per plant. IRsys lines of UT plants had slightly fewer seeds per capsule than UT WT plants (Fig. 1A bottom, right panel, ANOVA, Bonferroni post hoc test, $F_{2,61}$= 1.681, *P's* > 0.05), and in AZ, significant differences were detected between IRsys and WT plants. (Fig. 1A bottom, left panel, ANOVA, Bonferroni post hoc test, $F_{2,64}$= 5.920, *P's* ≤ 0.0126).

To determine if low seed numbers per capsule was due to deficiencies in pollen or ovule viability, we emasculated flowers and pollinated them by hand with pollen of the respective genotype. Hand pollination restored seed production of IRsys plants to WT levels in both accessions: AZ (Fig. 1B, left panel, ANOVA, Bonferroni post hoc test, $F_{2,29}$= 2.338, *P's* > 0.05) and UT (Fig. 1B, right panel, ANOVA, Bonferroni post hoc test, $F_{2,23}$= 0.7180, *P's* > 0.05). We also examined the viability of the pollen by staining it with fluorescine diacetate and found no differences in pollen viability among any of the genotypes (Suppl. Fig. 3).

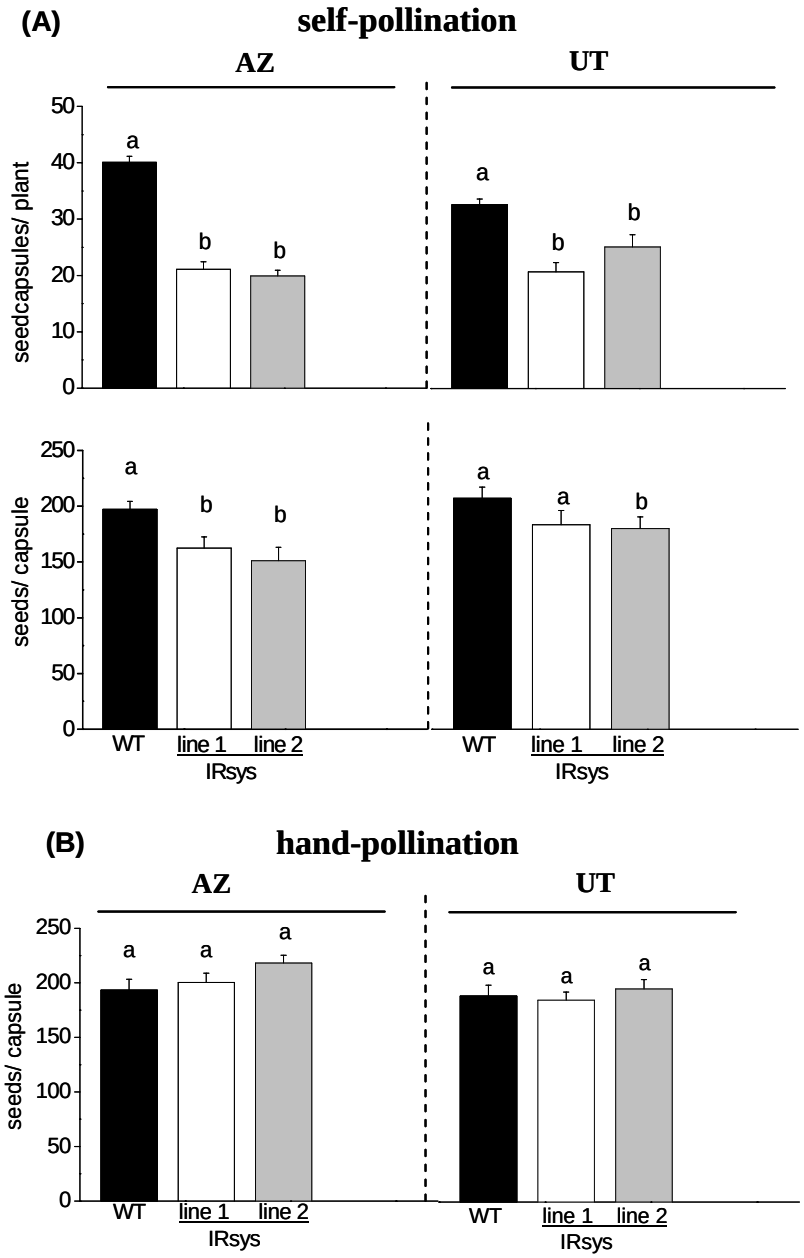

Figure 1: Silencing *NappHS* reduces the number of seed capsules produced in two accessions of *N. attenuata.* Mean ± SE number of mature seed capsules of 12-15 (A, top) wild-type (WT, black bars), IRsys line 1 (white bars), IRsys line 2 (gray bars) plants of the Arizona (AZ, left panels), and Utah (UT, right panels) accessions. Mean ± SE seed number per capsule (A, bottom) of the first 4 to 5 ripe self-pollinated capsules per plant (n=12) of each genotype and (B) of emasculated and hand-pollinated flowers. Different letters indicate significant differences from WT plants as determined by ANOVA followed by Bonferroni-corrected post-hoc test.

Silencing *Na*ppHS is associated with elongated pistils in *N. attenuata* flowers

Since reduced seed capsule and seed numbers in IRsys plants of both accessions was not due to reduced flower numbers or non-viable pollen or ovules, the differences may simply be the result of pollen limitation, which were equalized when emasculated flowers were hand pollinated. We observed that plants silenced in *NappHS* produce flowers with stigmas that protruded beyond the anthers (Fig. 2A). In contrast, in WT flowers of both accessions, the stigma is almost in the same plane as the anthers when the flower opens and the anthers dehisce, or even below it. Approximately 90% of the flowers in *NappHS*-silenced lines showed an elongated stigma, whereas only 24 % (AZ) and 26 % (UT) of the WT plants did (Suppl. Fig. 4, ANOVA, Bonferroni post hoc test, *P's* < 0.0001).

Measuring the distance between the stigma and the longest anther, we found a significantly difference in the IRsys flowers of both accessions (Fig. 2C, ANOVA, Bonferroni post hoc test, *P's* < 0.0001). As this difference could be either due to elongation of the stigma or shortening of the filaments, we measured the total length of pistil and anthers in all genotypes. We found longer pistils in the silenced lines of both accessions AZ (Fig. 2B, left panel, ANOVA, Bonferroni post hoc test, $F_{2,33}$= 53.519, *P's* < 0.0001) and UT (Fig. 2B, right panel, ANOVA, Bonferroni post hoc test, $F_{2,30}$= 5.035, *P's* ≤ 0.0109) compared to their respective WT. Anthers were not significantly shorter from WT in either ppHS-silenced lines (Fig. 2C, right panel, ANOVA, Bonferroni post hoc test, *P's* > 0.05). Finally, we found significantly longer anthers in the AZ IRsys line 2 (Fig. 2C, left panel, ANOVA, Bonferroni post hoc test, *P's* ≤0.0036).

Interestingly, pistils of both accessions had the highest abundance of *NappHS* transcripts compared to anthers and corollas (Fig. 2D left+ right panel) of the same flower. As Fig. 2D demonstrates, *NappHS* transcripts were significantly reduced in all flower parts of all transformed lines.

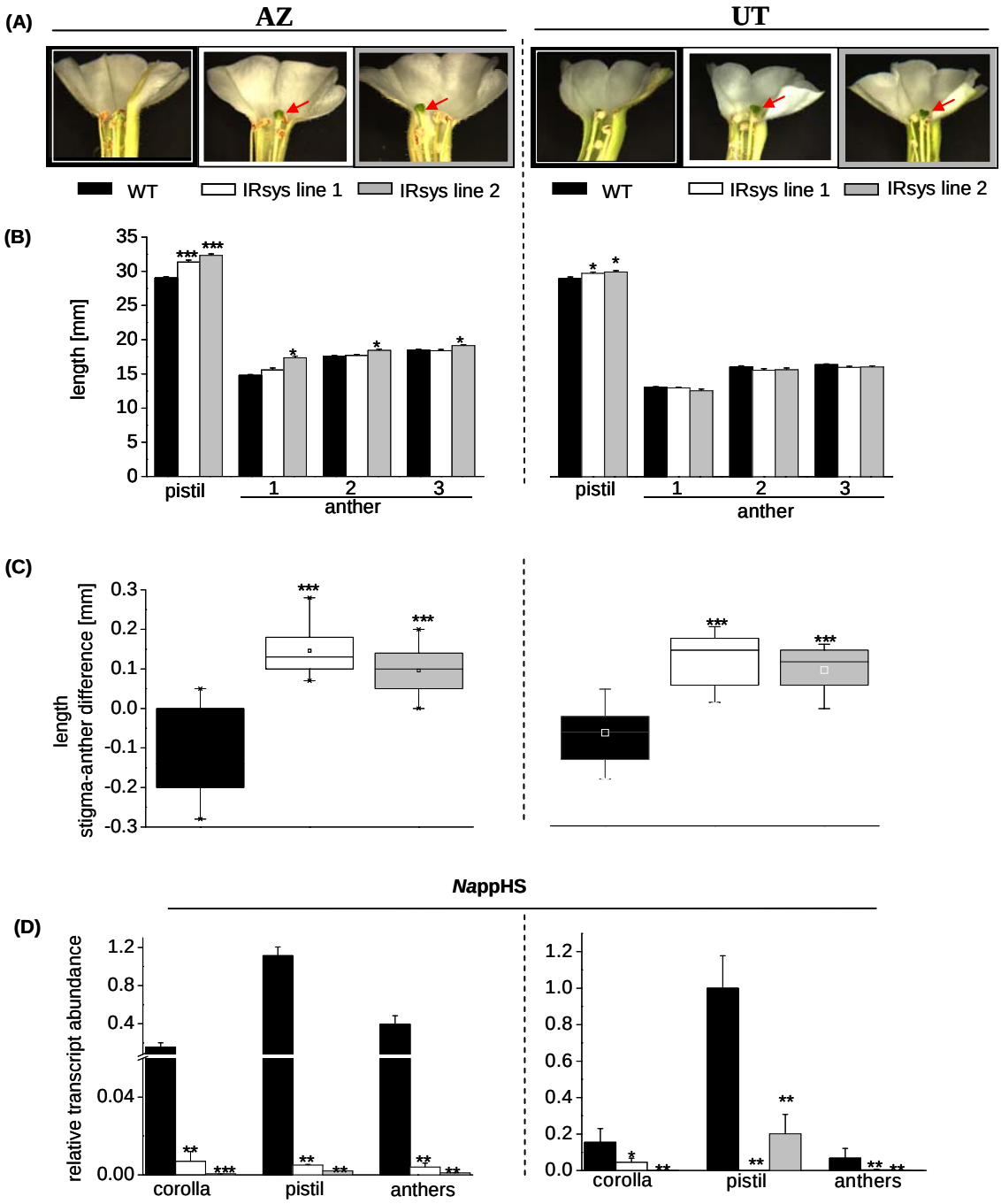

Figure 2: Silencing *NappHS* is associated with elongated pistils in *N. attenuata* flowers.

(A) Characteristic morphology of fully opened flowers of AZ WT, AZ IRsys line 1, AZ IRsys line 2 (left panel) plants, and of UT WT, UT IRsys line 1 and UT IRsys line 2 (right panel) plants. (B) Mean ± SE pistil and anther lengths [mm] of 12 flowers per genotype. Anther 1 is the shortest, 2 the middle length, and 3, the longest anther of the flower (C). Differences in length [mm] between the stigma and the longest anther of 10 to 24 fully opened flowers of each genotype. (D) Relative transcript abundance of *NappHS* in corolla, pistil and anthers of 3 to 5 replicate WT plants (black bars), IRsys line 1 (white bars) and IRsys line 2 (gray bars) plants of AZ (left panel) and UT (right panel) accessions. Asterisks indicate significant differences (*** < 0.001, ** < 0.005, * < 0.05); ANOVA followed by Bonferroni-corrected post-hoc test.

***Na*ppHS-silenced buds and flowers accumulate lower levels of jasmonates**

Many developmental processes are described to be orchestrated by jasmonates. Since there is evidence that the tomato systemin peptide is involved in amplifying a jasmonate-based mobile wound signal, the question arose: is the flower phenotype in the IRsys lines also mediated by jasmonates? Therefore we measured the JA and JA-Ile/Leu concentration in three different developmental stages of the flowers. All developmental stages of IRsys flowers contained less JA-Ile/Leu than their respective wild types (Fig. 3A, B, C). This was true for small buds (5 - 10 mm) of AZ IRsys lines (Fig. 3A, left panel ANOVA, Bonferroni post hoc test, $F_{2,12}$= 9.975, *P's* ≤ 0.0026) and of UT IRsys lines (Fig. 3A, right panel ANOVA, genotype, Bonferroni post hoc test, $F_{2,12}$= 21.718, *P's* ≤ 0.0035), buds (30 - 40 mm) (Fig. 3B, AZ IRsys lines; ANOVA, Bonferroni post hoc test, $F_{2,14}$= 15.731, *P's* ≤ 0.0003; and UT IRsys lines, ANOVA, Bonferroni post hoc test, $F_{2,12}$= 21.718, *P's* ≤ 0.0035) and flowers (Fig. 3C, AZ IRsys lines; ANOVA, Bonferroni post hoc test, $F_{2,14}$= 15.731, *P's* ≤ 0.0003; and UT IRsys lines, ANOVA, Bonferroni post hoc test, $F_{2,14}$= 20.260, *P's* ≤0.0003). JA-Ile/Leu contents in AZ and UT plants decreased throughout flower formation, with the lowest JA-Ile/Leu values in fully opened flowers. This was also found in the ppHS-silenced lines as well (Fig. 3C).

JA contents decreased even more drastically. Whereas small amounts were detectable in small buds (1000 ng g^{-1} FM in AZ and 800 ng g^{-1} FM in UT; Fig. 3D), fully opened flowers contained only minute amounts of JA (Fig. 3F). The JA content of bud and flower tissue in all the transgenic lines was significantly lower than that of the bud and flower tissue in UT WT plants: small buds (Fig. 3D, right panel, ANOVA, Bonferroni post hoc test $F_{2,12}$= 13.589, *P's* ≤ 0.0030), older buds (Fig. 3E, right panel, ANOVA, Bonferroni post hoc test $F_{2,14}$= 6.769, *P's* ≤ 0.0063), and flowers (Fig. 3F, right panel, ANOVA, $F_{2,12}$= 6.773, *P's* ≤ 0.030). Significantly lower JA levels than in AZ WT were detected in small buds (Fig. 3D, left panel, Bonferroni post hoc test ANOVA, $F_{2,12}$= 8.465, *P's* ≤ 0.0334) and in flowers (Fig. 3F, left panel, ANOVA, Bonferroni post hoc test $F_{2,11}$= 9.758, *P's* ≤ 0.0051) but not in medium buds (Fig. 3E , left panel, ANOVA, Bonferroni post hoc test $F_{2,14}$= 1.014, *P's* > 0.05)

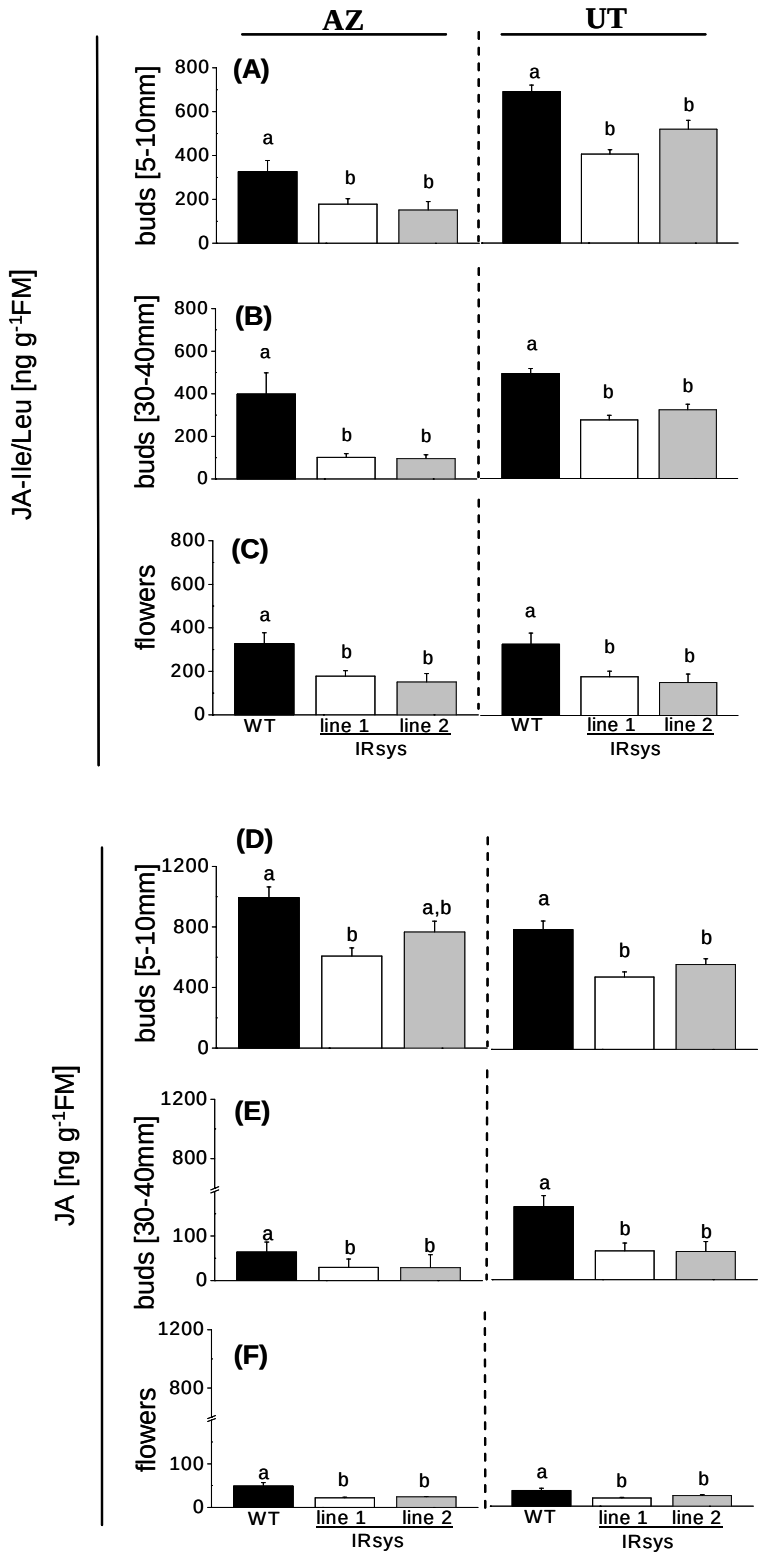

Figure 3: *NappHS*-silenced buds and flowers accumulate lower levels of jasmonates. Mean ± SE JA-Ile/Leu and JA contents of 4 to 5 small (A, D, 5-10mm), elongated (B, E 30-40mm) buds and flowers (C, F) of WT (black bars), IRsys line 1 (white bars), and IRsys line 2 (gray bars) of AZ (left panel) and UT (right panel) accessions. Different letters indicate significant differences (ANOVA followed by Bonferroni-corrected post-hoc test).

of AZ transgenic lines. Interestingly, the accumulation of ppHS transcripts during flower development did not vary significantly (Suppl. Fig. 5).

Since ABA (abscisic acid)'s role in flower senescence is known, and that ABA treatment increased ppHS transcripts in *N. tabacum* has been described (Rocha-Granados et al., 2005), we measured this phytohormone as well. We found no significant difference in the ABA content among the AZ (Suppl. Fig. 6A, B, C, left panel; ANOVA, Bonferroni post hoc test *P's* > 0.05) and UT plants (Suppl. Fig. 6A, B, C, left panel, ANOVA, Bonferroni post hoc test *P's* > 0.05), and their respective silenced lines.

Transcriptional profiling of jasmonate biosynthesis genes

To understand how silencing *ppHS* influences jasmonate accumulation in flowers, we isolated RNA from anthers, pistil and corolla and measured relative transcript abundances of *NaAOS, NaLOX3, NaJAR4, NaJAR6* and *NaTD* using real-time qPCR. *NaAOS*, the gene that encodes the allene oxidase synthase, and *NaLOX3*, the gene that encodes a lipoxygenase, are known to directly influence JA production. The relative abundance of both genes was not decreased in anthers, pistil and corolla of IRsys flowers (Suppl. Fig. 7A, *NaLOX3*, ANOVA, Bonferroni post hoc test, *P's* > 0.05; 4B *NaAOS*, ANOVA, Bonferroni post hoc test, *P's* > 0.05), indicating that the absence of ppHS in *N. attenuata* flowers does not impair the transcriptional regulation of enzymes involved in JA production. Since we found significantly lower levels of JA-Ile/Leu in buds and flowers of IRsys plants in AZ and UT, we measured transcript levels of *NaTD* and *NaJAR4*/ *NaJAR6*. TD, threonine deaminase, is involved in the synthesis of isoleucine, and *NaJAR4*/ *NaJAR6* genes encoding enzymes responsible for conjugating JA and isoleucine (Kang et al., 2006, Wang et al., 2007). We did not detect significant differences in *NaJAR4* transcripts between AZ IRsys and the AZ WT flower parts (Suppl. Fig. 7C, left panel, ANOVA, Bonferroni post hoc test, *P's* > 0.05), or between UT IRsys and UT WT flower parts (Suppl. Fig. 7C, right panel, ANOVA, Bonferroni post hoc test *P's* > 0.05). Similar results were obtained from real-time qPCR analysis of the second gene known to be responsible for the conjugation step, *NaJAR6* (Suppl. Fig. 7D, ANOVA, *P's* > 0.05). These data suggest that ppHS-silenced flowers do not differ from WT flowers in JA biosynthesis and JA conjugation activity.

However, differences in TD transcript levels were found. While *NaTD* levels in corolla and anther tissues did not differ among the genotypes, the pistils of IRsys-silenced flowers had significantly lower levels of *NaTD* transcripts in both accessions (AZ, Fig. 4,

left panel, ANOVA, *P's* < 0.0315; UT, Fig. 4, right panel, ANOVA, Bonferroni post hoc test, *P's* ≤ 0.0078).

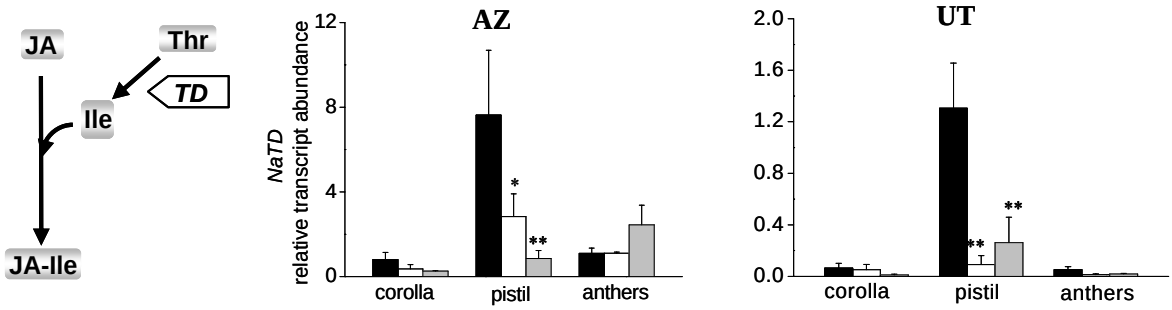

Figure 4: Silencing *NappHS* reduces TD (threonine deaminase) transcript abundance in corolla, pistil and anthers from WT plants (black bars), IRsys line 1 (white bars) and IRsys line 2 (gray bars) flowers of AZ (middle panel) and UT (right panel) accessions. TD expression is required for the production of JA-Ile. TD catalyzes the formation of α-keto butyrate from threonine (Thr), the first step in biosynthesis of Ile which is conjugated to JA (left panel).

Discussion

Both systemin and the hydroxyproline-rich glycopeptides (HypSys) belong to a functionally defined gene family whose members are supposed to amplify defense signaling pathways. HypSys peptides appear to be functionally diverse, as evidenced by studies in *S. lycopersicum*, *N. tabacum,* and *P. hybrida*. Recently, HypSys peptides in *S. lycopersicum* and *N. tabacum* were found to activate defense genes in response to wounding and herbivore attack (Pearce et al., 2001, Rochas-Granados et al., 2005), and transgenic cultivated tobacco plants overexpressing the HypSys precursor were found to be less susceptible to *Helicoverpa armigera* larvae (Ren and Lu, 2006). Although HypSys peptides isolated from petunia did not induce defense genes directed against herbivores, they did activate *defensin 1*, a gene involved in pathogen defense (Pearce et al., 2007). Surprisingly, we found that the *N. tabacum* prosystemin (ppHS) precursor homologue did not play a central role in *N. attenuata's* (accession Utah) anti-herbivore defense signaling, such as in TPI or nicotine production (Berger and Baldwin, 2007).

Due to the apparently rapid diversification of function among HypSys homologues, we hypothesized that *NappHS* may have lost its defensive function in UT recently and

extended our analysis to include a different accession, AZ, which unlike UT is unable to produce TPIs. Because TPIs are not present in AZ and because nicotine is a representative JA-induced defense compound that is present in both AZ and UT (Glawe et al., 2003), we measured how its production is affected by *NappHS*. From the fact that nicotine is elicited independently of *NappHS* in AZ (Suppl. Fig. 1D) and UT, we infer that *NappHS* is not directly involved in the anti-herbivore defense of either accession.

But if *NappHS* is not needed in the defense responses of *N. attenuata*, then why are *NappHS* transcripts expressed in aboveground tissue, and why are they particularly abundant in flowers (Berger and Baldwin, 2007)? It is known that hydroxyproline-rich glycoproteins are widely distributed in plants, and are believed to play fundamental roles in plant function. The expression of many hydroxyproline-rich glycopeptides is frequently tissue-specific and temporally regulated. Some are expressed in young leaves (soybean: Hong et al., 1987) and in sexual tissues (*N. alata* and *N. tabacum:* Sommer-Knudsen et al., 1998). We hypothesized that *NappHS* might play a role in reproduction. The hypothesis was corroborated by the fact that IRsys plants growing in the glasshouse produced fewer seed capsules and the capsules contained fewer seeds per capsule compared to WT plants (Fig. 1A). Silencing the HypSys precursor in *N. tabacum* would be an interesting follow-up experiment. If the silenced plants also had lower seed set than *N. tabacum* WT plants, then HypSys's of both *Nicotiana* species would share similarities in non-defensive functions. Moreover it would clarify whether the silencing of HypSys precursors causes fitness costs unrelated to herbivore induced defense metabolite production in *N. tabacum*.

How *NappHS* contributes to seed capsule production in *N. attenuata* needs to be clarified. Our growth data excluded differences in vegetative biomass as a possible reason for the observed differences in seed capsule production (Suppl. Fig. 2A, B). Dysfunctions in fertility were ruled out as hand pollination experiments revealed that neither a reduced pollen nor ovule viability could account for the reduced reproductive performance of IRsys plants (Suppl. Fig. 3, Fig. 1B). Since hand-pollination restored the numbers of seeds produced in IRsys plants to those of WT plants, we inferred that silencing *NappHS* reduces the self-pollination frequency under glasshouse conditions and consequently reduces the yield of seed capsule numbers and seed numbers per capsule. It seems likely that reduced self-pollination rates are the reason for the observed yield losses rather than an inhibition in the maturation of male and female flower organs, as seen in other flower mutants impaired in fruit and seed production. For example, the *jasmonic acid-insensitive-1* (*jai-1*) tomato mutant showed no growth phenotype, but was impaired in fruit and seed production similar

to IRsys plants. The performance of reciprocal crossing experiments with wild-type tomato and *jai-1* plants clearly revealed that the sterility of this mutant was based on dysfunctions in the female reproductive organs (Li et al., 2001).

N. attenuata flowers characteristically contain a stigma surrounded by five anthers as well as a long pair of anthers at the mouth of the corolla, a shorter pair of anthers, and a single anther shorter than the others (Goodspeed, 1954). During elongation of the corolla but before corolla opens, the stigma passes through the dehisced anthers, and the fertilization process is initiated. If the anthers do not dehisce or if dehiscence is delayed, the self-pollination is reduced. In the case of IRsys flowers, the stigma has already passed the anthers before the anthers dehisce and open flowers have a highly visible stigma that protrudes beyond the anthers (Fig. 2A). The greater distance between the male and the female parts of IRsys flowers was due to a longer pistil (Fig. 2B), the part of the flower with the highest *NappHS* transcript levels (Fig. 2D). Since IRsys anthers were not shorter than WT anthers, we propose that this distance is the result of morphological changes in the pistil.

Changes in flower morphology are commonly described in plants impaired or mutated in jasmonate signaling (Mandaokar et al., 2003; Sanders et al., 2000; Ishiguro et al., 2001; Park et al., 2002; Stinzi and Browse, 2000), and examples of plants with modified jasmonate contents are also known from *N. attenuata*. *N. attenuata* plants silenced in *COI1* are insensitive to jasmonic acid and plants silenced in *TD* are impaired in JA-Ile production. These two genotypes also exhibit abnormal flower phenotypes (Paschold et al., 2007; Kang, 2006) that resemble those of *NappHS*-silenced plants. JA and JA-Ile/Leu measurements revealed that the phenotypic changes of IRsys flowers are correlated with decreased levels of JA-Ile/Leu in flowers (Fig. 3C). In contrast to the reduced jasmonate levels in flowers, we found no differences in jasmonate content in IRsys leaves and also not 1 h after wounding and elicitation with oral secretions of *M. sexta* larvae (Berger and Baldwin, 2007). Therefore we suppose a tissue- and time-specific function for HypSys peptides in *N. attenuata*. Whether the strong *NappHS* transcript abundance in flowers might also reflect an involvement of *NappHS* in anti-pathogen defense to protect the reproductive organs is another interesting aspect that could be tested since HypSys peptides in petunia are involved in anti-pathogen defense.

Interestingly, JA levels decrease precipitously during flower development in both AZ and UT accessions, while JA-Ile levels decrease only modestly, (Fig. 3) suggesting that JA-Ile/Leu or other JA conjugates play a more important role in *N. attenuata* flower

development than JA itself. Similar findings in *S. lycopersicum* Mill cv. Moneymaker buds and flowers, which contain more JA-Ile than JA (Hause et al., 2000) are consistent with this view.

Changes in the jasmonate profiles of IRsys flowers are mirrored in the abundance of transcripts of JA and JA-Ile/Leu biosynthetic genes. Transcripts of JA biosynthesis and JA-Ile/Leu conjugation enzyme genes did not differ from those in WT flowers (Suppl. Fig. 7), but *TD* expression was reduced in the female parts of IRsys flowers (Fig. 4), suggesting that ppHS interfered with jasmonate signaling by regulation of TD expression. *N. attenuata* plants with reduced endogenous TD levels reveal several morphological changes including shortened pistils and petaloid anthers. Low levels of TD are correlated with low levels of JA-Ile/Leu in floral buds (Kang, PhD thesis, 2006), a pattern which was also seen in IRsys buds (Fig. 3A). While IRsys buds contain less JA than WT buds do, silencing TD was associated with a 1.3 fold increase of JA in buds (Kang, PhD thesis, 2006). We propose that the tissue-specific down-regulation of TD transcripts in pistils of IRsys flowers results in imbalances in the relative proportion of jasmonates in this floral organ. These imbalances might lead to the elongated pistils in IRsys flowers. The delicate balancing of time- and tissue-specific jasmonate profiles is important during flower development as demonstrated by Hause et al. (2000). Developmental phenotypes are notoriously difficult to complement or recover, which could explain why our attempts to restore the WT phenotype in IRsys flowers by stem feeding flowering stems with 0.1mM JA, JA-Ile or indanone (a JA-Ile mimic) or by dipping inflorescences in a 0.05 mM methyljasmonate solutions two times a day for three day were not successful.

Another hypothesis why JA complementation failed to restore the WT phenotype is that jasmonates only influence a particular aspect of the IRsys flower phenotype and that other, not-yet-identified factors play a role as well. In *Arabidopsis thaliana*, two paralogous auxin response transcription factors, ARF6 and ARF8 are known to regulate stamen and gynoecium maturation, and an *arf6 arf8* double-null mutant produces flowers with undehisced anthers and immature gynoecia. *Arf6 arf8* double-null mutants contain JA concentrations below the detection limit but endogenous application of JA could only restore anther dehiscence but not gynoecia maturation (Nagpal et al., 2005). Currently, little is known about the underlying mechanisms of the IRsys flower phenotype. The phenotype cannot be fully explained by a decrease in jasmonates, and more research is required to understand how *NappHS* regulates pistil length.

So the question remains why IRsys flowers possess longer pistils? Changes in style length in cultivated tomato flowers were recently attributed to a mutation of the promoter of *Style2.1*, a gene that encodes a putative transcription factor regulating cell elongation in developing styles. *Style2.1* was identified by QTL mapping as a major factor for flower development. The changes in style length lead to a switch from cross-pollination to self-pollination (Chen et al., 2007). Silencing *NappHS* results in longer pistils and to the ability to switch from self-pollination to out-crossing. Out-crossing increases genetic diversity and perhaps increases fitness in ephemeral plants such as *N. attenuata* that are adapted to germinate and grow in exceptionally unpredictable environments.

When paired WT and IRsys plants were planted into native habitats with native pollinators, they produced similar numbers of seed capsules which is likely due to the ability of pollinators to compensate for decreases in pollen loads in IRsys plants (Berger and Baldwin, unpublished data; Sime and Baldwin, 2003). We propose that the HypSys peptides encoded by *NappHS* act as environmentally elicited (or suppressed) signals that cause the plant to react to unfavorable environmental conditions (i.e. herbivore attack) by changing flower morphology so as to change its rate of out-crossing. This hypothesis would have to be tested in a field study that includes natural pollinators and plants from both accession, AZ and UT, as well as IRsys lines. The paternity of the seeds produced would have to be determined to specify out-crossing rates. This hypothesis would predict that out-crossing rates will be greater in IRsys plants and herbivore attack could increase the average stigma-anther distances in WT plants by *NappHS* suppression and thereby mediate out-crossing rates in response to herbivore pressure.

Materials and Methods

Plant material and growing conditions

Nicotiana attenuata Torr. Ex. Wats. (synonymous with *Nicotiana torreyana* Nelson and Macbr.) seeds from a Utah population originally collected near Santa Clara, Utah, USA, and inbred for 14 or 15 generations were used to conduct experiments and to generate transformed lines from this accession (UT). Seeds of the Arizona accession (AZ) were collected from a 20-plant population near Flagstaff, Arizona, USA (Glawe et al., 2003), and an inbred line to the 7th generation was established to conduct experiments and to generate transformed lines. For glasshouse experiments, seeds were germinated as

described in (Krügel et al., 2002). Plants were grown in 1-L pots at 26-28 °C under 16 h of light supplemented by Philips Sun-T Agro 400- 600W Na lights (Philips, Turnhout, Belgium).

Generation of IRsys lines

Cloning and sequencing the *Na*preproHypSys gene (*NappHS*), and the generation of IRsys lines for the Utah accession (line 1: A-04-366-11; line 2: A-04-464-1) are described in Berger and Baldwin (2007). IRsys lines of the Arizona accession (line 1: A-04-357-11; line 2: A-04-385-13) were generated with the same vector (pRESC5sys2), and they were tested for their ploidy level as described in Bubner et al. (2006).

Nucleic acid analysis

To determine the number of transformation vector insertions in the transformed lines of the AZ accession, isolated genomic DNA was hybridized with a PCR fragment of the *hptII* gene, the selection marker used on the transformation vector (pRESC). The PCR product for both probes was eluted from the gel using Geneclean Kit (BIO 101, Vista, CA, USA), labeled with $^{[32]}$P using a random prime labeling kit (RediPrime II, Amersham-Pharmacia, Little Chalfont, UK), and purified on G50 columns (Amersham-Pharmacia). After overnight hybridization, blots were washed one time with 2xSSPE at 62 °C and three times with 2xSSPE / 2% SDS, and analyzed with a phosphor-imager (model FLA-3000; Fuji Photo Film Co., Tokyo, Japan).

To quantify transcripts in leaves, flower tissue (anthers, corolla and pistil) and at different developmental flower stages, total RNA was extracted using TRI Reagent (Sigma, St. Louis, MO, USA) according to the protocol of (Chomczynski and Sacchi, 1987), with minor modifications for polysaccharide-rich plant tissue to minimize the co-precipitation of impurities. An additional salt buffer (1.2 M $NaCl_2$, 0.8 M Na citrate) was added to the isopropanol precipitation step.

For real-time quantification of the transcripts, cDNA was prepared from 200 ng total RNA with MultiScribe™ reverse transcriptase (Applied Biosystems). The gene-specific primers and probes for *NappHS*, *NaLOX3*, *NaAOS*, *NaJAR4*, *NaJAR6* and *NaTD* mRNA expression detection by qPCR are provided in Supplemental Table 1. The assays using a double dye-labeled probe were performed on an ABI PRISM® 7700 Sequence Detection System (qPCR™ Core Kit, Eurogentec). The expression of each gene was normalized to the expression of the endogenous control gene actin. Northern blot analysis

of ppHS transcripts in AZ wild type plants was conducted as described in Berger and Baldwin (2007).

Growth, fitness performance and flower morphology analysis

Stalk length measurements in elongating four-week-old plants were made over a period of two weeks to evaluate growth. First flowering, flower number and lifetime seed capsule numbers were determined as estimates of fitness. Freshly opened flowers were counted in 40-day-old plants and total seed capsule number was counted after irrigation was stopped. Above-ground tissue was collected and dried at 60 °C, and biomass was determined.

To determine the seeds per capsule of self-pollinated plants, the first four to five ripe seed capsules of each genotype were collected and dried, and seeds were counted. In an additional setup, flowers of each genotype were emasculated with forceps, and one day later pollinated by hand with pollen of the same genotype. After seed capsules had ripened, the number of seeds per capsule was counted.

Pollen grains from each genotype were stained with fluoresceine diacetate to determine their viability. A fluorescine diacetate stock (60 mg dissolved in 30 ml acetone) was added to a 10 % (w/v) sucrose solution until it turned milky. A drop of this solution was placed onto a glass-slide, and anthers were dipped into this solution before being incubated for five min. Viable pollen was visualized under a fluorescence microscope.

Fully opened flowers from each genotype were dissected with a scalpel, and the distances between the longest anther and the stigma, as well as the total length of each anther and the pistil, were measured with a caliper.

Phytohormone analysis

Approximately 150 mg tissue from five small buds [5 to 10 mm], green buds [30 to 40 mm] or fully opened flowers of each accession and genotype were harvested. The flash-frozen tissue was homogenized and extracted in FastPrep tubes containing 0.9 g of FastPrep Matrix (BIO 101, Vista, CA, USA), and 1 mL ethyl acetate containing methanolic 200 ng mL^{-1} [$^{13}C_2$] JA and 200ng mL^{-1} [D_6] ABA was added as the internal standards. JA-Ile concentrations were calculated based on an external standard dilution series of JA-Ile. The FastPrep tubes were shaken two times at 6.0 m sec^{-1} for 45 seconds. Samples were centrifuged at 13,000 rpm for 20 min at 4 °C and the supernatant was collected. The extraction step was repeated with 1 mL ethyl acetate. Both supernatants

were combined and evaporated in a SpeedVac concentrator. The dried samples were dissolved in 500 µL MeOH (70 %) and centrifuged for 10 min at 13,000 rpm at 4 °C. Analysis of the samples was performed using a Varian 1200L Triple-Quadrupole-LC-MS (Varian, Palo Alto, CA, USA) as described by Wang et al. (2007).

Nicotine

After AZ and AZ-IRsys leaves of the same position (defined as +2) were wounded with a pattern wheel, 20 µl deionized water was applied three times in a row, every 30 min. The nicotine content in unwounded control plants and in wounded AZ and AZ IRsys plants was determined as described in Keinänen et al., (2001) with the modification that approximately 100 mg frozen tissue was homogenized in 1 mL extraction buffer utilizing the FastPrep® extractions system (Savant Instruments, Holbrook, NY, USA).

Statistical Analysis

Data were analyzed with Statview 5.0 (SAS Institute, Cary, NC). Data were transformed if they did not meet the assumption of homoschedacity.

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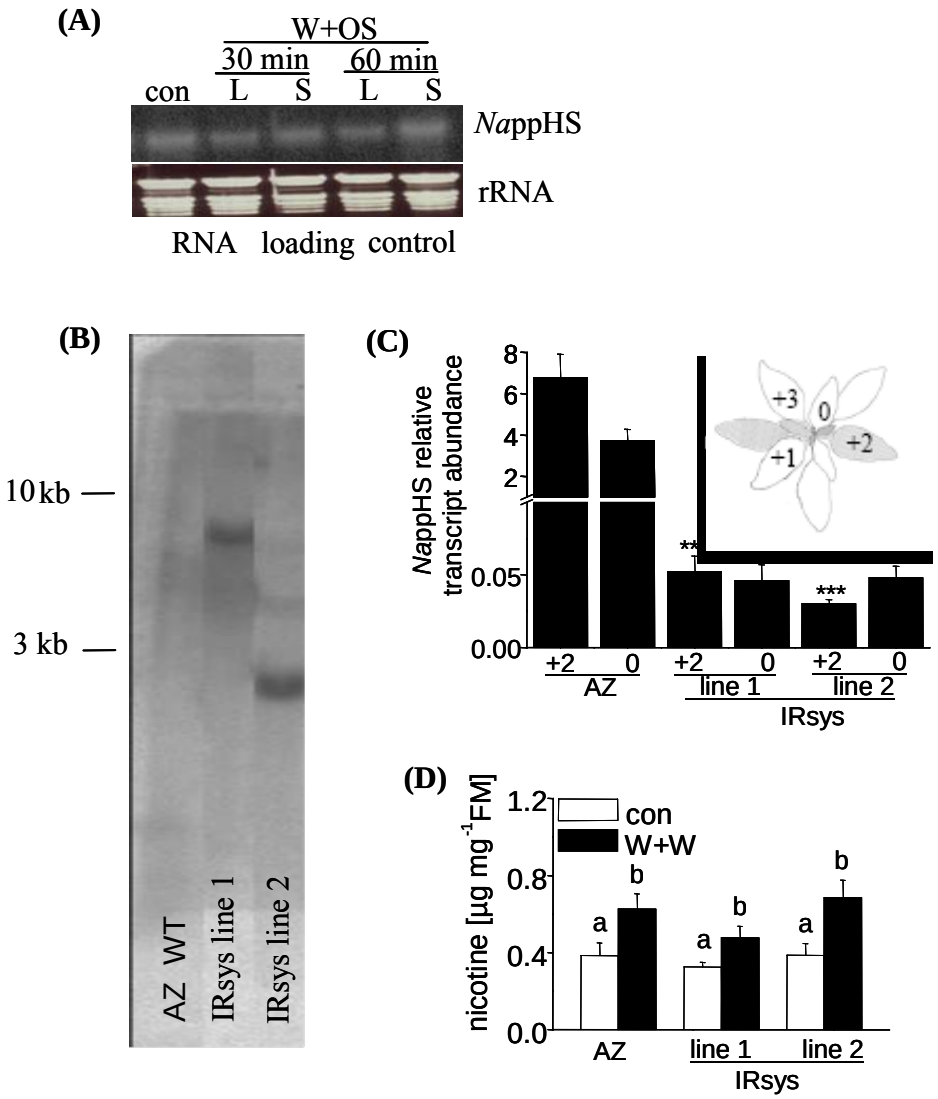
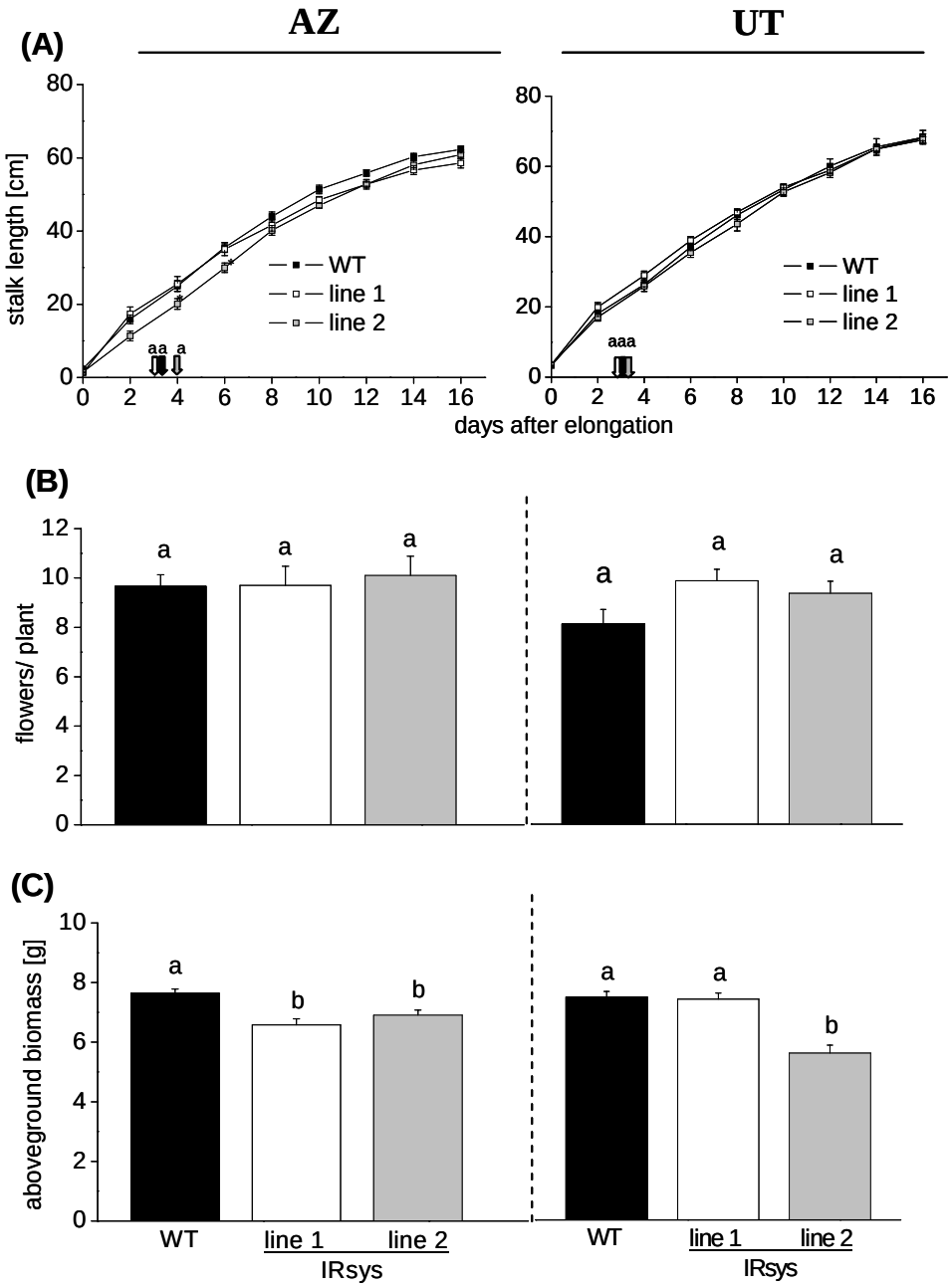
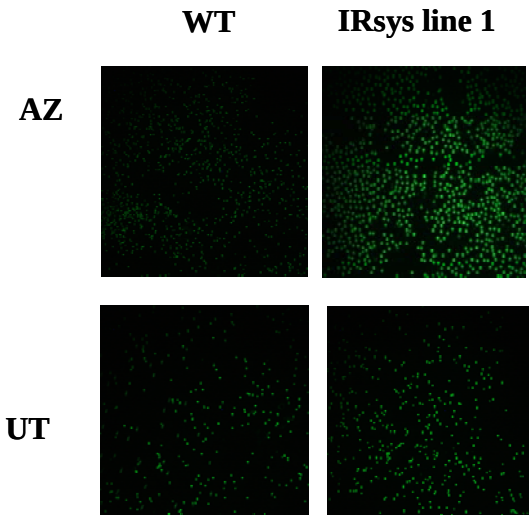
Supplementary Material



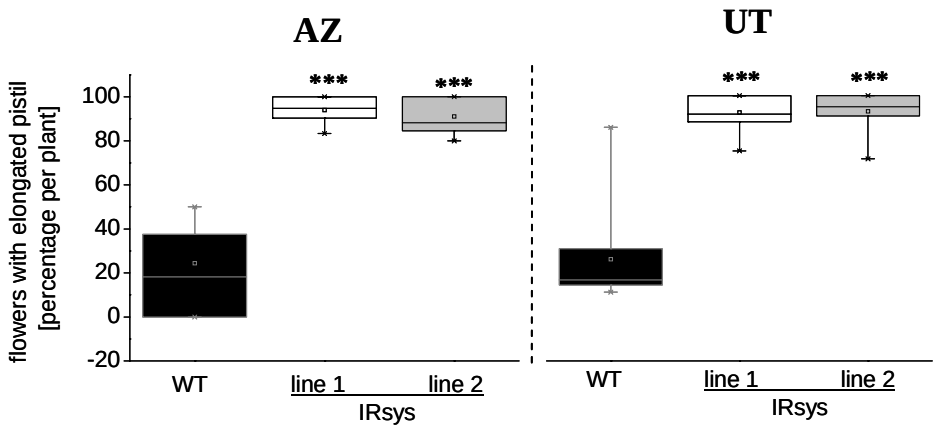
Supplemental Figure S1: (A) Northern blot analysis of *NappHS* transcripts in AZ wild-type plants. Rosette-stage leaves (position +2) were wounded with a pattern wheel (w), and 20 µl of 1:1 (v/v) diluted *M. sexta* oral secretions (OS) was applied to the resulting puncture wounds. Treated (L) and systemic (S) leaves from OS-elicited plants at position 0 were harvested after 30 min and 60 min, as well as unwounded (con) leaves at position +2 from five plants. **(B)** Southern blot analysis of AZ wild type, AZ IRsys line 1 and AZ IRsys line 2 plants. Genomic DNA (5µg) was digested with *BamH1*, blotted onto a nylon membrane, and hybridized with a PCR fragment of the *hptII* gene, a selection marker on the transformation vector pRESC. **(C)** Relative *NappHS* transcript abundance in local (+2) and systemic (0) leaves of W+OS treated Arizona wild type (AZ) and ppHS-silenced plants (IRsys line 1 and 2), (*** = $P < 0.001$, ANOVA). **(D)** Mean ± SE leaf (+2) nicotine content of 5 control (white bars) AZ wild type, AZ IRsys line 1 and AZ IRsys line 2 plants. Nicotine accumulation was additionally determined in rosette-stage leaves that were wounded, followed by the application of 20 µl water (3 in a row, every 30 min), and harvested four days after the treatment (black bars).



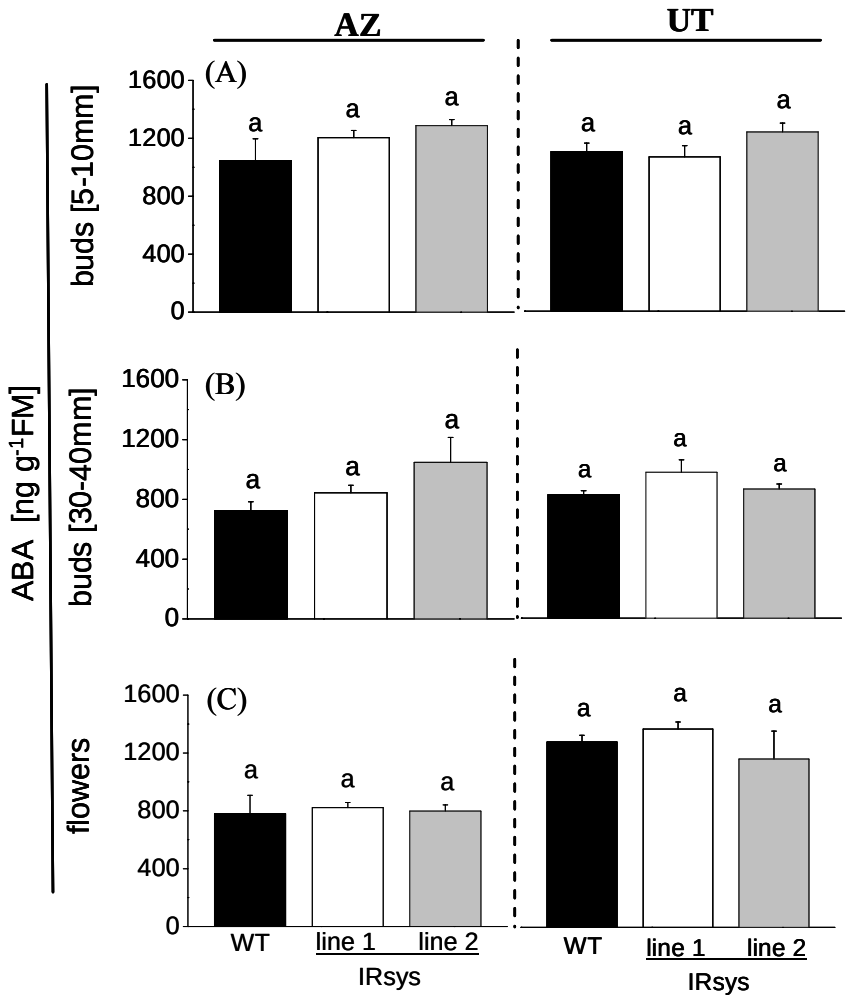
Supplemental Figure S2: *NappHS*-silenced plants do not differ from WT plants in flower production or stalk elongation. **(A)** Mean ± SE growth rates of 10 to15 WT (black squares), and the two lines of *Na*ppHS-silenced plants in two accessions: IRsys line 1 (empty squares) and IRsys line 2 (gray squares) of AZ (left panel) and UT (right panel) accession. Asterisks represent significant differences from WT values of each accession (as determined by ANOVA followed by Bonferroni-corrected post-hoc test). First flowering is indicated by arrows in both panels (WT: black arrows, IRsys line 1: white arrows and IRsys line 2: gray arrows). Number of flowers **(B)** and above-ground biomass **(C)** in wild type (black bars) and *Na*ppHS-silenced plants: IRsys line 1 (white bars); IRsys line 2 (gray bars) of the AZ (left panel) and the UT (right panel) accession. Bars represent means ± SE of 9 to 10 replicates. Different letters indicate significant differences among the genotypes.



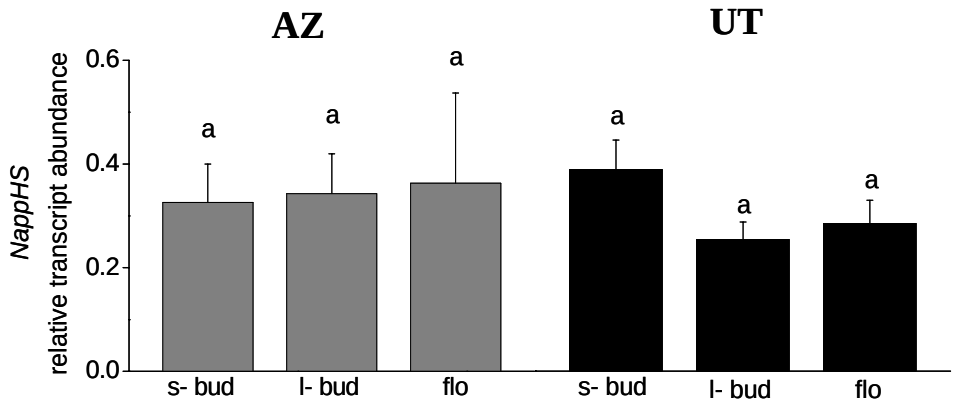
Supplemental Figure S3: Viability of pollen from WT and IRsys flowers. Pollen were stained with fluorescine diacetate and visualized using a fluorescence microscope.



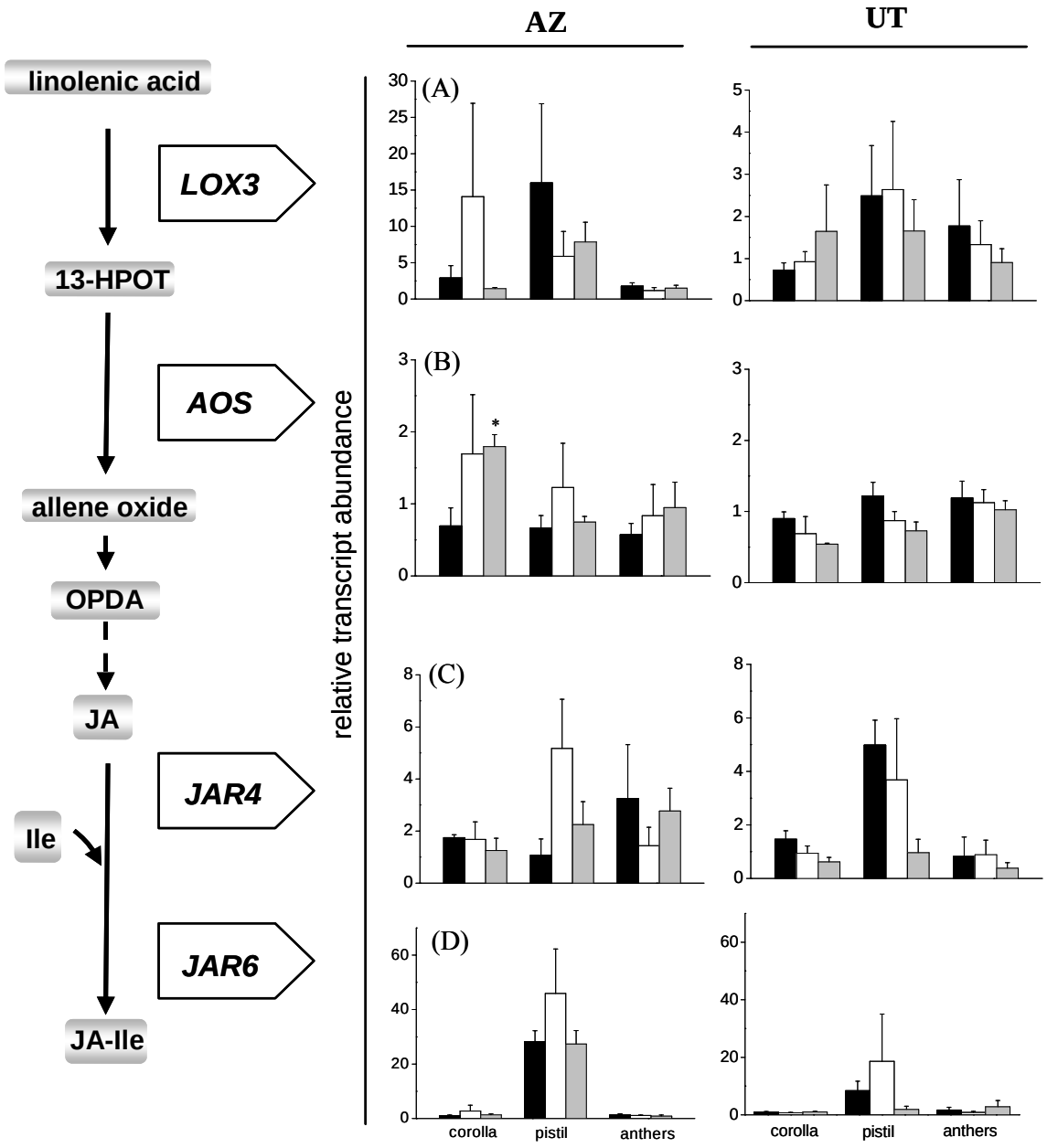
Supplemental Figure S4: Percentage of flowers with elongated pistils. Flowers with abnormal pistil growth in WT (black boxes), IRsys line 1 (white boxes) and IRsys line 2 (gray boxes) of AZ (left panel) and UT (right panel) were counted and divided by the total number of flowers per plant (n = 8 to10 plants/ per genotype).



Supplemental Figure S5: Mean ± SE abscisic acid (ABA) content of 4 to 5 small (A, 5-10mm), elongated (B, 30-40mm) buds and flowers of WT (black bars) and IRsys line 1 (white bars) and IRsys line 2 (gray bars) AZ (left panel) and UT (right panel) accessions. Different letters indicate significant differences among WT plants and their respective IRsys-silenced lines (ANOVA followed by Bonferroni-corrected post-hoc test).



Supplemental Figure S6: Relative transcript abundance of *NappHS* in small buds (s-bud), buds (l-bud) and flowers (flo) of AZ (gray bars) and UT (black bars) WT plants. Different letters indicate significant differences (ANOVA followed by Bonferroni-corrected post-hoc test, n= 5).



Supplemental Figure S7: Transcript accumulation of genes involved in jasmonate synthesis and in the conjungation step of JA with Ile in the oxylipin pathway. Left panel shows a simplified model of the oxylipin pathway: A 13-lipoxygenase (*LOX3*) oxygenates linolenic acid to 13-HPOT (13-hydroperoxide linolenic acid) which is further converted to allene oxide and OPDA (12-oxo-phytodienoic acid) by *AOS* (allene oxide synthase). OPDA is converted to JA,and the conjugation of JA to Ile is mediated by *JAR4* (jasmonate resistant 4) and *JAR6* (jasmonate resistant 6). Relative transcript abundance of **(A)** *LOX3*, **(B)** *AOS*, **(C)** *JAR4* and **(D)** *JAR6* in corolla, pistil and anthers of 3 to 5 replicated wild-type (black bars) and IRsys line 1 (white bars) and IRsys line 2 (gray bars) plants of AZ (left panel) and UT (right panel) plants. Asterisks represent significant differences from WT values of each accession (as determined by ANOVA followed by Bonferroni-corrected post-hoc test).

Gene	Primer 1 (5´- 3´)	Primer 2 (5´- 3´)	Taqman probe (5´- 3´)	Reporter	Quencher
NappHS	CGCACTGATGAGAAAACCTCTG	AACAGCTGATTAATGTTTTCCACTTG	ACACTGAGCTCGTGATTGCAACATTTGC	FAM	TAMRA
NaLOX3	GGCAGTGAAATTCAAAGTAAGAGC	CCCAAAATTTGAATCCACAACA	CAGTGAGGAACAAGAACAAGGAAGATCTGAAG	FAM	TAMRA
Na AOS	GACGGCAAGAGTTTTCCCAC	TAACCGCCGGTGAGTTCAGT	CTTCACCGGAACTTTCATGCCGTCG	FAM	TAMRA
NaJAR4	ATGCCAGTCGGTCTAACTGAA	TGCCATTGTGGAATCCTTTTAT	CAGGTCTGTATCGCTATAGGCTCGGTGATGT	FAM	BHQI
NaJAR6	TGGAGTAAACGTTAACCCGAAA	AGAATTTGCTTGCTCAATGCCA	TGCCCCCTGAGCTAGTCACTTATGCA	FAM	BHQI
NaTD	TAAGGCATTTGATGGGAGGC	TCTCCCTGTTCACGATAATGGAA	TTTTTAGATGCTTTCAGCCCTCGTTGGAA	FAM	BHQI
NaActin	GGTCGTACCACCGGTATTGTG	GTCAAGACGGAGAATGGCATG	TCAGCCACACCGTCCCAATTTATGAGG	Yakima Yellow	BHQI

Supplemental Table S1: Gene-specific primers and probes used for real-time qPCR.

4. Discussion

The goal of this thesis is to elucidate functional aspects of the hydroxyproline-rich glycopeptide systemin precursor (*Na*ppHS) in the diploid wild tobacco *Nicotiana attenuata*. *N. attenuata* is a well-established model system for studying plant-herbivore interactions. The study of *Agrobacterium*-mediated plant transformation presented here provides new insights into the occurrence of tetraploidy in transformed plants of an accession collected in Arizona (AZ) (also referred to as the Arizona genotype). Flow cytometry was adapted to determine ploidy levels in cells isolated from transformed *N. attenuata* plants. As polyploidy can significantly affect the plant phenotype, the selection of diploid plants for subsequent studies is important. Flow cytometry allows for this selection as exemplified in *Na*ppHS transformants (manuscript I). Comparative glasshouse- and field-studies of *N. attenuata* wild-type (WT) plants and diploid *Na*ppHS transformants refute the claim that *Na*ppHS plays a central role in anti-herbivore defense signaling. These results stand in contrast to data obtained on the *Na*ppHS sequence homologue from *N. tabacum* and on systemin from *S. esculentum* (manuscripts II and III). The present thesis demonstrates that an anti-herbivore defense function of HypSys peptides is apparently not conserved in the Solanaceae family. Instead, down-regulation of *Na*ppHS mRNA was associated with abnormally protruded pistils and reduced self-pollination rates in glasshouse-grown plants of two different accessions, AZ and UT (collected in Utah) (manuscript III). Under the assumption that a *Na*ppHS-regulated protrusion of pistils might occur in response to various stresses (i.e. herbivory), we hypothesize the following function: Protruding pistils may help *N. attenuata* populations to adapt to unpredictable environments, increasing genetic diversity by increasing out-crossing.

4.1. *Agrobacterium*-mediated transformation - a tool for gene function analysis

Plants have intricate regulatory pathways through which they control their interaction with abiotic (e.g. temperature, soil water availability, salinity, pH, nutrient balance, and UV radiation) and biotic factors (e.g. competitors, herbivores, predators, pathogens, and pollinators) present in their environment. The method of reverse genetics permits researchers to characterize the roles of single genes within these complex regulatory systems. Using the methods of reverse genetics, researchers alter the expression of individual genes and compare the resulting mutant phenotypes to the phenotypes of WT plants. These comparisons permit the contribution of specific genes to complex traits like plant evolutionary fitness and adaptive phenotypic plasticity to be quantified. Methods of

choice in reverse genetics include silencing gene expression by RNAi (RNA interference), or constitutively over-expressing genes under the control of a strong promoter. All techniques used to tune the expression of a gene require the introduction of recombinant DNA into the organism. *Agrobacterium tumefaciens*-mediated transformation is a widely used process for generating stable transformants in plant reverse genetics. This method usually depends on the regeneration of plantlets from a callus culture. The development of an *A. tumefaciens*-mediated transformation system for *N. attenuata* plants (Krügel *et al.*, 2002) provided the basis for analyzing gene function in this species.

In some cases, unintended consequences of the transformation process may confound the analysis of gene function. Testing the influence of the transgene integration site on the phenotype of transgenic plants is part of the standard transformant characterization procedure in our laboratory. In order to confirm the success of each transformation event, we conduct post-transformational antibiotic screens and DNA-blots (Southern blots) to ensure the selection of mutant lines which are homozygous for single insertions. Quantification of target gene transcript abundance by Northern blot analysis and quantitative real-time PCR determines the efficiency of silencing or over-expression. The approach of comparing defense-, growth-, and fitness-related phenotypic traits among multiple independently transformed lines ensures that commonly observed phenotypes are due only to the manipulated expression of the "gene of interest".

A recent study by Schwachtje *et al.* (2008) demonstrated that *Agrobacterium*-transformed lines of *N. attenuata* in fact do not show significant effects due to the integration of the transformation vector. Their study included multiple transgenic lines containing a control vector lacking an over-expression or silencing construct (empty vector control, EVC), isogenic WT plants, and a line transformed with a vector silencing the TPI (trypsin proteinase inhibitor) gene. EVC and WT lines did not differ in ecologically relevant traits, including the production of phytohormones and secondary metabolites and growth and fitness parameters. TPI-silenced plants, however, clearly differed in fitness and growth (traits predicted to be affected by silencing TPI), but not in the production of phytohormones and secondary metabolites (which should not be affected by silencing TPI). These findings were supported by a statistical power analysis demonstrating that possible side-effects caused by the transformation vector are not significant for the characterization of ecologically relevant phenotypes in *N. attenuata*.

Another set of unintended genetic changes that may arise from the transformation process are alterations in the ploidy level of plantlets re-grown from callus culture. Unlike

effects from vector integration, changes in ploidy level have been observed in *N. attenuata*. These changes, while usually not deadly for plants, may have unpredictable effects on the phenotype of an individual plant. Almost 70% of all flowering plants are thought to have undergone polyploidization during the course of their evolution (Masterson, 1994; Song *et al.*, 1995). Speciation caused by polyploidy is an intriguing field of study, especially as polyploid species can exhibit a greater ability to survive under unfavorable conditions (Song *et al.*, 1995). The selective breeding of crop plants, for example, benefits from artificially generated polyploidy, which is often associated with traits that are beneficial for commercial cultivation (Udall and Wendel, 2006). Polyploidization events are described for transgenic and nontransgenic plants regenerated from callus cultures, including the Solanaceous plants potato (Jacobs, 1981), tomato and pepper (Pozueta-Romero, 2001).

Polyploidization was also observed in transgenic *N. attenuata* plants of the AZ accession (manuscript I). Although polyploidization is an important factor for the evolution and selection of advantageous attributes in agricultural crops, it may cause undesirable effects in a gene of interest analysis, because of the resulting instability of the plant's genome and genetic regulation. A novel aspect of the occurrence of polyploidy in our system was that post-transformational tetraploidy was limited to only one of the *N. attenuata* accessions studied. The most likely explanation as to why no *Na*ppHS-silenced (IRsys) UT transformants, but 72 % of IRsys AZ transformants, were tetraploids is that polyploid UT calli may fail to regenerate into plantlets. The following reasons for the occurrence of tetraploidy in AZ were ruled out: tetraploid explant sources, tetraploidy triggered by the transgene itself, and differences in polysomaty of explant tissue. Given that the excluded causes are not directly related to callus-mediated transformation, we expected to find possible reasons for tetraploidy during callus formation and plant regeneration. This conclusion was recently supported in a study by Fras *et al.* (2007). Their investigation of polyploidization mechanisms during callus regeneration in *Arabidopsis thaliana* pointed to events such as endoreduplication, endomitosis, abnormal microtubule arrangement and DNA damage as factors that initiate polyploidization. The question as to why two accessions of one species might generate differentially viable polyploids is interesting in its own right, but the remainder of this thesis comprises a gene of interest study in both accessions. Diploid transformants generated from *Agrobacterium*-mediated transformation were selected via flow cytometry to determine the function of *Na*ppHS in *N. attenuata*. Many successful characterizations of defense traits in *N. attenuata* (Halitschke and Baldwin, 2003; Steppuhn *et al.*, 2004; Zavala *et al.*, 2004; von Dahl *et al.*,

2007, Schwachtje *et al.*, 2008), provide evidence that *Agrobacterium*-mediated stable transformation is a reliable tool with which to study *N. attenuata's* phenotypic plasticity in the glasshouse and the field.

4.2. *Na*preproHypSys - A missing piece in the solution of the plant defense puzzle?

In recent years, the understanding of defense responses in *N. attenuata* was advanced by expression profile studies on *N. attenuata´s* herbivory-induced transcriptome (e.g. Hermsmeier *et al.*, 2001; Heidel and Baldwin, 2004; Voelckel and Baldwin, 2004). Although detailed work on the proteome has only recently begun, it has already proven to be a useful complement (Giri *et al.*, 2006). Detailed loss-of-function studies with single gene transformants have synthesized this knowledge and illuminated the orchestration of herbivore-induced responses and tritrophic interactions in *N. attenuata* (Kessler and Baldwin, 2001). The resulting knowledge of *N. attenuata*'s anti-herbivore responses, particularly those to attack by the specialist lepidopteran herbivore *Manduca sexta*, is approaching the critical mass necessary to study system-wide effects of single genes and their products in this plant. In other words, it allows us not only to study single aspects of *Na*ppHS function, as was done on HypSys peptides and their precursors in *N. tabacum* (Pearce *et al.*, 2001, Rocha-Granados *et al.*, 2005, Ren and Lu, 2006), *Petunia hybrida* (Pearce *et al.*, 2007) and *Solanum esculentum* (Pearce *et al.*, 2003; Narvarez-Vasquez *et al.*, 2007), but also to unravel parts of the underlying cross-talk and interdependence among signaling and defense responses.

HypSys peptides from *N. tabacum* and *S. esculentum* are potent inducers of protease inhibitors (PIs) when applied to plants in minute amounts (Pearce *et al.*, 2001, Pearce and Ryan, 2003). Given that the activation of defense genes (e.g. PI) in response to herbivory and wounding is mediated by the octadecanoid pathway in *N. attenuata* (i.e. Halitschke and Baldwin, 2003), as it is in other plants in which prosystemin-like peptides have been studied, we examined the effect of *Na*ppHS on individual steps of this signaling pathway. Silencing (IRsys) or over-expressing (OVsys) the *Na*ppHS gene in the *N. attenuata* Utah accession produced surprisingly few differences in anti-herbivore defense responses when WT, IRsys and OVsys plants were compared in a comprehensive study conducted in the glasshouse and the field. The results obtained do not provide sufficient evidence to assume a central role for *Na*ppHS in induced anti-herbivore defense responses.

Phenotypic differences in the abundance of defense metabolites were limited to OVsys plants. *Na*ppHS over-expression resulted in constitutively higher levels of the

defense metabolites nicotine and TPI, which is consistent with the constitutively higher TPI levels seen in tobacco and tomato plants over-expressing the respective HypSys precursors (Ren and Lu, 2006; Narvarez-Vasquez *et al.*, 2007). TPI and nicotine levels elicited by *M. sexta* oral secretions, on the other hand, did not differ from those in WT plants. This slightly inconsistent defense phenotype obtained from Utah OVsys plants stands in stark contrast to the clear results obtained from IRsys plants of the UT and AZ accessions. Those plants do not exhibit any significant differences in investigated defense metabolites when compared to their respective WT counterparts (manuscripts II and III), which is not consistent with results obtained in the tomato system. PpHS-silenced tomato plants accumulate significantly less wound-induced TPI than WT plants do. The emerging picture from secondary metabolite data is corroborated by herbivore studies. In glasshouse experiments, the tobacco specialist *M. sexta* does not show growth differences when fed on *Na*ppHS transformants versus WT plants. In field studies, neither the *Solanaceae* specialist *Epitrix hirtipennis* nor the polyphagous insects *Trimerotropis spp*. and *Tupicoris notatus* exhibits drastic feeding preferences for either of the *Na*ppHS transformant types or the WT (manuscript II). Further support for the idea that *Na*ppHS does not regulate anti-herbivore defense responses comes from transcriptome assays comparing induced WT and IRsys plants. Microarray data revealed no differences in plant defense gene activation in response to wounding and the application of *M. sexta* oral secretions at 1 h and 8 h (Berger and Baldwin, unpublished data), a time window representing defense regulatory gene expression in *N. attenuata*. Functional aspects of *Na*ppHS clearly deviate from the commonly assumed roles for ppHS peptides in *N. tabacum* and *S. esculentum*; as anti-herbivore defense in two *N. attenuata* accessions with different defense strategies does not rely on *Na*ppHS, *Na*ppHS's true role remains to be discovered.

The observation that *Na*ppHS is not involved in anti-herbivore defense signaling is consistent with recent studies performed on HypSys peptides in *P. hybrida* (Pearce *et al.*, 2007). *Petunia* HypSys peptides were not able to induce secondary defense metabolites such as TPI and PPO (polyphenol oxidase) as they do in cultivated tobacco and tomato, but were shown to induce anti-pathogen defense genes instead. Would the ability to induce anti-pathogen defense genes and thereby defend *N. attenuata* against pathogens assign *Na*ppHS to the functionally-defined systemin family? – Yes, but whether *Na*ppHS is able to mediate pathogen induced systemic acquired resistance (SAR) instead of insect induced systemic resistance (ISR) in *N. attenuata* remains an open question. That tomato HypSys peptides are located in the cell wall matrix (Narvarez-Vasquez, 2005) indicates that they

may be involved in the response to pathogen attack. Hydroxyproline-rich glycoproteins (HRGPs) are structurally related to HypSys peptides and are also located in cell walls. HRGPs are known to accumulate in response to infections as an apparent defense mechanism. Examples of HRGP accumulation in *N. tabacum* have been documented for infections with TMV (tobacco mosaic virus) (Benhamou *et al.*, 1990; Ye *et al.*, 1992; Raggi, 1998) and *Erysiphe cichoracearum* (Raggi, 2000). Dramatic levels of infection by *Pseudomonas* sp. Strain 4 have been observed on *N. attenuata* transformants silenced for *NPR1* (non-expressor of PR1, a peptide mediating pathogen resistance) growing in their native habitat (Rayapuram and Baldwin, 2007). Leaves of *Na*ppHS-silenced plants growing in the same field plot exhibited no typical infection symptoms (personal observation). It therefore seems unlikely that NappHS functions in anti-pathogen defense. To categorically exclude this possibility, more detailed experimental work would be necessary, which was beyond the scope of this thesis.

In conclusion, in *N. attenuata*, HypSys peptides are demonstrated not to be a part of the defense response of herbivore-attacked plants.

4.3. Na*preproHypSys* and flower development

Although we did not find any essential function for *Na*ppHS in manuscript II, the gene is nonetheless expressed in all above-ground tissues of *N. attenuata.* The question as to why this occurs remains an intriguing question. A comparison of relative transcript abundance in different tissues showed that *Na*ppHS is most highly expressed in young, developing tissues, namely young leaves and flowers (manuscript II). With regard to function, its expression in flowers seemed to be particularly promising, as the relative transcript abundance in various flower organs was found to differ enormously (manuscript III).

Interestingly, glycoproteins containing hydroxyproline-rich amino acid sequences (HRGPs) are associated with reproduction. For example, arabinogalactan proteins (a HRGP subclass) are present in the pistil extracellular matrix of many species and are suggested to play a role in fertilization (Sedgley *et al.*, 1985; Bacic *et al.*, 1988). Other HRGP-like proteins, so-called extensin chimeras, are specifically expressed in *N. tabacum* pistils (Goldman *et al.*, 1992) and are further known to be important for the cell-cell recognition of potential mates in the green alga *Chlamydomonas* (Woessner and Goodenough, 1989). Thus it might be that the structurally similar hydroxyproline-rich

glycopeptides are recognized by related recognition systems in reproductive organs such as pistils, and that HypSys triggers processes involved in reproduction. In agreement with this hypothesis, seed capsule numbers and self-pollination rates are reduced in glasshouse-grown IRsys compared to WT plants; these reductions demonstrate that *Na*ppHS is indeed associated with reproduction (manuscript III). The reductions in self-pollination could not be attributed to impaired pollen or ovule viability because hand-pollinating IRsys plants with their own pollen restored seed production per capsule to levels found in WT plants. Changes in self-pollination rates which are not caused by alterations in pollen or ovule viability are observed in plants with a mixed-mating system. Hermaphrodite plant species with chasmogamous flowers are able to reproduce by self-pollination or out-crossing. Self-pollination promotes reproductive assurance in ephemeral plants growing in disturbed habitats, when opportunities for out-crossing are inadequate or unreliable, but it also generates genetic uniformity in a population. Cross-pollination, on the other hand, increases genetic variation and might contribute to the adaptive ability of plants, such as *N. attenuata*, that grow in exceptionally unpredictable environments.

One factor contributing strongly to the variance in self-pollination rates and the potential for out-crossing events is the distance between stigma and anthers (Carr and Fenster, 1994). Two consecutive field studies in *Datura stramonium*, an annual Solanaceous weed, compared out-crossing rates measured for flowers with various stigma positions in relation to the anthers. In each study, out-crossing rates were positively correlated with protruded stigmas (Motten and Stone, 2000). Reduced self-pollination rates were likewise accompanied by protruded stigmas in IRsys *N. attenuata* plants (manuscript III). The question of whether or not out-crossing is indeed positively correlated to stigma protrusion in *N. attenuata* can only be answered with field experiments, as out-crossing factors like wind or pollinators are missing in the glasshouse. Grounds for speculating about a positive correlation can be found in an observation made in 2006 on field-grown plants. WT and IRsys plants did not significantly differ in their seed capsule production in the presence of natural herbivores and of pollinators which are likely to compensate for decreases in pollen loads and self-pollination rates in IRsys plants (Berger and Baldwin, unpublished data). Hence, we hypothesize that HypSys peptides encoded by *Na*ppHS are factors that control pistil length and thereby change out-crossing rates in response to unfavorable environmental conditions such as herbivory. Variances in pistil length are known from field-grown *N. attenuata* plants attacked by herbivores (André Kessler, unpublished data). Another field study in which paternity was determined for seeds

produced by WT and IRsys plants from UT and AZ accessions in the presence and absence of natural pollinators would clarify the role of NappHS in out-crossing. Moreover, the following questions would be instructive to test under field conditions: Do *N. attenuata* WT flowers with protruded stigmas have suppressed *Na*ppHS transcripts in their pistils? Are protruded stigmas positively correlated with out-crossing rates? And if so, are out-crossing rates correlated to damage caused by herbivores, and do other abiotic and biotic factors play a role as well? Are there differences in out-crossing rates between AZ and UT accessions?

The underlying mechanisms and signaling cascades orchestrating flower development are quite complex, and usually regulated in a tissue- and time-specific manner. It has been demonstrated that jasmonic acid and octadecanoids play a role in flower development (Creelmann and Mullet, 1997; Wasternack and Hause, 2002). Moreover, OPDA, JA, and their derivatives occur in characteristic ratios within distinct flower organs (Hause *et al.*, 2000). Buds and flowers of *Na*ppHS-silenced plants contain less JA und JA-Ile (manuscript III), suggesting that jasmonates are involved in regulating pistil length in *N. attenuata* flowers. Whereas anther development is quite well-known, knowledge of pistil development is rather scarce. Most interesting was the down-regulation of TD (threonine deaminase) transcripts in pistils of IRsys plants, which is likely to contribute to the altered morphology and consequently to changes in self-pollination rates. TD is demonstrated to play a dual role in the defense and development in *N. attenuata* (Kang *et al.*, 2006; Kang PhD thesis, 2006). TD regulates the biosynthesis of isoleucine, which is important for plant growth (Sidorov *et al.*, 1981) and defense (Kang *et al.*, 2006). *Na*ppHS seems to impact jasmonates in a specific spatio-temporal manner. Furthermore, it is likely that signals other than jasmonates regulate pistil length because the WT flower phenotype could not be rescued by the application of jasmonates (manuscript III).

Other hormones such as auxins and ethylene regulate flower development as well. Auxins regulate the development of both anthers and pistils (Cecchetti *et al.*, 2004). The role of ethylene in pistil growth regulation was highlighted by Hibi *et al.* (2007). The TEIL (Tobacco EIN3-Like) gene is a tobacco homologue of the *Arabidopsis* Ethylene Insensitive 3 (EIN3), a gene encoding a key transcriptional factor in ethylene signaling. Over-expression of both genes in the respective species was associated with protruded pistils (Hibi *et al.*, 2007) similar to the flower phenotype found in *Na*ppHS-silenced *N. attenuata* plants (manuscript III). Interestingly, flowers silenced in TEIL also exhibit pistil protrusion, demonstrating that the underlying mechanisms may be complex and related to

differential perception of ethylene rather than increased or reduced sensitivity to this hormone. The answer to whether or not ethylene emission or perception is linked to *Na*ppHS expression in flowers could expand our knowledge of the tissue specificity of plant hormones.

How *Na*ppHS may regulate pistil length and the mating system in *N. attenuata* flowers is presented in Fig. 3.

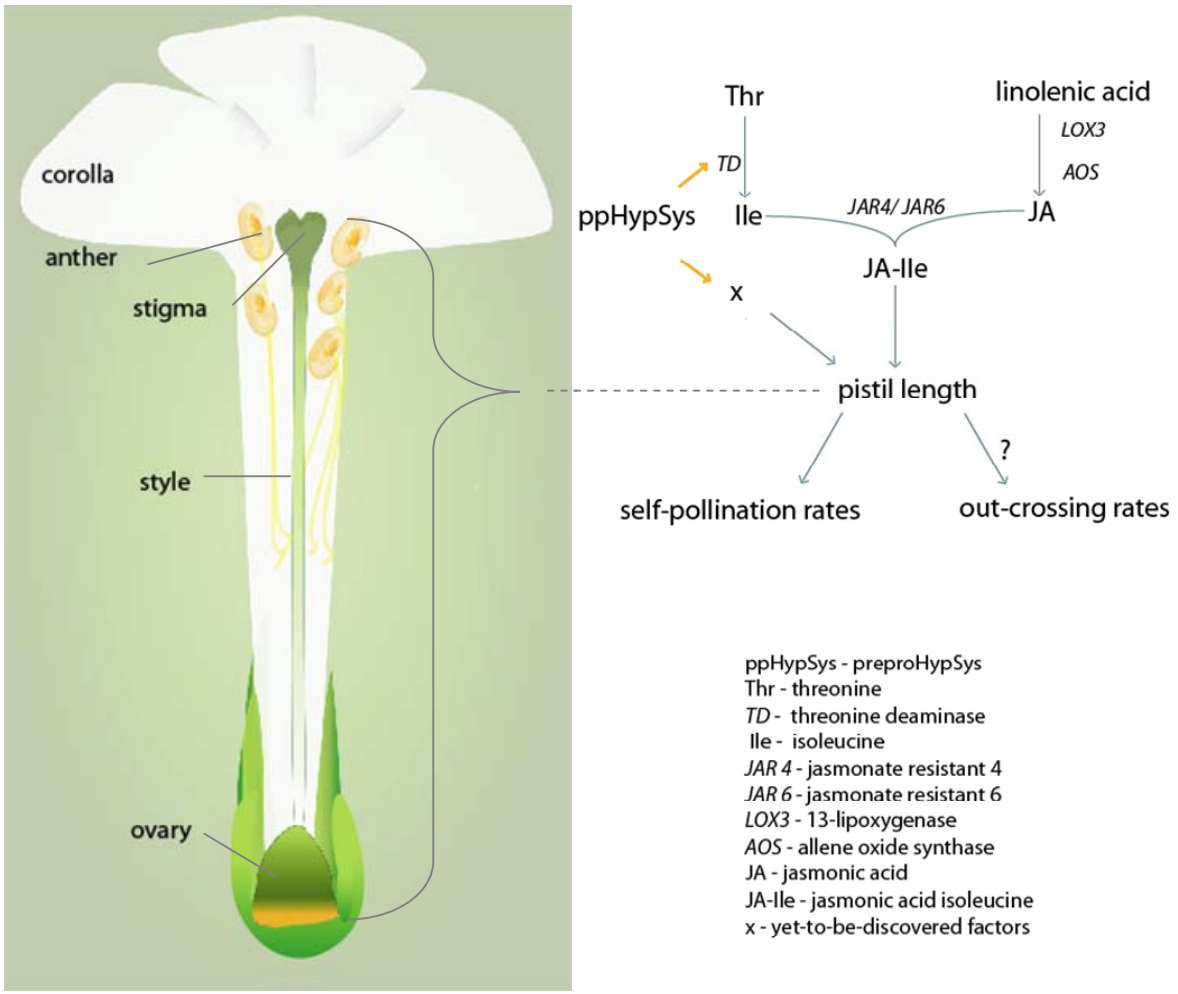


Figure 3: Putative regulation of pistil length and self-pollination rates in *N. attenuata* flowers mediated by *Na*ppHS.

Nicotine, which is regulated by a combination of jasmonic acid and ethylene in *N. attenuata* (Kahl *et al.*, 2000, Winz and Baldwin, 2001; von Dahl *et al.*, 2007), serves not only as foliar defense but also as a nectar defense compound (Kessler and Baldwin, 2007) and might also be spatially affected by *Na*ppHS. In the nectar of *N. attenuata*, nicotine reduces the attraction not only to nectar robbers but also to pollinators. Other compounds present in the nectar and flowers, like the sesquiterpene bergamotene, are highly attractive

to pollinating hawkmoths (Kessler and Baldwin, 2007), demonstrating the importance of nectar composition for pollination rates. Testing whether the nectar composition of IRsys flowers differs from that of WT flowers would be a logical next step given the assumption that IRsys flowers protrude their pistils to increase out-crossing rates. A combination of low nicotine and high bergamotene may help to attract pollinators and thereby contribute to increased out-crossing rates. *Na*ppHS is clearly not necessary for anti-herbivore defense in *N. attenuata* but could be shown to have distinct effects on flower morphology. Even without elucidating all mechanisms behind the changes in flower development, the emerging picture on out-crossing versus inbreeding in this ephemeral species is intriguing. Considering the influence that such a switch in mating strategy could have on evolutionary processes, we have demonstrated an important and novel function for this member of the HypSys peptide family.

4.4. Ongoing characterisation of *ppHypSys* in *Nicotiana attenuata* and relatives

Questions about the source of divergent HypSys functions arise from the observation that these functions do not seem to be conserved in the Solanaceae family. Do functional differences originate solely from sequence differences of the HypSys peptides? Are distinct posttranscriptional modifications and glycosyl residues main players in the recognition by as yet to be discovered receptors? A putative receptor such as the systemin SR160 receptor (Scheer and Ryan, 2002) is not known for HypSys peptides. But multiple proline, hydroxyproline, serine and threonine residues in HypSys peptides provide structural features that may interact with receptors (reviewed in Pearce and Ryan, 2003). Moreover, the rapid medium alkalinization responses of tobacco cell-culture suspensions in response to HypSys peptides (Pearce *et al.*, 2001) is a common response to peptide ligands, suggesting that the activities of these peptides are receptor-mediated. Interestingly, the systemin receptor SR160 of wild tomato (*Lycopersicon peruvianum*) was shown to have high sequence similarity to the BRI1 brassinosteroid receptor from *Arabidopsis* (Scheer and Ryan 2002). This observation stimulated research on the question of whether systemin is involved in the brassinosteroid-mediated regulation of growth and development.

Research on putative receptors and decoration properties of HypSys peptides from *Nicotiana* and other Solanaceous species could provide a deeper insight into the functional distinctiveness of the HypSys peptide family. Thus, the complex variety of HypSys peptides occurring in Solanaceae species stimulates many general plant evolutionary

questions. PreproHypSys genes were cloned from more than 20 different relatives of *N. attenuata* including diploid (i.e. *N. trigonophylla*) and tetraploid (i.e. *N. clevelandii, N. quadrivalis*) species. Conserved amino acid motifs (SPPS) were found in all species (Amy Lawton-Rauh, Jianqiang Wu and Ian T. Baldwin, unpublished data), and it is tempting to speculate about similar roles or binding motifs for putative receptors across the genus *Nicotiana*. Our results reveal that high sequence similarities between HypSys precursors and peptides of closely related species (e.g. between *N. tabacum* and *N. attenuata*) do not guarantee functional similarities (manuscripts II, III). The challenge is now to decode the preproHypSys sequence information obtained from various *Nicotiana* species in terms of putative receptor binding motifs, and/or to identify putative receptors. Knowledge about localization and expression patterns of putative HypSys receptors would accelerate the comprehension of HypSys peptides in *Nicotiana spp.* and subsequently in other members of the Solanaceae family.

4.5. Conclusion

The emerging picture on HypSys peptides in the *Solanaceae* describes a group of peptides that are highly variable in their functionality. In *N. attenuata* HypSys, peptides only marginally modulate phenotypic plasticity in the response to insect attack (manuscript II). This thesis postulates that *Na*ppHS is part of a signaling mechanism which controls self-pollination by regulating pistil length (manuscript III). The involvement of *Na*ppHS in flower development opens up a new branch of functional diversity for oligopeptide signals of the HypSys family. Thus, we are just beginning to understand how *Na*ppHS contributes to the big picture of *N. attenuata's* adaptation to environmental cues.

5. Summary

In plants, internal signaling is essential for the regulation of growth, reproduction and defense responses. The coordination of these functions is indispensable for normal growth as well as for adaptation to a changing environment. The coordination of these signalling cascades is achieved in part through plant hormones, which are generally derived from lipids or sterols. Recently, however, plant peptides with hormone-like signaling functions were described. Systemin, an 18-aa oligopeptide in tomato (*Solanum lycopersicum*), attracted interest as a signal that possibly transmits information from an herbivore-attacked site to distal parts of the plant. When applied to tomato plants, systemin induced the accumulation of proteinase inhibitors (PIs) even in plant parts distant from the application site. However, the idea that systemin itself acts as a mobile signal in anti-herbivore defense was later discarded. In the current model, systemin is instead supposed to amplify a jasmonate-derived mobile signal at the site of wounding. Tobacco (*Nicotiana tabacum*) does not possess a systemin homologue, but responds to wounding or herbivory with the elicitation of PIs. In this genus, hydroxyproline-rich glycopeptides (HypSys) are hypothesized to mediate the induction of PIs. Both systemin in tomato and HypSys in cultivated tobacco are, despite their lack of phylogenetic similarity, reported to fulfil similar functions in defense signaling. Similarities in defense signaling activities of systemin and HypSys have led to the inclusion of both in a functionally defined systemin family.

Nicotiana attenuata, a wild tobacco native to the south-western US, provides an excellent system to study herbivore resistance. This wild tobacco is characterized by natural genotypic variation in defense traits among accessions, typified by differences between two accessions collected in Utah (UT) and Arizona (AZ), respectively. *N. attenuata* is also known to tailor its wound response depending on the identity of the herbivore feeding on it. The present thesis focussed on the technical viability of stable plant transformation and the role of hydroxyproline-rich glycopeptide systemin precursors in *N. attenuata*, an undomesticated model system for herbivore-plant interactions.

I. *Agrobacterium*-mediated transformation can lead to tetraploidy in Arizona-, but not in Utah-accessions

Agrobacterium-mediated transformation, an indispensable tool for the analysis of single genes, may lead to unintended changes in plant ploidy levels. Flow cytometry was adapted to determine post-transformation ploidy levels in UT and AZ transformants. None

of the tested UT transformants but more than half of the AZ transformants were tetraploid, demonstrating that tetraploidization occurred in an accession-dependent manner. Studies on different tomato cultivars have shown that polyploidy in callus regenerants can correlate with polysomaty in the source tissue of the callus culture. The tissue specific distribution of polysomaty in hypocotyls, cotyledons and roots of *N. attenuata* was found to be independent of the accession and had no influence on the occurrence of polyploidy. Rather than differences in polysomaty producing more tetraploids in AZ, a failure of polyploid UT calli to regenerate back into plants may lead to the absence of tetraploid UT transformants. The origin of polyploidy in *N. attenuata* transformants is probably found during callus formation and plant regeneration.

II. *Na*ppHS does not play a central role in the anti-herbivore defense of *N. attenuata*

A comparison of WT and transformed plants, either containing a construct to silence (IRsys), or to constitutively express (OVsys) ppHS in *N. attenuata*, revealed that herbivory-associated signaling [salicylic-acid-induced protein kinase (SIPK) activity, jasmonic acid (JA), JA-isoleucine/leucine (JA-Ile/Leu), and ethylene production or perception] did not differ strongly among the lines. Only JA, JA-Ile/Leu, and ethylene were slightly higher in OVsys plants. Moreover, the performance of *Manduca sexta* larvae did not differ among the lines. OVsys plants suffered marginally less damage from the natural herbivore community. OVsys plants had marginally higher constitutive levels of PIs and nicotine compared to WT plants, while IRsys plants did not differ from WT plants. These differences disappeared after herbivory simulation, as the induced secondary metabolite levels in all three groups did not differ from one another. Similar results were seen in the secondary metabolite profiles of IRsys and OVsys plants transplanted into *N. attenuata´s* native habitat. Therefore we conclude that *Na*ppHypSys does not play a central role in anti-herbivore defense signaling in *N. attenuata*.

III. Silencing *Na*ppHS in two accessions of *N. attenuata* alters flower morphology and rates of self-pollination

IRsys plants of two different *N. attenuata* accessions (UT and AZ) showed reduced seed capsule numbers and seed number per capsule. Seed production per capsule in IRsys lines could be restored to WT levels by hand-pollination, demonstrating that the observed reductions were not due to impaired pollen or ovule viability. A more likely reason is decreased self-pollination efficiency in IRsys plants because flowers of IRsys plants have

pistils that protrude beyond their anthers. Changes in flower morphology are paralleled by reduced JA and JA-Ile/Ile levels at different floral developmental stages. In comparison to the WT, threonine deaminase (TD) transcripts were found to be significantly reduced in pistils of IRsys plants. TD supplies isoleucine for JA-Ile biosynthesis. We propose that *Na*ppHS influences the biosynthesis of JA-Ile during flower development, thereby altering the JA / JA-Ile balance and consequently changing flower morphology. Hence we suggest that the function of *Na*ppHS in *N. attenuata* may have changed during the course of evolution to modulate flower morphology and consequently self-pollination rates.

6. Zusammenfassung

In Pflanzen ist die koordinierte Weiterleitung von Signalen für deren Wachstum, Reproduktion und Abwehr von grosser Bedeutung. Die Weitergabe von Informationen kann dabei innerhalb (lokal), aber auch zwischen Geweben (systemisch) mittels chemischer Signale wie z.B. Phytohormonen erfolgen. Das Interesse an Peptiden mit Signalfunktion wurde durch die Entdeckung von Systemin, einem 18 Aminosäuren langem Oligopeptid aus Tomate geweckt. Systemin galt als möglicher Kandidat für die Rolle der Informationsweiterleitung zwischen verwundeten und unverwundeten Pflanzenteilen in der Tomate, weil es eine Aktivierung von Abwehrgenen wie Proteinase Inhibitoren (PIs) in unverwundeten Pflanzenteilen hervorrufen kann. Das Systemin selbst als systemisches Signal agiert, konnte jedoch nicht bestätigt werden. Seine tatsächliche Wirkung bei der Wundantwort wird vielmehr in einer lokalen Verstärkung eines von Jasmonsäure abgeleiteten Signals gesehen, welches dann zur Aktivierung von PIs in unverwundeten Pflanzenteilen führt. Kultivierter Tabak besitzt kein Systemin, ist aber dennoch in der Lage, auf Verwundung mit der Freisetzung von PIs in unverwundetem Gewebe zu reagieren. Dabei wird eine Induktion des PI Gens durch hydroxyprolinreiche Glykopeptide (HypSys) angenommen. Sowohl Systemin in Tomate als auch HypSys in Tabak haben offenbar trotz ihrer fehlenden phylogenetischen Ähnlichkeit Signalfunktionen in Abwehrprozessen. Deshalb wurden beide Peptide in einer funktionell definierten Systemin Peptidfamilie zusammengefasst.

Gegenstand der vorliegenden Dissertationsschrift war eine Erläuterung der Rolle des hydroxyprolin-reichen systemin-glykopeptide Vorläufers (ppHS) in der herbivorie-induzierten Abwehr und in der Entwicklung des wilden Tabak *Nicotiana attenuata*. *N. attenuata* ist im Great Basin Desert (USA) beheimatet und eignet sich aufgrund seiner Transformierbarkeit und der nahen Verwandtschaft zu Nutzpflanzen hervorragend als Modellsystem für ökologische Studien. Neben dem Einsatz von chemisch-analytischen und ökologischen Methoden spielt bei der Erforschung von spezifischen Aspekten der Abwehr und der Entwicklung von Pflanzen die Verwendung gezielt gentechnisch veränderter Organismen eine bedeutende Rolle. Eine Betrachtung der durch Transformation hervorgerufenen möglichen Veränderungen im Ploidiestatus transgener Pflanzen verschiedener Akkzessionen, die entweder reduzierte (IRsys) oder erhöhte (Ovsys)

Mengen an ppHS Transkripten aufwiesen ging den funktionellen Untersuchungen zu ppHS voraus.

Die in den Manuskripten gewonnen Daten können in folgenden Thesen zusammengefasst werden.

(I) *Agrobacterium* vermittelte Transformation kann zum Auftreten von Tetraploidie in transgenen *N. attenuata* Pflanzen des Genotypes Arizona führen.

- Infolge der *Agrobacterium* vermittelte Transformation kann es zu Veränderungen im Ploidiestatus von transgenen *N. attenuata* Pflanzen kommen. Das Auftreten von Tetraploidie (über 50 %) ist akzessionaspezifisch und begrenzt auf transformierte Pflanzen, die Nachkommen einer aus Arizona stammenden Populationen (AZ) sind. Transformierte Nachkommen von Samensammlungen in Utah (UT) zeigten dagegen keine Veränderungen in ihrem Ploidiestatus.
- Zwischen dem Auftreten von Tetraploidie und Polysomatie des zu transformierenden Gewebes, dem Inzuchtgrad der zu transformierenden Pflanze und dem jeweiligs zur Transformation verwendeten Vektors und Gen konnte kein Zusammenhang nachgewiesen werden.
- Durchflusszytometrie hat sich als eine geeignete Methode zur Bestimmung des Ploidiestatus in transgenen und isogenen *N. attenuata* Pflanzen erwiesen.

(II) Das hydroxyprolinreiche Systemin Glykopeptid und dessen Vorläufer nehmen keine zentrale Stellung in der Abwehr von *N. attenuata* gegen Insekten ein.

- Die Simulation von Herbivorie, Verwundung, oder die Applikation von Methyljasmonat (MeJA) führen in *N. attenuata* nicht zu einer Erhöhung des Gehaltes an hydroxyprolinreichem Systemin-Glykopeptid Vorläufertranskript (*Na*ppHS), dessen Vorkommen jedoch in allen oberirdischen Pflanzengeweben gezeigt werden konnte.
- Die zeitliche, sowie räumliche Ausbreitung des durch Herbivoriesimulation aktivierten „mitogen activated protein kinase“ (MAPK)-Signals erfolgt in *N. attenuata* Blättern unabhänging von endogenen *Na*ppHS Gehalten.

- Die Ethyleneproduktion in induzierten Blättern ist in transgenen Pflanzen mit reduzierten *Na*ppHS Transkripten (IRsys) nicht beeinträchtigt, aber in Pflanzen mit ektopischer *Na*ppHS Expression (Ovsys) gegenüber Wildtyppflanzen (WT) erhöht.
- Jasmonsäure (JA) und Jasmonsäure-isoleucin/leucin (JA-Ile/Leu) zeigen vorübergehende Unterschiede im Anstieg und Abfall nach Verwundung. Die Spitzenwerte, sowie die zeitliche Abfolge der Reaktion sind im Vergleich zwischen WT und transformierten Pflanzen jedoch nicht unterschiedlich.
- Transgene Pflanzen mit verringerten endogenen *Na*ppHS Gehalten weisen keine Beeinträchtigung in ihrer Fähigkeit zur Bildung induzierter Abwehrstoffe wie TPI und Nikotin auf.
- Ektopische Expression von *Na*ppHS führt zu einer konstitutiven Erhöhung von TPI und Nikotin, nicht aber zu einer höheren induzierten Produktion im Vergleich zum WT.
- Der Gehalt an *Na*ppHS in *N. attenuata* nimmt nur geringen Einfluss auf das Wachstum von Larven des Tabakschwärmers *Manduca sexta*, wenn diese an den Pflanzen fressen.
- In Freilandversuchen konnten keine Unterschiede im Frasschaden durch natürlich vorkommendende Insekten zwischen WT und IRsys Pflanzen nachgewiesen werden, wohingegen die ektopische Expression des Genes zu geringfügig weniger Frasschaden führte.

(III) Die Verringerung der endogenen Konzentration an *Na*ppHS führt zu Veränderungen in der Blütenmorphologie und zu reduzierten Selbstbestäubungsraten.

- Die Reduktion von *Na*ppHS Transkripten geht in *N. attenuata* mit einer Abnahme der Menge gebildeter Samenkapseln, sowie der Menge gebildeter Samen pro Kapsel einher. Unterschiede im Längenwachstum, sowie der Blütenanzahl waren nicht erkennbar.
- Handbestäubung der Blüten führt unabhänging vom *Na*ppHS Gehalt der Pflanze zur Ausbildung von Kapseln mit vergleichenbaren Samenzahlen. Somit sind die grundsätzlichen Funktionen der Blütenorgane nicht beeinträchtigt.
- Die grössten Mengen an *Na*ppHS Transkripten konnten in weiblichen Blütenorganen nachgewiesen werden.

- *Na*ppHS beeinflusst die Blütenmorphologie, denn Pflanzen mit reduzierten *Na*ppHS Transkripten zeichnen sich durch ein im Vergleich zu den Antheren deutlich verlängertes Pistill aus.
- Blütenknospen und Blüten von Pflanzen mit reduziertem ppHS Transkripten enthalten geringere Mengen an JA und JA-Ile/Leu.
- Die Expression von Threonine-desaminase, einem Gen das in die JA-Ile/Leu Produktion involviert ist, ist nur in den weiblichen Blütenteilen transgener Pflanzen reduziert.
- *Na*ppHS beeinflusst unmittelbar die Pistill-Länge und hierdurch mittelbar die Selbstbestäubungsraten von *N. attenuata*. Dies könnte einen wichtigen Schalter zwischen Selbst- und Fremdbestäubung in der Fortpflanzungsstrategie des wilden Tabaks darstellen.

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8. Selbständigkeitserklärung

Entsprechend der Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität (Nr. III, § 5, Ziff. 3) erkläre ich, dass mir die geltende Promotionsordnung der Fakultät bekannt ist. Die vorliegende Arbeit habe ich selbständig und nur unter der Verwendung der angegebenen Hilfsmittel, persönlichen Mitteilungen, Quellen und Literatur angefertigt habe.
Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen. Die vorliegende Arbeit wurde weder an der Friedrich-Schiller-Universität Jena, noch an einer anderen Hochschule als Dissertation eingereicht.

Beatrice Berger

9. Curriculum vitae

Personal Data

Name	Beatrice Berger
Date of Birth	December 30th, 1976
Place of Birth	Jena
Nationality	German

Education and Study

Oct. 1996-Nov. 2002	Diploma in Nutritional Sciences (Dipl. Trophologin), Friedrich-Schiller-Universität, Jena
Sept. 1995-Sept. 1996	Business studies/ Intercultural Management, Friedrich-Schiller-Universität, Jena
Sep. 1991-July 1995	High School Diploma, Staatliches Gymnasium Kahla, Kahla
Sep. 1983-July 1991	Polytechnische Oberschule ”Arthur-Becker”, Rothenstein

Professional and Scientific Career

Jun. 2003-present	Ph.D. thesis at Max Planck Institute for Chemical Ecology, Department of Molecular Ecology, Jena
Nov. 2002-Jun. 2003	Research assistant at Max Planck Institute for Chemical Ecology, Department of Molecular Ecology, Jena
Jan 2002-Nov. 2002	Diploma thesis at the Federal Institute for Agriculture, Department of Animal Nutrition, Braunschweig; “DNA-Abbau während der Rapsölgewinnung aus transgenem und isogenem Raps – Einfluss verschiedener Prozessbedingungen“

Practical training

May 1999	Internship at rehab hospital for oncology and rheumatology - nutrition counseling, Weissen, Germany
June 1999	Internship at TVL-thurigian-central laboratory for milk quality testing, Jena, Germany
August 1999	Willing worker on organic farms (WWOOF) – Reute, Austria
Sept. 1999-Oct. 1999	Internship at Zomba Central Hospital – nutrition ward, Zomba, Malawi
Oct. 2000	Willing worker on organic farms - Lanjaron, Spain

Publications

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

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Berger B., Aulrich K., Fleck, G., Flachowsky G. (2003) Influence of processing of isogenic and transgenic rapeseed on DNA-degradation. Proceedings of the Society of Nutrition Physiology, Band **12**, 108

Oral and poster presentations

Berger B. & Baldwin I.T. (2007) Prosystemin in *Nicotiana attenuata*: Defense or development? *3rd Plant Science Student Conference 2007*/ Leibniz Insitute of Plant Biochemistry, Halle, **poster**

Berger B. & Baldwin I.T. (2005) : Feind im Anmarsch! Systemin - Signal zum Gegenangriff der Pflanze?! *ICE Symposium/ MPI for Chemical Ecology*, Jena, **talk**

Berger B., Aulrich K., Fleck, G., Flachowsky G. (2003) Influence of processing of isogenic and transgenic rapeseed on DNA-degradation. *57. Tagung der Gesellschaft für Ernährungsphysiologie*, Göttingen, **poster**

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