

**Responses of Black Nightshade (*Solanum nigrum*) to insect
herbivory with a special focus on the 18-amino acid polypeptide
systemin**

Dissertation
zur Erlangung des akademischen Grades
doctor rerum naturalium (Dr. rer. nat)

vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät
der
Friedrich-Schiller-Universität Jena

Von Diplom-Biologin
Silvia Schmidt

geboren am 17. Juni 1977 in Neunkirchen

Gutachter

Prof. Dr. Ian T. Baldwin (Max-Planck-Institut für Chemische Ökologie, Jena, Deutschland)

Prof. Dr. Erika Kothe (Friedrich-Schiller-Universität Jena, Deutschland)

Prof. Dr. Clarence A. Ryan (Washington State University, Pullman, USA)

Tag der Doktorprüfung: 22.10.2007

Tag der öffentlichen Verteidigung: 16.11.2007

Contents

1 General Introduction.....	1
2 Thesis outline - List of manuscripts and authors' contribution.....	11
3 Manuscripts	15
Manuscript I	
<i>Solanum nigrum – a model ecological expression system</i>	15
Manuscript II	
<i>Solanaceous responses to herbivory</i>	40
Manuscript III	
<i>Systemin does not mediate direct resistance in S. nigrum</i>	61
Manuscript IV	
<i>Down-regulation of systemin is associated with root growth in S. nigrum</i>	80
4 General Discussion.....	94
5 Summary.....	103
6 Zusammenfassung.....	106
7 References.....	110
8 Acknowledgments.....	127
9 Eigenständigkeitserklärung.....	129
10 Curriculum vitae.....	130

1 General Introduction



itness, defined in the classical ecological sense, is a measure of an individual's reproductive success or its success in passing its genes on to future generations. Estimated relative to the reproductive output of other genotypes in the same environment, fitness is the ultimate cause of an organism's evolutionary success. The frequency with which an individual's genotype is represented in the gene pool of the next generation is supposed to be the product of the individual's survival and fecundity. Maximizing fitness by increasing both lifetime and fecundity should therefore be adaptively favored by natural selection. Consequently, unfavorable conditions that either reduce the possibility of survival or decrease the fecundity of an individual impair its fitness. The spectrum of unfavorable conditions is multifarious, spanning disadvantageous environmental factors, poor nutrition, competition, diseases, pathogens, predators or herbivores. In order to counteract such fitness-imperiling stresses, organisms are able to respond behaviorally, morphologically or physiologically. Such adaptive traits can either be constitutively manifested or expressed only when actually needed. The latter, known as phenotypic plasticity, describes the ability of an organism with a given genotype to change its phenotype in response to environmental changes.

For plants, the attack of phytophageous insects can have detrimental effects on fitness, which explains the enormous variety of adaptations plants have against insect herbivores. Plant defenses against herbivory are generally classified into two main groups: resistance and tolerance.

1.1 Resistance against insect-herbivory

Resistance is commonly defined as any plant trait that reduces the preference or performance of herbivores, thereby limiting the amount of damage a plant incurs (Strauss, Watson & Allen 2003). Constitutive traits offer plants the potential to keep herbivores away, whereas induced traits generally reduce the performance of attacking insects. Both constitutive as well as induced mechanisms can be direct or indirect, resulting in four defined categories which are commonly used to group resistance mechanisms of plants against insect herbivores.

Typical examples of direct constitutive resistance traits are trichomes (uni- or multi-cellular epidermal outgrowths), thorns (sharp outgrowth from a stem other than at a node), spines (modified stipules or sharp branchlets found in a leaf axil or on the margin of a leaf) or leaf waxes. By involving a third interaction partner, plants have evolved indirect constitutive resistance mechanisms. Central American *Acacia* species, for example, are known to be kept free from herbivores by ants of the genus *Pseudomyrmex*. As a reward for this service, the plants provide the ants with nesting space in their hollow spines as well as with food, which is offered as specialized lipid-rich cells called Beltian bodies at the tips of the leaflets.

In contrast to these constitutively expressed resistance traits, induced resistance responses are activated upon attack by an herbivorous insect. A well-studied direct induced resistance trait is the production of protease inhibitors. These proteins are able to deactivate both endo- and exopeptidases including proteolytic digestive enzymes of the phytophage, thereby decreasing the digestibility of the ingested food (Ryan 1990; Jongsma & Bolter 1997). Herbivores feeding on transgenic *Nicotiana attenuata* plants silenced in the expression of protease inhibitors were shown to grow faster and having a higher survivorship than those feeding on untransformed wild-type plants (Zavala *et al.* 2004). Consequently, the induction of protease inhibitors may reduce the herbivore's growth, most likely resulting in less plant damage. Indirect induced resistance mechanisms again involve a third interaction partner. The inducible extrafloral nectar (EFN) of Lima bean (*Phaseolus lunatus*), for example, has been recently reported to attract ants, wasps and flies. The increased presence of these insects reduced the amount of leaf damage of plants in which EFN availability was experimentally increased (Kost & Heil 2005). Likewise, the emission of volatile organic compounds (VOCs) following herbivory attracts arthropod predators (Kessler & Baldwin 2001) and parasitoids (Van Poecke, Posthumus & Dicke 2001), which may kill the herbivore and thereby limit damage to the plant.

1.2 Tolerance to insect herbivory

In contrast to resistance, tolerance mechanisms -- defined as all plant characteristics that reduce the detrimental effects of herbivore damage on plant fitness without affecting the herbivore (Tiffin 2000) -- have been largely neglected. Generally, tolerance is expressed as the degree to which plant fitness is affected by herbivore damage relative to the individual's fitness in the undamaged state (Strauss & Agrawal 1999). As a consequence, tolerance can

only be estimated from a group of related plants as it is not possible to examine the fitness of an individual in both damaged and undamaged states. When damage levels are continuous, tolerance is measured as the slope of the linear regression between plant fitness and damage levels (Fig. 1). If the slope is 0, the plant is able to fully compensate for the damage (C). In

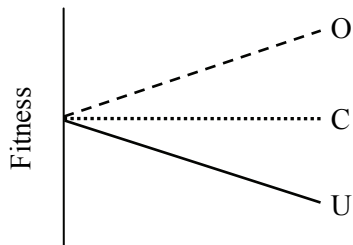


Figure 1: Reaction norm approach for depicting the degree to which plant fitness is affected by herbivore damage.
 O = overcompensation
 C = compensation
 U = undercompensation

cases where the slope is > 0 , plants are overcompensating for herbivory (O); if the slope is < 0 , undercompensation (U), meaning no tolerance, occurs. Tolerance mechanisms result from the interaction of traits which are either genetically or developmentally determined (i.e. intrinsic factors) with environmental characteristics (extrinsic factors) such as the availability of resources to support regrowth (Rosenthal & Kotanen 1994). Among the intrinsic factors is the increased photosynthetic activity in remaining tissues after partial defoliation, which however, seems to be unaffected by leaf miners or phloem sap suckers (Welter 1989).

Intriguingly, increased photosynthesis may also be necessary to support induced resistance traits (Karban & Baldwin 1997). Furthermore, herbivore damage may result in compensatory growth and activation of dormant meristems (McNaughton 1979; Paige & Whitham 1987), thereby allowing the plant to replace some or all tissues removed by the herbivore. Moreover, some plant species seem to be able to escape from herbivory by allocating increasing resources to the roots while aboveground herbivores are present, thereby positively affecting their root-shoot ratio. These belowground reserves allow the plant to regrow when the attack has ceased (Van der Meijden, Wijn & Verkaar 1988; Schwachtje *et al.* 2006). Alternatively, plants may respond phenologically (Marquis 1988), delaying their growth and reproduction when partially defoliated or damaged by herbivores.

Besides the given characteristics that only occur after herbivore damage and are thus classified as induced traits, tolerance like resistance can also result from constitutive mechanisms. Individuals with constitutively high root masses in relation to their shoot masses may be more tolerant as they already have the foundation for acquiring more nutrients to regrow and compensate for tissue losses. Alternatively, as photosynthetic activity of reproductive structures may commonly contribute more than 20% of the carbon needs of

developing fruits and seeds (Bazzaz, Carlson & Harper 1979), genotypes with a higher proportion of photosynthetic surfaces in stems and fruits may be less dependant on photoassimilates provided by leaves and thus more tolerant of folivory (Tiffin 2000). Any discussion of tolerance should include the possible interaction and interdependence of the different mechanisms and be aware of the fact that different kinds of damage can result in disparate tolerance responses even in the same species (Sadras 1996; Rosenheim *et al.* 1997). However, one may generalize tolerance as the capacity of a plant to regrow and reproduce despite or after herbivory.

1.3 Elicitation of induced defense responses

While tolerance in plants remains nearly uncharacterized on the molecular level, the signals and signal pathways leading to herbivore resistance have been extensively studied. Induced resistance responses are generally initiated by primary wound signals such as mechanical tissue damage and the introduction of the herbivore's oral secretions into the site of wounding.

Mechanical damage has been demonstrated to lead to much weaker emissions of volatile organic compounds (VOCs) than damage by herbivores (Mattiacci, Dicke & Posthumus 1994; Paré & Tumlinson 1997). One hypothesis for this phenomenon is that plants are able to discriminate between a single wounding event as it is usually performed in such comparative studies and the continuous feeding of an herbivore. Using an artificial caterpillar (MecWorm), Mithöfer *et al.* (2005) were able to mimic the time and leaf area of herbivore-caused tissue damage sufficiently to induce the emission of VOCs qualitatively similar to those known to be induced by real herbivores. In tomato (*Solanum lycopersicum*), mechanical tissue damage has been shown to systemically induce the expression of systemin (McGurl *et al.* 1992), a polypeptide exclusively found in Solanaceae. Systemin, which has been proposed to be one of the early signals that play a central role in tomato, has received exceeding attention; however, to date it has been mainly studied in crop plants.

Regarding the second primary wound signal, the introduction of the herbivore's oral secretions into the site of wounding, constituents of the oral secretions of *Pieris brassicae* and *Spodoptera exigua* - β -glucosidase and volicitin - have been demonstrated to elicit the release of parasitoid-attracting volatile organic compounds (Mattiacci, Dicke & Posthumus 1995; Alborn *et al.* 1997). Similarly, all measured direct and indirect resistance responses of wild

tobacco (*Nicotiana attenuata*) can be attributed to the two most abundant fatty-acid amino-acid conjugates present in the oral secretions of the tobacco hornworm *Manduca sexta* (Roda *et al.* 2004). Recently, Maischak *et al.* (2007) were able to show that oral secretions of eight lepidopteran larvae exhibit ion channel-forming activities, presumably leading to intracellular calcium influx and depolarization of the cell membrane, both of which considered secondary signals of the plant.

Such secondary signals are commonly generated after the primary signals (tissue damage and the introduction of the herbivore's oral secretions into the site of wounding) have been perceived at the outer membranes of the damaged cell layers (Zimmermann *et al.* 1999; Maffei *et al.* 2004). Other secondary signals suggested to be involved in the induction of induced resistance responses are reactive oxygen species (Orozco-Cardenas, Narvaez-Vasquez & Ryan 2001) and the activation of kinase cascades (Kodama *et al.* 2000). The orchestration of these secondary wound signals activates the octadecanoid-pathway via a yet to be fully elucidated interaction (Fig. 2). Starting with the release of linolenic acid from the cell membrane, this lipid-based pathway produces the plant hormone jasmonic acid (JA), which together with its derivatives, represents the best characterized class of signals mediating direct and indirect resistance responses to wounding and herbivory (reviewed in (Halitschke & Baldwin 2004). By silencing the expression of the *lipoxygenase3* (*lox3*) gene in wild tobacco (*Nicotiana attenuata*), Kessler *et al.* (2004) were able to elegantly demonstrate that plants impaired in the production of JA are more susceptible to herbivores and even attract novel herbivore species, highlighting the key role of the octadecanoid pathway in the regulation of plants' anti-herbivore resistance responses.

Even though these signaling pathways occur locally in the damaged tissue, plants are well known to also show resistance responses to herbivory in distal, undamaged leaves. The signals mediating these so-called systemic responses have been extensively studied.

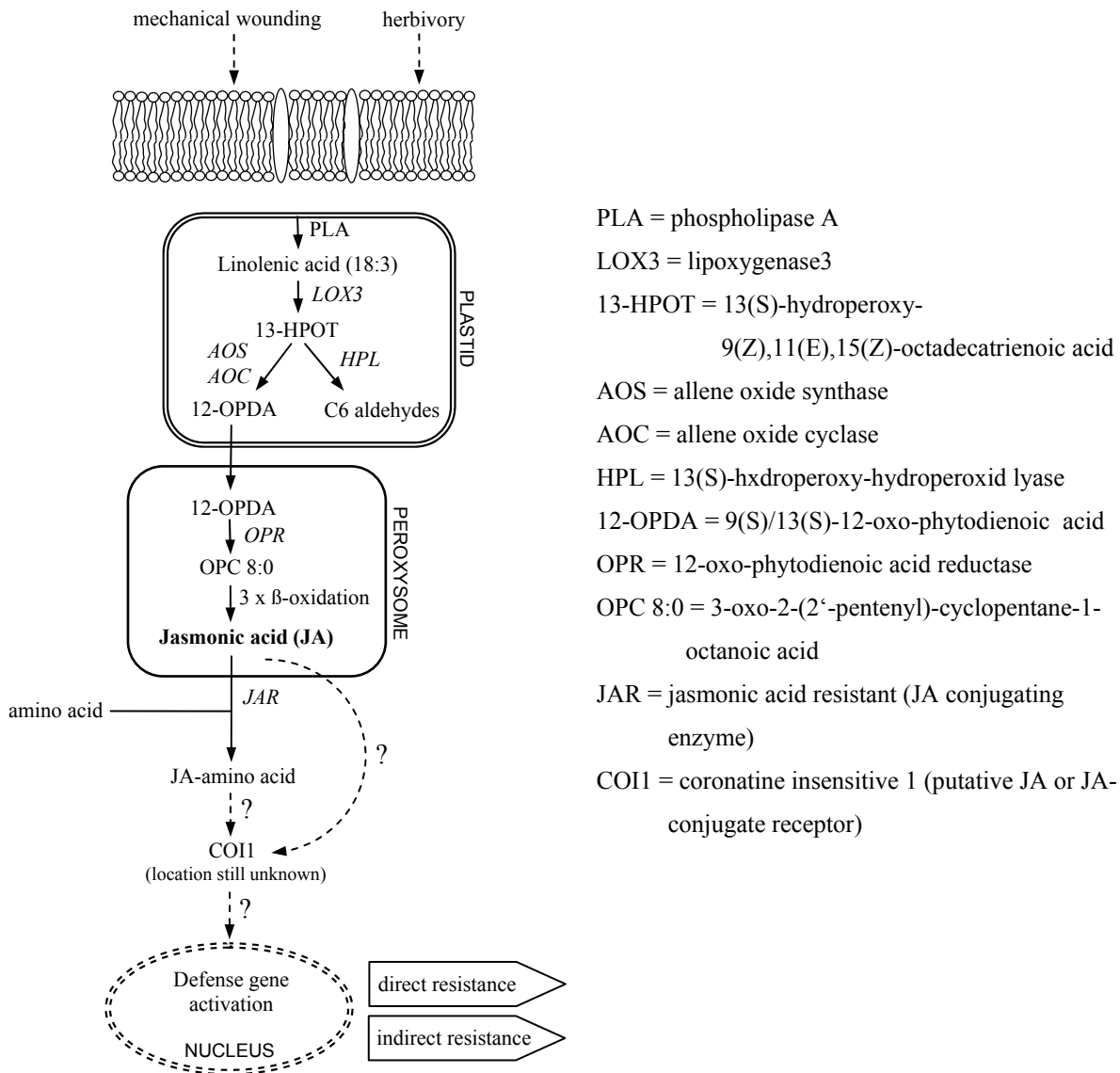


Figure 2: Schematic overview over the octadecanoid pathway including proposed signaling steps downstream of jasmonic acid which are currently under investigation in several plant species.

1.4 Systemin(s) and the systemic wound response: a little history

A multitude of different signals has been proposed as capable of transmitting the information from herbivore attack from the site of wounding to the rest of the plant. Among them are electrical impulses (Chessin & Zipf 1990), oligosaccharide fragments of damaged plant cell walls (Ryan 1987), chitin and chitosan fragments from fungal cell walls (Walker-Simmons, Hadwiger & Ryan 1983), jasmonic acid and its derivatives (Farmer & Ryan 1990), the plant hormones salicylic acid and abscisic acid (Doherty, Selvendran & Bowles 1988; Pena-Cortez *et al.* 1989) as well as systemin, which has been studied in the most detail.

Systemin, an 18-amino acid polypeptide, was first isolated and purified from tomato leaf extracts by Ryan and coworkers; they demonstrated its mobility in the phloem by treating fresh wounds of tomato plants with ^{14}C -labeled synthetic systemin (Pearce *et al.* 1991). The mature peptide is processed from its larger precursor prosystemin, which is synthesized and processed in the vascular phloem parenchyma cells (Narvaez-Vasquez & Ryan 2004). Scheer and Ryan (1999; 2002) purified and identified a 160 kDa leucine-rich repeat receptor-like kinase (LRR-RLK) from wild tomato (*Lycopersicon peruvianum*) to be the systemin receptor (SR160). Astonishingly, cloning revealed that it was homologous to the brassinosteroid receptor BRI1 (Scheer & Ryan 2002). Wounding tomato leaves systemically induced prosystemin mRNA as well as proteinase inhibitor (PI) I mRNA (McGurl *et al.* 1992), and supplying young tomato plants with low concentrations of systemin through their cut stems led to the accumulation of PI I and PI II (Pearce *et al.* 1991). Supporting the positive correlation between systemin and PIs, the systemic induction of PI I and II has been shown to be almost completely suppressed in transgenic tomato plants silenced for the expression of prosystemin (McGurl *et al.* 1992). As a consequence, *Manduca sexta* larvae consumed more leaf material and gained three times more weight when grown on plants silenced in their prosystemin expression as compared to wild-type tomato plants (Orozco-Cardenas, McGurl & Ryan 1993). Transgenic plants overexpressing the prosystemin gene constitutively produced PI I and II proteins and accumulated more PIs in local and systemic leaves after wounding than did wild-type plants (McGurl *et al.* 1994). Grafting experiments using prosystemin-overexpressers as root stocks led to the constitutive production of PIs in wild-type scions (McGurl *et al.* 1994), supporting former observations suggesting that systemin was the mobile wound signal. The situation changed when Howe and Schilmiller (2004; 2005) reciprocally grafted wild-type tomato plants and jasmonic acid biosynthesis mutants (*spr2* mutants) or wild-type tomato plants and systemin signaling mutants (*spr1* mutants). With these elegant experiments they could show that both JA biosynthesis and the presence of systemin are needed in the local, damaged leaf to produce a systemic signal and hence to induce PIs in distal, unwounded leaves. Moreover, neither JA biosynthesis nor systemin seemed to be required in undamaged leaves to produce PIs. With these findings the role of systemin in the wound response of tomato had to be reconsidered; according to the current model, systemin acts at or near the site of wounding by amplifying the JA-derived mobile wound signal. Evidence that systemin acts upstream of JA, at the top of the octadecanoid

pathway, as proposed earlier by Farmer and Ryan (Farmer & Ryan 1992) is given by Chen and Stenzel (2003; 2006). They reported higher constitutive as well as induced JA-levels in leaves of plants overexpressing prosystemin compared to wild-type plants.

The grafting experiments performed by Howe and Schilmiller (2004; 2005) mentioned earlier strongly suggest that JA or a related compound is the systemic wound signal; the location of OPDA biosynthesis enzymes in vascular bundles as well as the preferential formation of JA in the vasculature supports such a hypothesis (Hause *et al.* 2000; Hause *et al.* 2003; Stenzel *et al.* 2003). Furthermore, intact phloem is required for a systemic wound response and the timing of the systemic response fits the rate of transport occurring in the phloem (Schilmiller & Howe 2005). Recently, JA-isoleucine has been shown to play an important role in the defense response of *Nicotiana attenuata* to herbivory by activating the production of the most effective direct resistance traits, PIs and nicotine (Kang *et al.* 2006). As these findings are supported by the work of Wang *et al.* (2007), who demonstrated three JA-amino acid conjugates formed by the action of JAR 4 and JAR 6 to induce PIs in *Nicotiana attenuata*, it is tempting to speculate that one of these conjugates might be the actual systemic wound signal.

Besides the ‘classical’, proline-rich tomato systemin described by Pearce *et al.* (1991), three other hydroxyproline-rich glycopeptides have been isolated so far from tomato (TomHypSys I, II and III) and described to induce the synthesis of PI proteins (Pearce & Ryan 2003). Interestingly, all three peptides are derived from the same, wound-inducible precursor, which is supposed to be synthesized through the secretory pathway, in which it is hydroxylated and glycosylated. The amino acid sequence of this precursor exhibited weak identity to the precursor of two hydroxyproline-rich glycopeptides found in tobacco plants (TobHypSys I and II), which are likewise potent inducers of PIs (Pearce *et al.* 2001) and therefore included in the systemin family, which is a functionally defined family of peptide signals that regulate defensive genes in solanaceous species (Ryan & Pearce 2003). A homolog to the tobacco precursor has recently been described in wild tobacco (*Nicotiana attenuata*) by Berger & Baldwin (2007), and the two encoded hydroxyproline-rich glycopeptides (NaHypSys I and II) were shown not to play a central role in the plant’s anti-herbivore defense response. Also quite recently, Ryan and coworkers discovered a wound-inducible homolog of the tomato and tobacco HypSys precursors in *Petunia hybrida*, which contains three hydroxyproline-rich glycopeptides (Pearce *et al.* 2007). Intriguingly, the

peptides do not induce resistance responses to herbivores but could be shown to activate the expression of *defensin I*, a gene known to be involved in defense responses to pathogens. A similar response has been observed in *Arabidopsis thaliana*, where a peptide called *AtPep 1*, which is derived from an inducible precursor, has been isolated (Huffaker, Pearce & Ryan 2006).

Homologs of the ‘classical’, proline-rich tomato systemin have been described in three closely related species of the Solanaceae family: bell pepper, *Capsicum annum*; potato, *Solanum tuberosum*; and black nightshade, *Solanum nigrum* (Constabel, Yip & Ryan 1998).

1.5 Black nightshade (*Solanum nigrum*) as a model plant system

The black nightshades form a complex group of plants in the section *Solanum* of the genus *Solanum* that are still not completely resolved taxonomically (Defelice 2003). As the section *Solanum* centering around *Solanum nigrum* L. is one of the largest and most variable species groups of the genus, it is often referred to as the *Solanum nigrum* complex. Causes of this taxonomic complexity may be the phenotypic plasticity of the *Solanum* species (particularly its vegetative features), floral and vegetative genetic variations, polyploidy levels ranging from diploid to octoploid, as well as inter- and intraspecific hybridization (Edmonds & Chweya 1997). Although black nightshades occur on most continents, the center of diversity appears to be South America. Interestingly, *Solanum* species native to South America are diploid, whereas all polyploid species are introduced. Conversely, species native to the old world are tetra- and hexaploid, and all diploids are introduced.

The hexaploid species *Solanum nigrum* has been suggested to genetically originate from an allopolyploidy event involving the tetraploid *S. villosum* and the diploid *S. americanum* or conspecific taxa (Edmonds 1979). *S. nigrum*, originally distributed throughout Eurasia, was introduced to North America, Australia, and New Zealand. A herbaceous plant, it prefers moist environments and dry areas where crops are under irrigation, growing as a pioneer plant in open woodlands, waste areas, rubbish dumps, gardens, and cultivated fields (Edmonds 1979; Edmonds & Chweya 1997).

Resistance responses to insect herbivory and the underlying signal pathways have been nearly exclusively studied in crop plants such as tomato. An important question arising from this fact is whether the findings also apply to related but undomesticated species such as *Solanum nigrum*. *S. nigrum* may be regarded as a model system for studying plant-insect

interactions focusing on the plant's responses to insect herbivory on the basis of two main characteristics: First, as a wild species it has never been cultivated and thus still exhibits its natural responses to herbivores, which is not the case for tomato and potato. Second, it belongs to the same genus as potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) for which several molecular tools such as microarrays or transformation systems to silence the gene expression are readily available. This phylogenetic proximity most likely allows the adoption of the mentioned methods to study *S. nigrum* and its interaction with its biotic and abiotic environment.

1.6. Aim of this thesis

To address the question of whether the mainly crop-plant-based findings regarding herbivory resistance also apply to an undomesticated species, the responses of *Solanum nigrum* to attack by phytophagous insects should be studied. As systemin, of which a homolog has been found in *S. nigrum*, is known to play a central role in the wound response of tomato, the function of this polypeptide should be elucidated in particular. The following questions are addressed on detail:

- (1) What are the natural herbivores of *S. nigrum* in the field and is it possible to effectively study their interactions by developing and adopting molecular tools, thus establishing *S. nigrum* as a model plant system (manuscript I)?
- (2) How does *S. nigrum* respond to herbivory by a solanaceous specialist and is the resulting transcriptional pattern comparable to that of another solanaceous species (manuscript II)?
- (3) Does systemin play a crucial role in *S. nigrum* by mediating the plant's direct resistance responses as it does in tomato (manuscript III)? And if not,
- (4) What is the actual role of systemin in *S. nigrum* (manuscript IV)?

2 Thesis outline – List of manuscripts and authors' contribution

Manuscript I

Solanum nigrum: A model ecological expression system and its tools

Dominik D. Schmidt, André Kessler, Danny Kessler, Silvia Schmidt, Michelle Lim,
Klaus Gase and Ian T. Baldwin

Molecular Ecology (2004) 13, 981-995

This manuscript describes the establishment of Black Nightshade (*Solanum nigrum*) as a model system to study plant-insect interactions. By developing new tools and adopting tools that have previously been established for other solanaceous species to quantify and manipulate the responses of *S. nigrum* to herbivory, this manuscript sets the methodological stage for all further studies of this thesis.

Dominik D. Schmidt was responsible for the planning, realization and analysis of the experimental work. The field experiments were supported by André Kessler and Danny Kessler who primarily adapted the volatile measurements to *S. nigrum* and identified the insect herbivores. I optimized a Southern Blot procedure to characterize the asRuBPCase lines by checking for the number of inserted transgenes. The transgenic lines were generated by Michelle Lim who transformed *S. nigrum* with the pRESC2RUB vector which was provided by Klaus Gase. The manuscript was written by Dominik D. Schmidt, optimized after suggestions of Ian T. Baldwin.

Manuscript II

Specificity in ecological Interactions. Attack from the same lepidopteran herbivore results in species-specific transcriptional responses in two solanaceous host plants

Dominik D. Schmidt, Claudia Voelckel, Markus Hartl, Silvia Schmidt, and Ian T. Baldwin

Plant Physiology (2005) 138, 1763-1773

In this manuscript, the transcriptional responses of *Solanum nigrum* and *Nicotiana attenuata* to the attack by leaf-chewing *Manduca sexta* larvae are compared by means of a 10k-cDNA microarray. Despite some commonly regulated genes involved in resistance against herbivores, the responses of both species were quantitatively and qualitatively distinct from one another and exceeded the anticipated differences in alkaloid biosynthesis.

Dominik D. Schmidt and Claudia Voelckel were responsible for the planning, realization and analysis of the *Manduca sexta* experiments. Markus Hartl and me conducted and analyzed the methyl jasmonate experiments. The writing of the manuscript was a joint effort of Dominik D. Schmidt and Claudia Voelckel; I contributed to the materials and methods section (MeJA treatment). The first draft of the article was refined after suggestions of Ian T. Baldwin.

Manuscript III

Systemin in *Solanum nigrum*. The tomato-homologous polypeptide does not mediate direct defense responses

Silvia Schmidt and Ian T. Baldwin

Plant Physiology (2006) 142, 1751-1758

In this manuscript the role of the 18-amino acid polypeptide systemin in the defense response of *Solanum nigrum* against herbivory is studied. In field and glasshouse experiments, wild-type (WT) plants were compared to transgenic plants transformed with an inverted repeat prosystemin construct (IRSys) to silence the expression of the endogenous *S. nigrum* prosystemin gene. Neither the accumulation of proteinase inhibitors as a direct defense mechanism, nor the performance of herbivores or the levels of elicited jasmonic acid levels differed between WT and IRSys plants. Thus, we concluded that the tomato-homologous polypeptide does not mediate direct defense responses in *S. nigrum*.

I planned and realized all field and glasshouse experiments. The data evaluation including the statistical analyses was done by me. The manuscript was written by me and optimized after suggestions of Ian T. Baldwin.

Manuscript IV

Down-regulation of systemin after herbivory is associated with increased root allocation and competitive ability in *Solanum nigrum*

Silvia Schmidt and Ian T. Baldwin

Submitted to *Functional Ecology* (date of submission: 09/08/2007)

In this manuscript, the hypothesis that the down-regulation of systemin after elicitation helps the plant to tolerate rather than resist herbivory was examined. Growth experiments revealed that both elicited wild-type (WT) plants and transgenic plants silenced in prosystemin expression (IRSys) had significantly more root mass than untreated WTs. IRSys plants produced significantly more berries than did WT competitors. Berry production of elicited and unelicited WT plants did not differ, but when elicited WTs were additionally treated with systemin, plants produced fewer berries than did unelicited WT competitors. We proposed that the rapid down-regulation of systemin after herbivory is associated with increased root allocation which allows plants to more effectively compete with conspecifics and may allow plants to compensate for tissue losses during herbivory

In agreement with Ian T. Baldwin I planned and realized the experiments. I did the data analyses and wrote the manuscript, which was refined after suggestions of Ian T. Baldwin.

Manuscript I

***Solanum nigrum*: a model ecological expression system and its tools**

Dominik D. Schmidt, André Kessler, Danny Kessler, Silvia Schmidt, Michelle Lim, Klaus Gase and Ian T. Baldwin*

Molecular Ecology (2004) 13: 981-995

Max Planck Institute for Chemical Ecology
Department of Molecular Ecology
Beutenberg Campus
Hans-Knoell-Str. 8
D-07745 Jena, Germany

* Corresponding author:

Ian T. Baldwin
Max Planck Institute for Chemical Ecology
Department Molecular Ecology
Beutenberg Campus
Hans-Knoell-Str. 8
D-07745 Jena, Germany
Phone: ++ 49-3641-571100
Fax: ++ 49-3641-571102
E-mail: baldwin@ice.mpg.de

Abstract

Plants respond to environmental stresses through a series of complicated phenotypic responses, which can be understood only with field studies because other organisms must be recruited for their function. If ecologists are to fully participate in the genomics revolution and if molecular biologists are to understand adaptive phenotypic responses, native plant ecological expression systems that offer both molecular tools and interesting natural histories are needed. Here, we present *Solanum nigrum* L., a Solanaceous relative of potato and tomato for which many genomic tools are being developed, as a model plant ecological expression system. To facilitate manipulative ecological studies with *S. nigrum*, we describe: (i) an *Agrobacterium*-based transformation system and illustrate its utility with an example of the anti-sense expression of *RuBPCase*, as verified by Southern gel blot analysis and real-time quantitative PCR; (ii) a 789-oligonucleotide microarray and illustrate its utility with hybridizations of herbivore-elicited plants, and verify responses with RNA gel blot analysis and real-time quantitative PCR; (iii) analyses of secondary metabolites that function as direct (proteinase inhibitor activity) and indirect (herbivore-induced volatile organic compounds) defences; and (vi) growth and fitness-estimates for plants grown under field conditions. Using these tools, we demonstrate that attack from flea beetles elicits: (i) a large transcriptional change consistent with elicitation of both jasmonate and salicylate signalling and (ii) increases in proteinase inhibitor transcripts and activity, and volatile organic compound releases. Both flea beetle attack and jasmonate elicitation increased proteinase inhibitors and jasmonate elicitation decreased fitness in field-grown plants. Hence, proteinase inhibitors and jasmonate-signaling are targets for manipulative studies.

Key words: *Agrobacterium*-mediated transformation, herbivore-induced volatile production, indirect and direct defenses, oligonucleotide microarray, plant fitness, proteinase inhibitor

Introduction

Molecular techniques are widely used in all biology, but their incorporation into ecological studies has largely been confined to the characterization of population structure and species distributions. As a consequence, the extraordinary advances that molecular techniques have, permitted most biological disciplines, namely the ability to identify the genetic basis of a biological phenomenon and manipulate it, have yet to be realized in ecology. Many reasons underlie the nonparticipation of ecologists in the genomics revolution, but the limited availability of appropriate model systems has played an important role. Molecular tools developed for one model system can be difficult to transfer to other systems without substantial investment in technique development. Most techniques have been developed for agronomically and economically important organisms and cannot be readily applied to wild relatives. Transformation systems, in particular, can be difficult to use with near relatives. *Agrobacterium*-mediated gene transfer protocols are available for a number of higher plants and fungi, and allow the manipulation of the expression of genes mediating ecological interactions if these transformation systems have been adapted for native species. Plants and herbivores account for the majority of all higher species (Strong *et al.*, 1984), and their interactions structure many of the planet's biological processes. The transformation of autotrophs allows for the 'bottom-up' manipulation of ecological interactions and thereby provides a powerful tool for studying community and ecosystem processes.

Once the ability to manipulate the expression of individual genes in a native species is available, the next task is to decide which genes to manipulate and how to interpret the responses to the manipulations. Microarrays allow biologists to examine the expression of hundreds of genes simultaneously, and their use in combination with elicitation studies provides a powerful means of identifying 'suspect' genes relevant for ecological interactions (Hui *et al.*, 2003; Korth, 2003). Alternatively, various differential display procedures (Voelckel, Baldwin, 2003) allow researchers to 'ask the organism' to identify transcripts relevant in a given ecological interaction. Once a gene or a suite of genes has been selected for manipulation, the next challenge lies in interpreting the fitness consequences of the manipulation. Laboratory bioassays are not likely to provide a full functional understanding of many traits elicited by biotic interactions as is illustrated by the traits mediating plant-herbivore interactions.

Plants are known to recruit components of their community, as is graphically illustrated by the elicitation of indirect defenses (volatile organic compound [VOC] emissions, extrafloral nectar production, etc.) that plants use to enlist the natural enemies of herbivores in their defense against herbivores. A functional understanding of these complex responses is possible only in the context of the selective forces under which these responses evolved, namely their natural environments. Moreover, ecologists have long known that competition from other plants and herbivore pressure represent the two most important selective forces determining relative plant fitness in natural habitats (Begon *et al.*, 1996). As a consequence, traits mediating competitive ability and herbivore resistance are likely intertwined, and understanding the genetic basis of these responses will require experimental manipulations in natural environments. Lastly, since environmental performance is a whole-plant trait best measured by various surrogates of Darwinian fitness (seed set, male reproductive success), an understanding of how the expression of a gene product contributes to a plant's Darwinian fitness is required.

The choice of an ecologically relevant organism is crucial for the study of plant-environment interactions. Adaptive responses are mediated by complex polygenic traits (Simms, Rausher, 1992), and because agricultural plants have long been under intense selection for particular yield-enhancing traits, genetic associations mediating adaptive traits are likely to have been altered during agricultural selection and hence are difficult to interpret in these plants. We introduce a suite of molecular tools for the native plant *Solanum nigrum* that should facilitate the identification and manipulation of the genes that mediate these complex environmental responses. *S. nigrum* was selected not only because of its phylogenetic proximity to the agricultural species tomato and potato for which substantial genetic tools are available, but also because its particular natural history makes it ideal for studying the interaction of competition and herbivore resistance. *S. nigrum* is attacked by various herbivores from different feeding guilds and grows in association with many other species. As an annual, it colonizes nitrogen-rich agricultural and disturbed habitats at a wide range of altitudes throughout its pan-arctic distribution (Edmonds, Chweya, 1997).

If *S. nigrum* is to become a model ecological system, a minimum number of molecular tools are necessary. Most important, it must be readily transformable so that hypotheses about the ecological function of particular genes can be falsified. Here, we present such a transformation system for *S. nigrum* using an antisense (as) construct of the photosynthetic

gene *RuBPCase*. cDNA libraries of environmentally elicited plant tissues provide a means of cloning genes and microarrays allow biologists to examine the expression of hundreds of genes simultaneously. Here we present such a library and a 789-oligonucleotide microarray, representing 558 genes of ecological interest. We analyze traits thought to be important for plant performance that are quantifiable in complex environments and offer a means of measuring their correlation with *S. nigrum*'s Darwinian fitness. We have selected a direct defense and an indirect defense for the analysis. Proteinase inhibitors (PIs) are among the best-studied induced direct defense chemicals in plants (Heath *et al.*, 1997; Jongsma *et al.*, 1994; Koiwa *et al.*, 1997; Ryan, 1990), which function by inhibiting particular digestive proteinases of herbivores, such as chymotrypsin and trypsin. High PI content has been found to reduce herbivore growth in plants that were transformed with heterologous PI genes (Rahbe *et al.*, 2003; Xu *et al.*, 1996). (Xu *et al.*, 2001) characterized two PIs of the *pin2* family (*SaPIN2a* and *SaPIN2b*) in *S. americanum*, a species belonging to the taxonomically diverse *S. nigrum* group (Edmonds, Chweya, 1997). We isolated the *SaPIN2b* homologue from *S. nigrum* (*SnPIN2b*) and used it to verify the responses observed on the array with a RNA-gel blot and real-time quantitative polymerase chain reaction (qPCR). We present a technique for quantifying the herbivore-induced VOC emissions from plants and illustrate its use with measurements from field-grown plants. VOCs are known to attract predators to the herbivore-damaged plant and therefore play a key role in plant-insect interactions (Baldwin *et al.*, 2002; Dicke *et al.*, 2003; Kessler, Baldwin, 2001).

Materials and Methods

Plant growth

The hexaploid *Solanum nigrum* L. inbred line, Sn30, of seeds collected from the field site in Jena, Germany (voucher specimens of the Sn30 line are deposited in the Max Planck Institute for Chemical Ecology branch of the Herbarium Haussknecht [JE], Jena) was used in all experiments. Seeds were incubated in 3.5mM Ca(NO₃)₂ overnight at 4°C and germinated in a peat-based substrate with clay additions (Tonsubstrat). Plants were grown in individual 400-mL pots for 3 weeks in the greenhouse (26 °C / 16 h light; 25 °C / 8 h dark). After 5 days of acclimatization to outside conditions, plants were randomly planted into monoculture plots at the experimental field site. The site is a former agricultural field with alluvial loam as substrate and located north of Jena, Germany. For the greenhouse experiments, plants were

grown in 2 L pots with supplemental lighting from 400 W Na-vapour HID lamps and watered once a day.

Agrobacterium-based transformation

A fragment of the *Nicotiana attenuata* gene for *RuBPCase* (Hermsmeier *et al.* 2001) was PCR amplified. After digestion with *XhoI* and *BstEII* the resulting fragment (323 bp) was cloned in pRESC20 (Zavala *et al.* 2004), yielding the transformation vector pRESC2RUB (10.0 kb) which was used for the transformation of *S. nigrum*.

S. nigrum seeds (inbred line Sn30) were used for transformation. Seeds were sterilized for 5 min in a 5-mL aqueous solution of 0.1 g dichloroisocyanuric acid (Sigma) with 50 μ L of 0.5 % (v/v) Tween-20 (Merck). Seeds were washed three times with sterile water and incubated for 3 days at 4 °C in an aqueous solution of 3.5mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (Merck). Subsequently, seeds were washed 3 times with sterile water and transferred onto a germination medium containing Gamborg's B5 with minimal organics (Sigma) and 0.6% (w/v) phytigel (Sigma). The plates were maintained in a growth chamber (Percival) at 26°C/16h light with 155 $\mu\text{m}^2/\text{s}$ PAR at shelf height and 24°C/8h dark. *Agrobacterium tumefaciens* strain 4404 was maintained and cultivated for transformation as described in Krügel *et al.* (2002). Hypocotyls of sterile 1-week-old seedlings were excised with a scalpel dipped into the *Agrobacterium* suspension, and co-cultivated as described in Krügel *et al.* (2002). Within a month, explants on callus induction media developed calli followed by green shoot primordia and shoots, so that sub-culture onto maturation media (described in Krügel *et al.* 2002) was necessary only until plantlets formed. Plantlets were subsequently sub-cultured onto maturation media, which also served as rooting media in this case, until roots appeared. Further selection of the putatively transformed plants, including segregation analysis of T₁ plants using germination bioassays on hygromycin-containing media, is described in Krügel *et al.* (2002).

Microarray hybridization and analysis

Pooled leaf samples were ground under liquid nitrogen and total RNA was extracted with TRI REAGENT™ (Sigma) according to the manufacturer's instructions. The herbivore-infested test samples were labeled with Cy3 and the corresponding control (reference) samples with Cy5 according to the procedure described in (Halitschke *et al.*, 2003). The labeled samples were hybridized to the microarray (789 50-mer oligonucleotides spotted onto an epoxy-coated glass slide; Quantifoil Microtools) according to the published procedure (Halitschke *et al.*, 2003).

An Affymetrix 428™ Array Scanner (Affymetrix) was used to scan the hybridized microarrays with sequential scanning for Cy5 cDNA and then for Cy3-labeled cDNA at a maximum resolution of 10 µm/pixel with a 16-bit depth. The images were evaluated with the AIDA IMAGE ANALYZER (Raytest Isotopenmeßgeräte GmbH) software. Each image was overlaid with a grid to assess the signal strength (quantum level = QL) for both dyes from each spot. The background correction was calculated with the 'non spot' mode of the AIDA software package.

The microarray-specific normalization factor was calculated based on the Cy5 / Cy3 total fluorescence ratio (Halitschke *et al.*, 2003). The ratios of normalized fluorescence values for Cy3 and Cy5 of each individual spot (expression ratio = ER) and the mean of the four replicate spots for each cDNA were calculated. A transcript was defined as being differentially regulated, if the following three criteria were fulfilled: (i) the average expression ratio for the 4 spots exceeded the thresholds (0.67 and 1.5); (ii) the individual expression ratios were significantly different from 1, as determined by a *t*-test; (iii) the combined signal fluorescent intensity from both Cy3 and Cy5 averaged over the 4 spots was > 1000 QL. A complete list of all signal ratios (\pm SE) and details on all spotted genes is available from the authors. To evaluate these criteria, we hybridized two microarrays with the same cDNA pools and found that 84% of the genes had the same regulation (Heidel, Baldwin, 2004).

RNA gel blot analysis

RNA samples (20µg) were size-fractionated by 1.2% (w/v) agarose formaldehyde gel electrophoresis and capillary blotted onto a nylon membrane (GeneScreenPlus; NEN-DuPont) as described in the manufacturer's instructions. Ethidium bromide staining of the gel

prior to blotting revealed rRNA bands, which served as the loading control. After blotting and UV-crosslinking, ^{32}P -labeled probes specific for *PIN2b* were used for detection. The probe for *PIN2b* was obtained by PCR of *S. nigrum* cDNA with primers specific for *SaPIN2b* from *S. americanum* (Xu *et al.*, 2001). This fragment was used to screen a *S. nigrum* leaf cDNA library (Lambda ZAP II kit, Stratagene), and the longest resulting *PIN2b* sequence (679 bp; *SnPIN2b*; AY422686) was used as a probe to detect *PIN2b* transcripts.

Real-time quantitative PCR (qPCR)

Total RNA was reverse transcribed into cDNA using SuperScriptTM II RNaseH- Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The amount of cDNA template used per well was reverse transcribed from 10 ng total RNA; each sample was replicated three times. The following sequences were used for the design of primers specific for *PR-1*, *SnPIN2b*, *psbA* and *RuBPCase*: *Lycopersicon esculentum* PR-1 (Tornerio *et al.*, 1997), *S. nigrum* PIN2b (see above), *S. nigrum* photosystem II D1 protein (Zhu *et al.*, 1989) and *L. esculentum* RuBPCase LESS17 (McKnight *et al.*, 1986). 18S RNA (template for primers: *S. tuberosum* gene for 18S RNA, GenBank Accession no. X67238) was used for quantitative normalization. The ABI PRISM[®] 7700 Sequence Detection System (1997) was used for the SYBR Green I-based assay. The qPCRTM Core Kit for SYBR[®] Green I (Eurogentec) was used according to the manufacturer's instructions with the following cycler conditions: 10 min at 95°C; 40 cycles: 30 s at 95°C and 30 s at 60°C. To ensure the specificity of the PCR, a melting curve analysis was conducted using the ABI PRISM[®] 7700 Dissociation Curve Software. To detect *asRuBPCase* transcripts amplicons specific for the *as*-construct were designed ((Halitschke *et al.*, 2003). The assay using a double dye-labeled probe was performed on an ABI PRISM[®] 7700 Sequence Detection System (qPCRTM Core Kit, Eurogentec) with 18S RNA for normalization (TaqMan[®] Ribosomal RNA Control Reagents, Applied Biosystems). The relative expression of the target genes was determined by using standard curves (Applied Biosystems 1997).

Isolation and blotting of genomic DNA

Plant genomic DNA was prepared from leaves of *S. nigrum* using CTAB (Reichhardt, Rogers, 1994). DNA samples were restriction digested with *EcoRV*, size-fractionated by 0.8 % agarose gel electrophoresis, and Southern blotted onto a nylon membrane with high-salt

buffer (Brown, 1995). The blot was analyzed with ^{32}P -labelled probe specific for the hygromycin resistance gene (*hph*).

Plant treatments

MeJA-induction: We applied 250 μg of methyl jasmonate (MeJA; Sigma) in 20 μL lanolin (Sigma) to the stem of the 5-week-old plants ($n = 62$) above the third leaf node. To exclude possible lanolin effects, we treated control plants ($n = 55$) with 20 μL lanolin.

Flea beetle damage: Flea beetles (*Epitrix pubescens*) were abundant at our field site. To compare uninfested control plants with flea-beetle-infested plants, we sprayed the controls ($n = 28$) with the pyrethroid-based insecticide Spruzit (0.1 %, Neudorff) and the infested plants ($n = 31$) with water directly after planting each day until tissue was harvested for microarray and PI analysis. The average flea beetle load of the water-sprayed plants for the duration of the experiment was ~ 40 adults per plant compared with 1-5 adults per plant for pyrethroid-sprayed plants. In a comparable field experiment, Baldwin (1998) detected no influence of pyrethrin treatment on inducible defences (nicotine) in the Solanaceous plant, *Nicotiana attenuata*.

For the microarray analysis, we harvested and pooled fully expanded leaves of eight individual plants 48h after exposure to flea beetles, flash-froze them in liquid nitrogen, and stored the leaf samples at -80°C until RNA extraction. For the PI analysis, a systemic leaf near the youngest node of all control and elicited plants was harvested 3 days after induction (MeJA or flea beetle), flash-frozen in liquid nitrogen, and stored at -80°C until protein extraction.

Herbivore comparison: *Leptinotarsa decemlineata* originated from wild populations on *S. tuberosum*, *Acherontia atropos*, from a laboratory population reared on *S. nigrum*. To achieve approximately the same leaf area damage among herbivore treatments, we placed two *L. decemlineata* adults or two second-instar *A. atropos* larvae on individual 5-week-old plants (five replicates per treatment). After 24h of continuous feeding, we collected VOCs from the differentially treated plants (see VOC analysis).

Trypsin-PI analysis

Harvested leaves were ground in liquid nitrogen. Proteins were extracted according to the protocol used for *Nicotiana attenuata* (Van Dam *et al.*, 2001) and protein content was measured by the method of (Bradford, 1976) with IgG (Sigma) as standard. We determined the activity of trypsin inhibitors by the radial immunodiffusion assay (Jongsma *et al.*, 1993). A series of soybean trypsin inhibitor (STI, Sigma) solutions was used to obtain a reference curve. Trypsin-PI activity is expressed as nmol/mg of total protein.

VOC analysis

In an open-flow trapping system, VOCs of one fully-expanded stem leaf were collected (see Fig. 3A). To confine insects to a single leaf and to trap volatiles from the same leaf, leaf and insects were enclosed in 400-mL polystyrene chambers fitted with holes at both ends. Air was pulled through the chamber at 450-500 mL min⁻¹ (measured by a mass flow meter: Aalborg Instruments) and subsequently through a charcoal air-sampling trap (ORBOTM-32; SUPELCO) by a portable vacuum pump. Each charcoal trap was spiked with 300 ng tetraline as an internal standard (ISTD) for quantification, eluted with 750µL dichloromethane, and analyzed by GC-MS according to (Halitschke *et al.*, 2000).

Results

Herbivore community

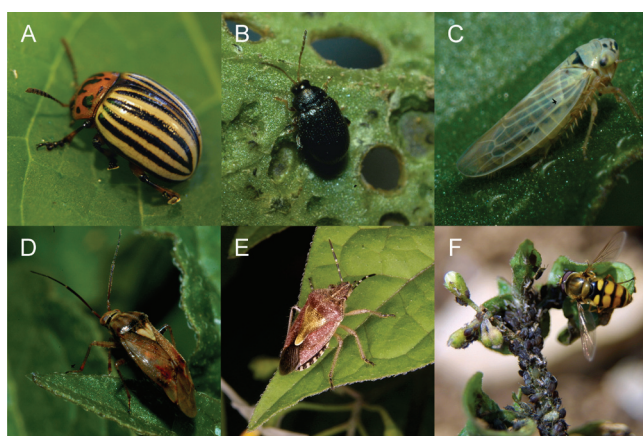


Fig. 1: Herbivores on *Solanum nigrum*: (A) Colorado potato beetle (*Leptinotarsa decemlineata* Say); (B) flea beetle (*Epirix pubescens* Koch); (C) cicada (*Macrostelus sexnotatus* Fallèn); (D) mirid bug (*Lygus wagneri* Remane); (E) pentatomid bug (*Dolycoris baccarum* Linnè); (F) bean aphids (*Aphis fabae* Scopoli) and syrphid fly.

Table 1: List of herbivore species observed feeding on *Solanum nigrum* in experimental field plots in Jena, Germany, during two growing seasons. Species are classified by feeding guild (C- leaf chewing, PP-piercing sucking on phloem, PM- piercing sucking on mesophyll) and whether they are specialized on Solanaceous plants.

Species	Order	Family	Feeding	
			guild	Specialization
<i>Epitrix pubescens</i>	Coleoptera	Chrysomelidae	C	Specialist
<i>Leptinotarsa decemlineata</i>	Coleoptera	Chrysomelidae	C	Specialist
<i>Barypeithes pellucidus</i>	Coleoptera	Curculionidae	C	Generalist
<i>Plutella xylostella</i>	Lepidoptera	Plutellidae	C	Generalist ?
<i>Lygus pratensis</i>	Heteroptera	Miridae	PM	Generalist
<i>Lygus rugulipennis</i>	Heteroptera	Miridae	PM	Generalist
<i>Lygus wagneri</i>	Heteroptera	Miridae	PM	Generalist
<i>Stenodema sericans</i>	Heteroptera	Miridae	PM	Generalist
<i>Dolycoris baccarum</i>	Heteroptera	Pentatomidae	PM	Generalist
<i>Holocostethus vernalis</i>	Heteroptera	Pentatomidae	PM	Generalist
<i>Corizus hyoscyami</i>	Heteroptera	Rhopalidae	PM	Generalist
<i>Philaenus spumarius</i>	Auchenorrhyncha	Cercopidae	PP	Generalist
<i>Balclutha punctata</i>	Auchenorrhyncha	Cicadellidae	PP	Generalist
<i>Evacanthus interruptus</i>	Auchenorrhyncha	Cicadellidae	PP	Generalist
<i>Empoasca spec.</i>	Auchenorrhyncha	Cicadellidae	PP	Generalist
<i>Eupteryx aurata</i>	Auchenorrhyncha	Cicadellidae	PP	Generalist
<i>Macrosteles sexnotatus</i>	Auchenorrhyncha	Cicadellidae	PP	Generalist
<i>Aulacorthum solani</i>				
<i>langei</i>	Sternorrhyncha	Aphididae	PP	Generalist
<i>Aphis fabae</i>	Sternorrhyncha	Aphididae	PP	Generalist

During the 2002 and 2003 growing seasons, we sampled different native and planted populations of *Solanum nigrum* near Jena for insects. In 2002, the main collection sites were a planted population in Jena-Isserstedt (~100 plants) and native and planted populations in Jena-Nord (~500 plants); 2003 specimens were collected mainly from a planted population (~140 plants) on the Jena/Beutenberg-Campus. Populations were sampled at least once a week from May to September of each year. We classified insects as being herbivores on *S. nigrum* only if the adult insects or their larvae were repeatedly observed to feed on *S. nigrum* in both years (Table 1). Many additional insect species were observed on the plants, but only a small subset was observed to consistently feed on this plant. For example, only 3 of at least 24 Coleopteran species repeatedly found on the plants were classified as *S. nigrum* herbivores. The phytophagous insects belong to different groups according to their feeding behavior and host-plant specialization. Two Solanaceous specialists, both leaf-chewing beetles, namely the Colorado potato beetle *L. decemlineata* and the flea beetle *E. pubescens*, were repeatedly found on *S. nigrum* (Fig. 1). The flea beetles infested the plantation and native *S. nigrum* plants heavily throughout 2002 in Jena-Nord. *L. decemlineata* appeared in August, having dispersed from small potato fields. In addition to the leaf-chewing species, we found a variety of piercing-sucking insects, including bugs, cicadas, and aphids. *Aphis fabae* colonized *S. nigrum* plants from May until September. Later in the season (July/August) mirid bugs of the genus *Lygus* were the most abundant herbivores. The rich herbivore fauna attracted a large variety of parasitoids and predatory species [syrphids (Fig. 1F), chrysopids, coccinellids, etc].

Transformation

We developed an *Agrobacterium*-based transformation procedure for *S. nigrum*, which requires approximately 5-6 months from the transformation to the production of T₀ plants bearing mature fruit. Transformation and regeneration of plantlets (2 cm in size) that can be transferred into soil requires 2 months. Callus generation in *S. nigrum* occurred at a lower rate than it does in *Nicotiana attenuata*, but the calli that did generate, did so at a higher rate (M. Lim unpublished results). Short tissue culture times are helpful in reducing somaclonal variation, which limits the production and utility of transgenic plants (Beaujean *et al.*, 1998). In comparison to the published *S. nigrum* protoplast transformation procedure using

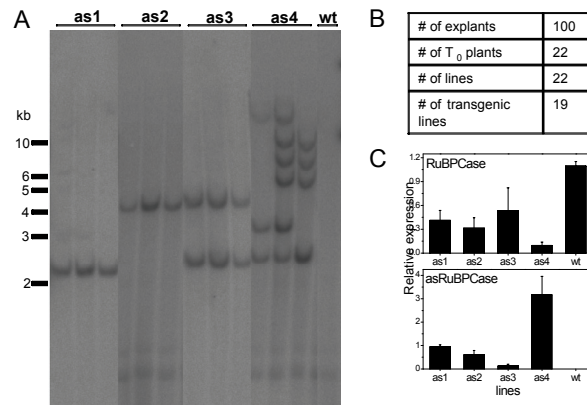


Fig. 2: Characterization of *asRuBPCase S. nigrum* lines. (A) DNA gel-blot analysis of three *asRuBPCase* T₁ lines (as1, as2, as3, as4; three replicates each) and one wildtype, untransformed line (wt). Genomic DNA was digested with *EcoRV* and the blot was hybridized with a probe specific for the hygromycin resistance gene (*hph*). The lines as1 and as2 have single copy insertions of the transgene, while as3 has two copies and as4 has multiple copies. The wt DNA is shown as a negative control. (B) Summary of transformation efficiency. Twenty-two independent lines were examined in detail, 19 were found to be transformed as verified by antibiotic selection and PCR. (C) Relative expression of *asRuBPCase* (lower panel) and *RuBPCase* (upper panel) in *as*-lines (as1-as4) and wt, as determined by real-time qPCR. The *as*-lines showed expression of the *as*-transcript in different amounts and had reduced amounts of *RuBPCase* transcripts in comparison to the amounts measured in wt plants ($r = -0.549$, $P = 0.0326$). Transformation clearly resulted in differential silencing of the endogenous *RuBPCase* transcripts.

Agrobacterium rhizogenes (Wei *et al.*, 1986), our protocol proved to be more efficient in the regeneration of plants. Also, the use of Ri-based vectors increases the frequency of phenotypically abnormal and infertile plants in comparison to the T-DNA based vectors used here (Davey *et al.*, 1987). Hygromycin resistance (*hph*) proved to be a reliable selectable marker that could be incorporated into the germination media and subsequently allowed seedlings to be rapidly selected. The efficiency of the procedure is high (Fig. 2 B inset): 19 of the 22 haphazardly selected *asRuBPCase* lines (86 %) could be verified as harboring the transgene by means of antibiotic selection and PCR. Remarkably, the transgenes in the hexaploid *S. nigrum* line segregated as in a diploid, in accordance with findings for transgenes in the tetraploid *Arabidopsis suecica* (Lawrence & Pikaard 2003), indicating that chromosome pairing occurs among homologues. For further characterization, we selected 10 T₁ lines and examined them by DNA gel blot analysis (four lines are shown in Fig. 2A). All lines contained the transgene, and 2 of the 10 lines tested contained the transgene as a single copy

insertion. Real-time qPCR supported the successful incorporation of the transgene into *S. nigrum*'s genome (Fig. 2C). As expected no antisense (*asRuBPCase*) transcripts were detected in wildtype (wt) plants, whereas these transcripts were abundant in the transformed lines. The quantity of *asRuBPCase*-transcripts in transformed lines correlated negatively with the quantity of *RuBPCase*-transcripts (Pearson's correlation coefficient $r = -0.549$, $P = 0.0327$), demonstrating that the transformation had successfully reduced the expression of this important photosynthetic gene.

Microarray analysis

Results of the oligonucleotide microarray analysis are summarized in Fig. 3 and the complete list of all regulated genes, their expression ratios, and annotations can be obtained from the authors. The hybridization compared *E. pubescens*-infested plants with plants treated with insecticide to protect from damage by flea beetles. We found a total of 155 genes to be significantly regulated (27 % of 568 genes on the microarray). The regulated genes were assigned to different putative functional categories (Fig. 3A). Several genes involved in the biosynthesis of defense-related secondary metabolites were downregulated (tropinone reductase, *TRII*; phenylalanine ammonia lyase, *PAL*). A suite of PI genes were strongly upregulated (*SaPIN2a*, *SaPIN2b*, *pin2*, *PI-WuSP*), which correlated with the observed increase of trypsin-PI activity in flea beetle-infested plants (Fig. 4C). Amongst defence-related genes, we found an α -dioxygenase (*PIOX*; cv57.4) to be upregulated, in addition to several pathogenesis-related proteins (*PR-1*, *PR-2*, *PRP4*, *PRP5*, *PRP-6*, *PRp27*). Photosynthesis-related genes were generally downregulated (e.g. different subunits of RuBPCase). Genes involved in defense-signaling processes were strongly upregulated in flea beetle-infested plants (octadecanoid pathway: lipoxygenases *LOX*; alleneoxide synthase *AOS*; 12-oxophytodienoate reductase *opr*). The microarray results were verified by RNA gel blot analysis with a *SnPIN2b* probe and real-time qPCR for the genes *SnPIN2b*, *PR-1*, and *psbA* (Fig. 3 B-E).

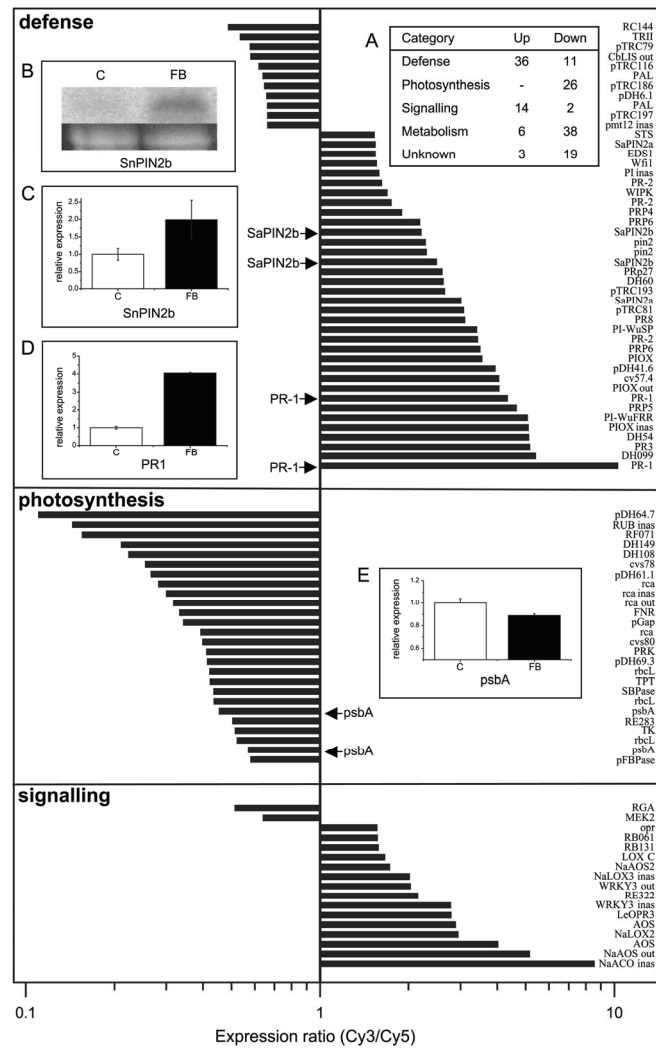


Fig. 3: Expression of *S. nigrum* genes in response to attack from flea beetles, *Epitrix pubescens*, by microarray, RNA gel-blot, and real-time quantitative PCR analysis (C = control, FB = flea-beetle-infested). Microarray analysis revealed gene regulation in different categories and independent gel-blot and real-time PCR analyses verified expression patterns of individual genes from the array. The expression ratios (normalized mean Cy3/Cy5 ratio) for three categories (defense, photosynthesis, signalling) from an array hybridized with fluorescently labeled cDNA from attacked and unattacked plants are depicted. Inset table (A) summarizes the numbers of up- and down-regulated genes in the categories (see Supplementary Table 1 for a complete list of regulated genes and gene descriptions). Arrows identify genes used for verification of the expression data (panel B-E). (B) RNA gel blot analysis hybridized with an *SnPIN2b* probe (18S RNA is shown as a control for equal loading). (C-E) Real-time qPCR for the genes (C) *SnPIN2b*, (D) *PR1*, and (E) *psbA*. The expression level in the control sample equals 1.

Proteinase inhibitors

We assayed systemic leaves of MeJA- and flea beetle (*E. pubescens*)-infested plants for their trypsin-PI content. Greenhouse-grown plants showed a significant increase in trypsin-PI activity (Student's t-test, $t = -15.239$, $P = 0.001$) after treatment with 250 μ g MeJA (Fig. 3A). A similar increase was found in field-grown plants (Fig. 3B; Student's t-test, $t = 5.301$, $P <$

0.001), but the constitutive trypsin-PI-levels of field plants were higher than those of greenhouse plants at a similar developmental stage. Trypsin-PI-levels in field-grown *S. nigrum* plants were significantly higher in response to attack from the naturally occurring herbivore, *E. pubescens* (Fig. 3B; Student's *t*-test, $t = 2.397$, $P = 0.0199$). The increase of PIs was similar in MeJA-elicited and flea beetle-infested plants.

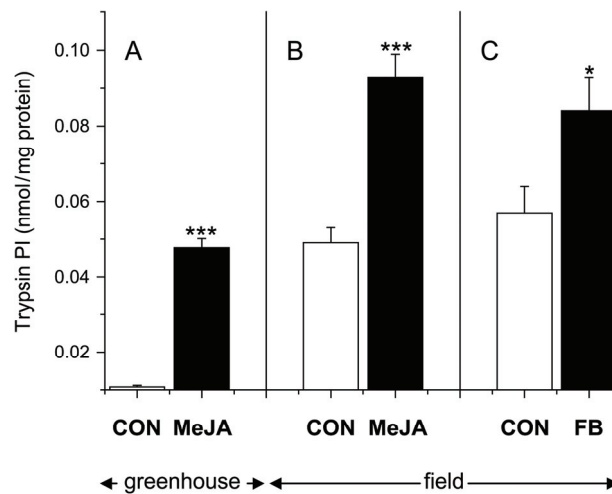


Fig. 4: Trypsin-PI protein levels (mean \pm SE) in MeJA-treated (250 μ g/plant) and flea beetle-infested plants. (A) Greenhouse-cultivated *S. nigrum* plants, MeJA-treated; (B) field-grown *S. nigrum*, MeJA-treated; (C) field-grown *S. nigrum*, flea beetle-infested. All treatments significantly increased Trypsin-PIs in the leaves of *S. nigrum*. Trypsin-PI-levels correlated with expression of Trypsin-PI genes (Fig. 3A, B, G).

Volatile organic compounds

In a field experiment, we collected VOCs emitted from plants in response to attack from phytophagous insects. We used an open-flow trapping system (Fig. 5A), which allowed us to synchronously sample all experimental replicates and to maintain the sampled leaves under physiological conditions. We allowed two herbivore species (*L. decemlineata* and *A. atropis*) to feed separately on *S. nigrum* for 24h and compared the composition of VOCs trapped with those trapped from uninfested control plants. Emissions of several compounds, ranging from monoterpenes (e.g. 3-carene, β -myrcene, +/-limonene) to green-leaf volatiles (*cis*-3-hexenyl acetate, *cis*-3-hexen-1-ol) and sesquiterpenes (longifolene, *trans*- β -caryophyllene), was increased in insect-attacked plants (Fig. 5B).

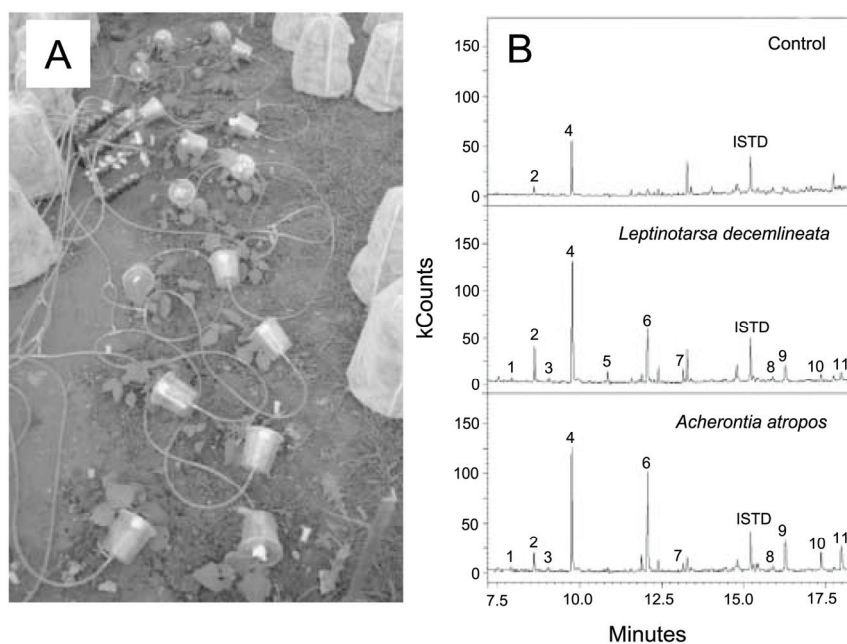


Fig. 5: Volatile organic compounds (VOCs) released in response to attack from different herbivore species. (A) VOCs were collected from 15 field-grown plants, using an open-flow trapping system. (B) Representative Total Ion Chromatograms of the headspace volatiles eluting from a GC column of an undamaged leaf from an undamaged *Solanum nigrum* plant (control), a leaf damaged by *Leptinotarsa decemlineata*, and a leaf damaged by an *Acherontia atropos* hornworm (from separate plants). The labels represent an unknown monoterpene (1), 3-carene (2), β -myrcene (3), +/-limonene (4), unknown compound (5), *cis*-3-hexenyl acetate (6) *cis*-3-hexen-1-ol (7), longifolene (8), *trans*- β -caryophyllene (9), unknown sesquiterpene 1 (10), unknown sesquiterpene 2 (11).

Fitness

We evaluated the fitness consequences of MeJA-elicitation under field conditions in the plants previously assayed for trypsin-PIs. *S. nigrum* is extremely plastic in its growth form and is able to adjust its morphology to diverse conditions. In botanically precise terminology, the growth shape varies from decurrent with plagiotropic to fastigate branching to excurrent with orthotropic branching. Plant size at reproductive maturity varies from 5 cm to over 1 m. We manipulated plant size in a greenhouse experiment by planting *S. nigrum* into different sized pots and found that the dry biomass correlated strongly with the number of fruits produced ($R^2 = 0.665$). Pot size was used to manipulate above-ground biomass, but also likely influenced the root architecture and biomass and the available rooting space may fundamentally influence plant fitness. In the field, we did not find morphologically detectable changes in response to a single elicitation with MeJA, nor was the number of flowers produced (data not shown) significantly influenced by elicitation. However, fruit number and

seed production differs significantly between induced and uninduced plants. MeJA-treated plants produced $\sim 40\%$ fewer fruits than did control plants (Fig. 6), suggesting that MeJA-elicited responses result in large fitness costs for an individual plant.

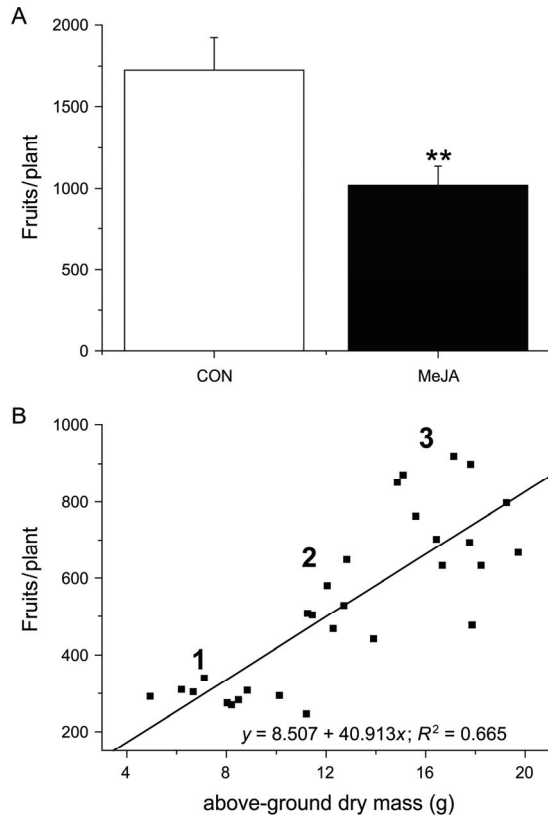


Fig. 6: Fitness estimates of *S. nigrum*. (A) Fruit number: Comparison of MeJA-elicited vs. control plants. Fruit numbers were significantly lower in plants that received a single treatment of $250\ \mu\text{g}$ MeJA (Student's *t*-test, $t = 3.256$, $p = 0.0038$). One fruit contained 77 ± 1.45 (SEM, $n = 33$) seeds; hence control plants produced approximately 130,000 seeds, while elicited plants produced 80,000 seeds per plant. (B) Fruit number is linearly correlated with the above-ground dry mass of greenhouse-grown plants. Different plant sizes were obtained by growing plants in (1) 1-L pots, (2) 2-L pots, or (3) 3-L pots.

Discussion

To facilitate the identification of genes mediating responses to ecological interactions, and to allow for the manipulation of their expression, we present the following tools for the *Solanum nigrum* expression system: (i) an *Agrobacterium*-based transformation system; (ii) an oligonucleotide microarray, enriched with ecologically relevant genes; (iii) measures of direct (PIs) and indirect defences (VOC emission) under both field and laboratory conditions; and (iv) measures of Darwinian fitness. These tools have been optimized to analyze responses that are rapidly elicited by ecological interactions. We illustrate their utility by analyzing responses to attack by a native herbivore of *S. nigrum* and compare the elicited responses with those elicited by MeJA treatment. The analysis links changes in transcript abundance

with phenotypic changes, which, in turn, are correlated with changes in the fitness of plants grown under field conditions.

An initial survey of the differential gene expression of *S. nigrum* revealed a large-scale change in the plants' transcriptome in response to flea beetle attack: 27% of the monitored genes showed a significant change in expression pattern. Genes involved in important primary processes, such as carbon fixation and metabolism, were largely down-regulated, while defence genes, including genes involved in defence-related signalling, were largely upregulated. The octadecanoid signalling cascade with its key compound, jasmonic acid (JA), is known to play an important role in triggering many of the insect-induced responses of a plant (Blee, 2002; Farmer *et al.*, 2003). Consistent with a regulatory role for oxylipins in the elicitation of defense-related genes in *S. nigrum*, oxylipin biosynthetic enzymes (AOS, allene oxide synthase; LOX, lipoxygenase; OPR, 12-oxophytodienoate reductase) were strongly upregulated, as were the transcripts of the JA-elicited PI defence genes (e.g. *SaPIN2a*, *SaPIN2b*, *pin2*). PIs are known to adversely affect the performance of herbivorous insects (Rahbe *et al.*, 2003; Xu *et al.*, 1996), and the causal associations among JA signalling, PI elicitation, and insect resistance were recently established with *Nicotiana attenuata* plants in which JA signaling was silenced by expressing *LOX-H3* in an antisense orientation (Halitschke *et al.*, 2003). In this study herbivore performance was enhanced on *asLOX* plants most likely due to attenuated PI and nicotine defence responses.

Several pathogenesis-related (PR) proteins (PR-1; PR-2, β -1,3-glucanase; PR3, PR4 both chitinases; PRP6; Prp27), which are known to be elicited by pathogen attack or salicylic acid (SA) treatment, were among the strongest upregulated transcripts. From this, we deduce that herbivore attack to *S. nigrum* may elicit both SA- and JA-related signalling and the commonly invoked tradeoff between systemic resistance of plants to microorganisms and resistance to insect herbivores (Felton *et al.*, 1999; Thaler *et al.*, 2002) may not apply to this plant species. Some transcripts may be co-regulated by both insects and microorganisms. For example, α -dioxygenase (α -DOX; formerly PIOX, pathogen-induced oxygenase), an enzyme which catalyzes the conversion of linolenic acid to its 17-hydroperoxy-derivative (Hamberg *et al.*, 1999), is upregulated in *S. nigrum* in response to flea beetle feeding. In *N. tabacum*, α -DOX can be induced by bacterial elicitors (de Leon *et al.*, 2002; Sanz *et al.*, 1998), and in *N. attenuata*, by attack from *Manduca sexta* larvae (Hermsmeier *et al.*, 2001). Although the

biological function of α -DOX remains unclear, its transcriptional behaviour illustrates the likely interactions of pathogen and herbivore signalling under natural conditions.

Adaptive responses are likely to be the result of complex interacting signal networks rather than single signal cascades (Genoud *et al.*, 2001; Reymond, Farmer, 1998), and transcription factors may play an important role in coordinating the responses from these many signal cascades. In plants attacked by flea beetles, a transcription factor of the WRKY family (WRKY3) was strongly upregulated, as it was shown to be in *N. attenuata* attacked by *M. sexta* larvae (Hui *et al.*, 2003). WRKY transcription factors occur in large gene families and are known to regulate numerous stress-related genes, including those responsive to pathogens and wounding (Eulgem *et al.*, 2000). Transcription factors may coordinate large-scale patterns of transcriptional changes and deserve more attention in the regulation of environmental responses.

The coordination of transcriptional responses to flea beetle attack extends beyond the upregulation of stress-responsive transcripts to include a coordinated downregulation of growth-related transcripts, which is most clearly seen in the negative correlation between the expression of photosynthetic and that of defense genes (Hermsmeier *et al.*, 2001; Schittko *et al.*, 2001). The herbivore-induced suppression of *RuBPCase* transcripts (pDH64.7, RUB inas, *rbcL*) and of additional elements of the photosynthetic machinery (e.g. photosystem proteins: *N. tabacum* PSI, *N.t.* PSII precursor, *S. nigrum* PSII D1) might benefit the plant by redirecting carbon flux toward the production of defenses. RuBPCase activase (*rca*), a stromal protein catalyzing the dissociation of inhibitory sugar biphosphates from uncarbamylated and carbamylated RuBPCase in an ATP-requiring process (Portis, 1995; Robinson, Portis, 1989), may play an important role in regulating *RuBPCase* transcripts and perhaps other photosynthetic proteins, and was downregulated in attacked plants. It has been shown that the light-dependent regulation of RuBPCase is controlled by *rca* (Zhang *et al.*, 2002) but recently, (Voelckel, Baldwin, 2003) demonstrated that the expression of *rca* in *N. attenuata* increased in response to attack by the mirid bug, *Tupiocoris notatus*. Hence it is possible that *rca* participates in the herbivore-induced downregulation of photosynthetic metabolism. In addition to the down-regulation of photosynthetic genes, genes involved in cell wall metabolism (*XTH4*, xyloglucan endotransglycosylase), glycolysis (DH63, homologous to *Petunia hybrida* triosephosphate isomerase; DH123 and *cGap*, both homologous to *N. tabacum* glyceraldehyde-3-phosphate dehydrogenase), and nitrogen metabolism (*nir*, nitrate

reductase; *GOGAT*, glutamine oxoglutarate aminotransferase; etc.) were also down-regulated. These alterations suggest many hypotheses about the regulation of primary metabolism in response to herbivore attack and require additional work.

Even though only a few of the sequences on this ‘Solanaceous’ microarray were designed from *S. nigrum* specific-sequences, it is clear from the verifications of selected array responses by RNA gel blot analysis and real-time qPCR (Fig. 3B-E) that the microarray provided valuable information about differential gene expression in *S. nigrum*. Several studies have established the utility of using sequence information of related species for monitoring gene expression. (Kane *et al.*, 2000) demonstrated that expression patterns derived from oligonucleotide (50-mer) microarrays reflected those from cDNA (~300-400bp PCR products) microarrays and that 50-mer oligonucleotides are specific, if the target sequences shared 80% or more with the oligonucleotide. (Izaguirre *et al.*, 2003) analyzed the transcriptome of *N. longiflora* with a cDNA microarray consisting of *N. attenuata* sequences and (Held *et al.*, 2004) used the same microarray to characterize transcriptional responses in *N. clevelandii* and *N. quadrivalvis*. (Girke *et al.*, 2000) compared gene expression in developing seeds of *Arabidopsis thaliana* and *Brassica napus* with an *Arabidopsis*-specific microarray. Regardless of whether the array is designed from homologous or heterologous sequences, responses should always be verified before a hypothesis about the functional significance of a gene is pursued. The need for verification is particularly acute when microarray studies suggest a lack of response, as negative results can be caused by small sequence differences between the oligonucleotides and the targeted transcripts. When a microarray produces signals, the spotted oligonucleotides will likely function as probes for screening *S. nigrum* cDNA libraries, thus facilitating the verification procedure. Once a transcriptional response is verified, the next step in an ecological analysis is to determine if the response correlates with a change in phenotype, not only in the laboratory but also in the field.

The hypothesis from the microarray analysis that flea beetle attack elicited JA signalling and thereby increased PI production was supported by the observations with both field- and greenhouse-grown plants that beetle attack and MeJA treatment significantly increased PI levels. The absolute levels of PI activity were higher in field-grown plants (Fig. 4), suggesting that plants growing in the rough-and-tumble of the natural world may be partially induced in comparison to the coddled plants grown in the glasshouse, which

excludes normal solar UV-B radiation. PIs are probably elicited by exposure to UV-B, as shown by (Izaguirre *et al.*, 2003), who used phenotypic measures and microarrays to compare transcriptional patterns in *N. longiflora* elicited by UV-B with the pattern elicited by *Manduca sexta* herbivory. In this study, UV-B exposure not only increased PI transcripts and activity but also downregulated photosynthesis-related transcripts in a manner similar to herbivore-elicited responses, suggesting that common regulatory elements had been recruited by this pair of abiotic and biotic stressors.

In response to herbivore attack, plants frequently activate indirect defense responses that complement the function of the direct defences (Kessler, Baldwin, 2002). By releasing VOCs in response to herbivore attack, plants attract predators and parasitoids of herbivorous insects (Dicke *et al.*, 2003; Kessler, Baldwin, 2001; Turlings *et al.*, 1990). When *S. nigrum* plants were attacked by herbivores from two different Orders of insects in the field (Fig. 6), the composition and the quantities of the VOCs trapped from the headspace of leaves significantly differed from those of unattacked plants. The fact that an induced response was observed in field-grown plants is significant, particularly in light of recent reports on *Zea mays* demonstrating just how significantly abiotic factors such as soil nutrition, air humidity, temperature, and light can influence the herbivore-induced VOC response (Gouinguene, Turlings, 2002; Schmelz *et al.*, 2003). The large and diverse predator community (syrphids, chrysopids, coccinellids and braconid wasps) we observed during our field studies of *S. nigrum* could respond to these VOC emissions. Whether any of these potential predators actually respond to increased VOCs can be readily tested by adding components of or the entire herbivore-induced volatile blend to plants containing a ‘predator-monitor’ (an herbivorous insect or egg used to score predation events: (Kessler, Baldwin, 2001).

Whether or not a trait can be formally considered to be a defense depends on whether it increases a plant’s Darwinian fitness in environments with aggressors. The best surrogate measures for Darwinian fitness are determined by a plant’s life history, but for selfing annual plants such as *S. nigrum*, lifetime seed production is likely to be an adequate measure. Fruit and seed production were found to be strongly correlated with plant above-ground dry mass (Fig. 6B), and these are an important measures for the resource partitioning to growth, reproduction and defense in response to biotic interactions (Bazzaz *et al.*, 1987; Givnish, 1986; Mauricio *et al.*, 1993). The reproductive output of plants that have been under strong artificial selection for particular yield components will frequently be strongly buffered from

variations in canopy size. For example, tomato plants are strongly buffered from leaf area loss from herbivores and do not decrease fruit number in response to herbivore attack (Thaler, 1999). We found that jasmonate elicitation of *S. nigrum* did not reduce plant size, a conclusion (Thaler *et al.*, 1996) had reached with regard to jasmonate treatment of tomato. Moreover, we found that jasmonate elicitation in *S. nigrum* significantly reduced lifetime fruit and seed production, although this is not observed in tomato (Thaler, 1999), which likely reflects differences between agronomic and native species in their selective history. Jasmonate elicitation is known to significantly decrease lifetime seed production in *N. attenuata* (Baldwin, 1998), and a large fraction of these fitness costs can be attributed to the induced production of PIs (Zavala *et al.*, 2004). The underlying mechanisms of *S. nigrum*'s fitness costs remain to be explored.

Direct manipulation of the genetic basis of an observed response is the most powerful means of falsifying functional hypotheses available to biologists. *Agrobacterium*-based transformation systems provide the means to produce stably transformed lines in which particular genes are silenced or over-expressed. Independently transformed lines are typically variable in their phenotypes due to the random insertion of the transgene into the genome, which, in turn, leads to differences in transcriptional activity ('positional' effects). Krügel *et al.* (2002) demonstrated that *N. attenuata* lines, transformed with an *asLOX* construct exhibited up to 71% reduction in wound-induced JA accumulation. This genetically determined phenotypic variation is enormously useful, because it allows the fitness consequences of a trait to be quantitatively analyzed. Also, possible pleiotropic effects resulting from single genetic changes must to be considered in the analysis of transgenic lines, and such effects require that multiple independently transformed lines with the same transgene must be examined before the phenotype can be attributed to the expression of the transgene. The *S. nigrum asRuBPCase* lines showed a clear reduction in *RuBPCase* transcripts (Fig. 2C), yet they did not reveal differences in their growth phenotype in comparison to wildtype plants in the glasshouse. Whether this lack of growth phenotype persists when these plants are grown under field conditions will be interesting to determine. Experiments in realistic environments may reveal why plants appear to be 'over-engineered' with respect to their RuBPCase pools (Matt *et al.*, 2002; Quick *et al.*, 1991). The rapid development of new transformation vectors that allow for more efficient silencing of endogenous genes (RNAi with inverted repeat elements: (Waterhouse, Helliwell, 2003), will

make the process of producing transformants with fully silenced genes more efficient, thereby facilitating the search for phenotypes of plants that are grown in complex environments.

Most of the tools presented in this paper have been used only in controlled laboratory experiments. The results obtained from such experiments can be different from those seen in plants growing in nature. For example, although constitutive PI levels were higher in field-grown plants than in greenhouse-grown plants, field-grown plants (Fig. 4) were still inducible. The combination of several stresses often provokes a potentiation of a response, leading either to an increase or a decrease of subsequent responses to the same or other stresses (Zimmerli *et al.*, 2000). Therefore the plant may recruit similar fundamental cellular reactions in response to various stresses, which may be what is happening in response to UV-B irradiation and herbivory (Izaguirre *et al.*, 2003) or cold and drought stress in comparison to disease resistance (Singh *et al.*, 2002). Experimentation with field-grown plants in which a variety of stresses are factorially manipulated will elucidate both the amount of cross-talk that occurs among environmental responses and also the fitness consequences of the different selective forces for plants whose ability to respond is selectively impaired.

While molecular biology has provided the ability to manipulate the expression of individual genes, understanding the functional consequences of these manipulations will require additional ecological tools to dissect the complex interplay of selective forces that all organisms face in nature. Multivariate and path analyses provide a means to examine correlations among the different levels of analysis that occur from gene expression to the formation of a phenotype with a given Darwinian fitness. Ecologists have been successful in using such approaches to evaluate complex correlations and to recognize interrelationships among the biotic and abiotic factors that structure ecosystems. The challenge remains to find a way to incorporate the powerful manipulative and descriptive molecular methods into this “big picture” analysis so as to harvest the fruits of the molecular revolution.

Acknowledgments

We thank Susan Kutschbach and Thomas Hahn for invaluable assistance in microarray processing and data analysis, Dr. Jörg Perner for assistance in determining beetles, Dr. Wolfgang Weisser for assistance in determining aphids, Dr. Tamara Krügel for valuable

support and advice and the Max-Planck-Gesellschaft and the Deutsche Forschungsgemeinschaft (project BA2138/1-1) for financial support.

Manuscript II

Specificity in ecological interactions. Attack from the same Lepidopteran herbivore results in species-specific transcriptional responses in two Solanaceous host plants

Dominik D. Schmidt, Claudia Voelckel, Markus Hartl, Silvia Schmidt, and Ian T. Baldwin*

Plant Physiology (2005) 138, 1763-1773

Max Planck Institute for Chemical Ecology
Department of Molecular Ecology
Beutenberg Campus
Hans-Knoell-Str. 8
07745 Jena, Germany

* Corresponding author:

Ian T. Baldwin
Max Planck Institute for Chemical Ecology
Department Molecular Ecology
Beutenberg Campus
Hans-Knoell-Str. 8
D-07745 Jena, Germany
Phone: ++ 49-3641-571100
Fax: ++ 49-3641-571102
E-mail: baldwin@ice.mpg.de

Abstract

Model systems have proven enormously useful in elucidating the biochemical function of plant genes. However their ecological function, having been sculpted by evolutionary forces specific to a species, may be less conserved across taxa. Responses to wounding and herbivore attack differ among plant families and are known to be mediated by oxylipin, ethylene and systemin signaling networks. We analyzed transcriptional responses of two native Solanaceous species to the attack of an herbivore whose elicitors are known not to be influenced by diet. With the TIGR 10k-cDNA potato microarray, we compared the transcriptional responses of *Nicotiana attenuata* with those of black nightshade (*Solanum nigrum*) when both were attacked by the Solanaceous generalist herbivore, *Manduca sexta*. Based on an *ndhF* (NADH dehydrogenase subunit F) phylogeny, *S. nigrum* is more closely related to potato than *N. attenuata*, but responded significantly less to *M. sexta* attack. Apart from transcriptional differences anticipated from their differences in secondary metabolism, both species showed distinct transcriptional patterns (with only 10% overlap in significantly regulated genes), which point to fundamental differences in the signaling cascades and downstream genes mediating herbivore resistance. The lack of transcriptional response of *S. nigrum* could not be attributed to its inability to respond to elicitation, because MeJA elicitation of *S. nigrum* resulted in a strong transcriptional response. Given that attack from the same herbivore elicits profoundly different responses in two Solanaceous taxa, we conclude that “blueprints” for commonly regulated responses to plant-herbivore interactions appear unlikely.

Introduction

Understanding the genetic basis of plant secondary metabolism will require multiple model systems (Kutchan 2001) because clades of plant species are biochemically specialized to produce particular classes of secondary metabolites (e.g. Brassicaceous taxa emphasize glucosinolates, while Solanaceous plants produce steroidal, tropane or pyridine alkaloids; Fraenkel 1959). Such metabolic specialization is thought to have evolved in response to selection pressures from the plant's enemies (Ehrlich and Raven 1964, Fraenkel 1959, Jones and Firn 1991). For example, the model Brassicaceous and Solanaceous plants, *Arabidopsis* and tomato, respectively, have each evolved a different arsenal of biochemical weapons that can be activated upon attack from herbivores (Walling 2000). While it has long been clear that the downstream defense responses of a plant would differ among taxa, the mechanisms that activate the defense responses are thought to be more conserved, and it has been assumed that *Arabidopsis* would provide a "blueprint" for the mechanisms that elicit ecological responses in higher plants (Mitchell-Olds 2001). However evidence is emerging that this "blueprint" has undergone some revisions at the family level.

Three plant hormones, jasmonic acid (JA), ethylene (ET), and salicylic acid (SA), mediate responses to wounding and attack from herbivores and pathogens in most taxa studied, but research on "crosstalk" among these signals has identified important differences at the family level. For example in *Arabidopsis*, wounding leads to the activation of two pathways: an oligosaccharide-dependent and a JA-dependent pathway in the damaged leaves (Walling 2000, Leon et al. 2001). The former produces ET, which antagonizes the elicitation of JA-responsive genes in the wounded leaves, but not in unwounded leaves from the same plant. In contrast, both ET and JA are required for the elicitation of wound-inducible genes in tomato (Walling 2000, Leon et al. 2001). In addition to differences in "crosstalk" among the same signal molecules, Brassicaceous and Solanaceous plants differ in the signal molecules that are recruited. Systemin, a polypeptide hormone, which mediates local and systemic responses to wounding, has only been found in Solanaceous species to date (Ryan 2000). In addition to these family-level differences, differences have also been found between taxa within the same family.

Within-family differences have been observed in the crosstalk between JA and ET signaling in the elicitation of defense responses. While JA and ET interact synergistically to

elicit proteinase inhibitor expression in tomato, attack from *Manduca sexta* larvae in *Nicotiana attenuata* elicits an ET burst, which antagonizes the JA-mediated increase in nicotine (Kahl et al. 2000, Winz and Baldwin 2001). Within-family differences in signals have also been found. Systemins of potato, bell pepper and black nightshade are structurally similar and differ each in three (of 18) amino acids from the tomato systemin and their ability to elicit tomato proteinase inhibitors (Constabel et al. 1998). Two tobacco systemins that elicit the accumulation of proteinase inhibitors have been identified, but these are structurally dissimilar to the tomato systemin (Pearce et al. 2001). Tobacco does not produce peptide signals that activate the tomato systemin receptor and does not react to tomato systemin (Scheer et al. 2003).

Given this evidence for differences in defense signaling among several Solanaceous taxa, we were interested in a more thorough characterization of the potential differences in responses to herbivore attack within a plant family. For this analysis, we compared the herbivore-induced transcriptome of two native Solanaceous species – *Nicotiana attenuata* and *Solanum nigrum* - to attack from the same native herbivore, *M. sexta* larvae. Three aspects of this analysis make this a valuable comparison. First, we analyze the responses of two native plants, in which the responses observed to herbivore attack are not confounded by a history of artificial selection for yield-associated traits. Second, we measure the responses to attack by a shared native herbivore (Fraenkel 1959), which is particularly well-studied with regard to how it elicits responses in its host plants. *M. sexta* larvae produce a suite of 8 fatty acid-amino acid conjugates (FAC) which are thought to be introduced into wounds during feeding and are necessary and sufficient to account for all of the observed changes in the plant's wound response - including defense metabolites, signals and transcriptional responses - that are elicited by larval feeding (Halitschke et al. 2001, 2003; Roda et al. 2004). Simply adding these FACs to plant wounds simulates the plant's responses to herbivore attack. Moreover, *M. sexta*'s FAC profile is not substantially altered when it feeds on different host plants (Alborn et al. 2003) and specifically does not quantitatively or qualitatively change when it feeds on *N. attenuata* or *S. nigrum* (D.D. Schmidt, A. Steppuhn and R. Halitschke, unpublished results). Third, the secondary metabolites produced by both species are well-studied and provide a valuable backdrop against which to compare their herbivore-elicited transcriptomes. *N. attenuata* and *S. nigrum* constitutively produce nicotine and glycoalkaloids (Baldwin 1999, Dopke et al. 1987, Ridout et al. 1989), respectively, and both species are capable of

producing proteinase inhibitors in response to wounding and release monoterpenoid and sesquiterpenoid volatiles in response to herbivore feeding (Van Dam et al 2001, Kessler and Baldwin 2001, Schmidt et al. 2004). In summary, for this pair of hostplants, attack by *M sexta* larvae is likely to provide a standardized elicitation of the responses to herbivore attack, which, in turn, are likely sculpted by natural selection.

In order to provide an unbiased comparison of the herbivore-regulated transcriptome in both species, we used a microarray with over 10,000 potato cDNAs (representing approximately a third of the potato genome). We were particularly interested in the scope of the response (how many genes are involved?) and the specificity of the response (how many transcripts are commonly and specifically regulated?). The potato microarray was established through the NSF Potato Functional Genomics project (<http://www.tigr.org/tdb/potato>) and is available from The Institute of Genomic Research (TIGR, Rockville, Maryland, USA). The scope and specificity of the response could be influenced by the taxonomic similarity between the source of the genes on the array (*S. tuberosum*) the samples used in the hybridizations (*S. nigrum* and *N. attenuata*). To clarify the phylogenetic relationship among the three species, we analyzed sequence similarities among the plastidial *ndhF* gene of several Solanaceae species. Based on this analysis, *S. nigrum* is more closely related to *S. tuberosum* than *N. attenuata*, which generated the expectation that the array analysis would likely reveal a stronger response from *S. nigrum* than from *N. attenuata*. However, we found the opposite to be true - *S. nigrum* showed a weaker response to *M. sexta* than *N. attenuata*. To test the hypothesis whether *S. nigrum* is generally less responsive, we examined the transcriptional responses of *S. nigrum* after elicitation by methyl jasmonate (MeJA), the volatile derivative of JA. MeJA is known to be both a product and elicitor of the oxilipin signaling pathway, the signal cascade that mediates many defense responses to herbivore and pathogen attack (Walling 2000).

Results

Phylogenetic analysis

To clarify the phylogenetic relationship between *N. attenuata* and *S. nigrum*, we analyzed sequence similarities among the plastidial *ndhF* gene of several Solanaceae species. Based on this comparison, *S. nigrum* is more closely related to *S. tuberosum* than *N. attenuata*, as

demonstrated by a 97% homology between *S. nigrum* and *S. tuberosum* and 94% between *N. attenuata* and *S. tuberosum* (Fig. 1).

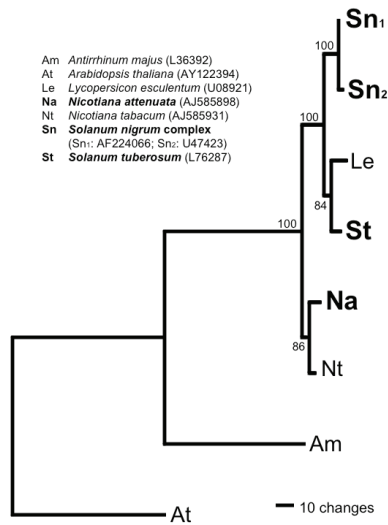


Figure 1: The single most parsimonious tree recovered using sequences of *ndhF* (length=372, CI=0.949, RI=0.835). The two species of interest (*N. attenuata* and *S. nigrum*) are in bold as is the source species for the cDNA clones spotted onto the TIGR potato array (*S. tuberosum*). Branch lengths correspond to parsimony steps and the numbers above branches indicate parsimony bootstrap support values for that clade.

Microarray analysis of *M. sexta*-induced responses

Three replicate TIGR chips were hybridized with RNA from thrice replicated *M. sexta*-infested *N. attenuata* plants and *M. sexta*-infested *S. nigrum* plants. Of 11,243 cDNAs, a total of 754 were regulated (mean ratio >1.5 or <0.67 for up- and down-regulated genes, respectively) in either *N. attenuata*, *S. nigrum* or both species in response to *M. sexta* attack (Fig. 2). Interestingly, there were more genes up-regulated (561) than down-regulated (203) and only 75 cDNAs (10%) were equally regulated in both species (Fig. 2). When the expression ratios of the 754 responsive cDNAs were subjected to a cluster analysis, the three *N. attenuata* arrays were clearly separated from the three *S. nigrum* arrays and patterns of commonly and specifically regulated genes were discernable (Supplementary Fig.1). Based on the annotations of the 754 regulated cDNAs, we grouped the genes into functional categories (Supplementary Table 1) and in the following use this classification to discuss some striking differences in gene up-regulation that occur between these two Solanaceous species. For the details (expression ratios, annotations, categories) of gene down-regulation see Supplementary Table 1.

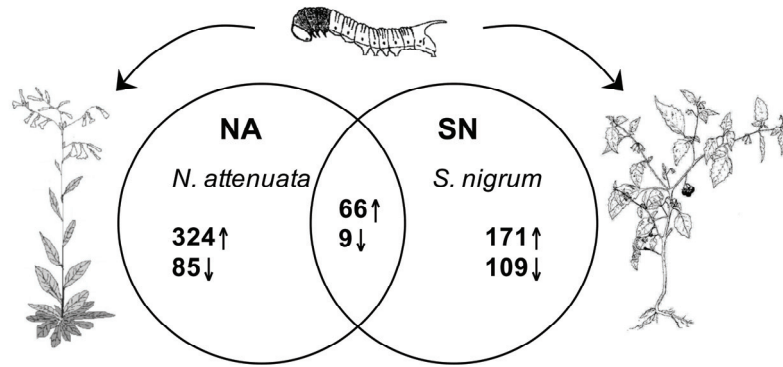


Figure 2: Venn diagram of the numbers of overlapping and non-overlapping significantly up-regulated (↑; ER>1.5) or down-regulated (↓; ER<0.67) genes in *N. attenuata* and *S. nigrum* that are elicited by *M. sexta* herbivory. In summary, 561 and 203 genes were found to be up- and down-regulated, respectively, but only 10% had the same regulation in both plant species. (*S. nigrum* from: http://www.rmc.sierraclub.org/outings/images/weeds_blacknightshade_drawing.jpg)

Signal transduction

Additionally to a common up-regulation of lysophospholipase and lipoxygenase (oxylipin signaling), *N. attenuata* and *S. nigrum* activate different signal cascades in response to leaf-chewing *M. sexta* larvae. Increases in genes coding for a G-protein-coupled receptor, a GTP-binding protein, phospholipase C, diacylglycerol kinase, calmodulin, annexin, a Ca²⁺-activated kinase and a Ca²⁺-activated ion channel indicate calcium- and inositol phospholipid-based signaling, as well as, G-protein-mediated signaling in *N. attenuata*. Moreover, the generation of 2-hydroperoxides (α -dox) and glucosylated salicylate (UDP-glucose:SA glucosyltransferase) appears to be specific to *N. attenuata*, while a zeatin-glucosyltransferase and a 12-oxo-phytodienoate reductase seem to have in role in signaling in *S. nigrum*. An auxin-amino acid hydrolase and several kinases (calcium-dependent kinases, receptor kinases, and MAP kinases) are activated in both species.

Proteolysis

Among the genes coding for proteolytic enzymes, a leucine aminopeptidase (LAP), which catalyzes the release of N-terminal residues from proteins and peptides, was up-regulated up to 20-fold in *S. nigrum*. In contrast, increases in ubiquitin-mediated proteolysis (polyubiquitin, ubiquitin-conjugating enzyme) are specific to *N. attenuata*. An up-regulation

of protein disulfide isomerase and peptidylprolyl isomerase was found in both species, but the response of the latter was stronger in *S. nigrum*.

	Both	NA	SN
Signal transduction (hormone synthesis, second messenger synthesis, receptors, kinases)	auxins (IAA-amino acid hydrolase 2x) oxylipins (lipoxygenase 6x) kinases 12x lipases (lysophospholipase 2x)	calcium 7x (calmodulin, calcium-dependent kinase 2x, calmodulin-binding ion channel, annexin 2x) DAG (diacylglycerol kinase) lipases 3x (patatin, phospholipase C 2x) G proteins (GTP binding protein, G protein-coupled receptor) oxilipins 2x (a-dox 2x) SA (UDP-glucose:SA glucosyltransferase)	cytokinins 3x (zeatin O-glucosyltransferase 3x) oxilipins (OPR1)
DNA binding proteins (putative trans-activating factors)	5x (SPF1, AT-hook DNA binding protein, SCARE-CROW gene regulator, zinc finger protein)	6x (MADS box protein, bHLH factor, NDX1, WRKY 22, homeobox protein)	8x (WRKY65, RING protein, KNAT3 homeodomain protein, Yabby2, zinc finger protein, heat shock factor, dehydration-responsive element binding protein)
transcription, translation, proteolysis, protein metabolism	tl (RNA helicase, RNA binding protein) prot 4x (cysteine protease 3x, protease related) protm (peptidylprolyl isomerase 4x, protein disulfide isomerase 5x)	tl (40S ribosomal protein S2) prot 9x (polyubiquitin 3x, ubiquitin conjugating enzyme 2x, serine carboxypeptidase 2x, subtilisin, Ftsh protease)	tc (sigma factor 2) tl 8x (elongation factor 2x, initiation factor, ribosomal protein S9, RNA binding protein F6N18, 17 A. thaliana 4x) prot 4x (leucine aminopeptidase 3x, endopeptidase)
Secondary metabolism (putative direct and indirect defense metabolites, e.g. polyphenol oxidases, terpenoids, polyamines, green leaf volatiles, shikimates, digestibility reducers, phenylpropanoids, flavonoids, green leaf volatiles, sterols)	pa (spermidine synthase 3x) terp (HMGR2) glv (HPL 2x)	PPOs 8x (PPOA 2x, PPOB, catechol oxidase 4x) shik 8x (chorismate synthase 3x, chorismate mutase, dehydroquinate synthase 3x, shikimate kinase) phepro 9x (PAL 2x, 4CL 2x, C4H, coumaryl shikimate hydroxylase 2x, cinnamoyl CoA reductase) pa 6x (SAMDC 6x) terp 11x (IPP isomerase 4x, DOX reductoisomerase 4x, DOX synthase, linalool synthase) flav 3x (rhamnosyltransferase 2x, dihydroflavonol reductase)	flav (leucanthocyanidin dioxygenase-like protein) terp 11x (FPPS1 3x, sesquiterpenes synthase 1, sesquiterpenes synthase 2 2x, germacrene C synthase 2x, HMGS, HMGR1 farnesyltransferase, sabinenesynthase) dig red (proteinase inhibitor 1 3x) ste 4x (sterol C-14 reductase 2x, 7-dehydrocholesterol reductase, 3-beta hydroxysteroid dehydrogenase)
Primary metabolism (cell wall, carbohydrates, amino acids, nucleotides, photosynthesis, precursors for secondary metabolites)	cw (PME 5x, extensin 5x, glucan glucosidases 3x) carb (fructose biphosphate aldolase 2x, GAPDH) lipids (acyl-CoA synthetase 2x)	putrescine 6x (ODC 2x, ADC 4x) SAM 10x (SAM synthetase 1 and 3) acetyl CoA 2x (acetyl CoA synthase) malonyl CoA (acetyl CoA carboxylase) cw (XET 2x, glucan endo glucosidase polygalacturonase inhibiting protein) carb 21x (formate dehydrogenase 2x, starch synthase 4x, ATP citrate lyase 3x) aa 4x (glutamate synthase 2x, aspartate transaminase, aminotransferase) nuc 7x (APRT 3x, nucleotide sugar epimerase 2x, UPRT, nucleoside triphosphatase)	cw 2x (expansin, cellulose synthase) aa 6x (tryptophan decarboxylase, serine acetyltransferase, amino acid transporter, N-acetylornithine deacetylase 2x) lipids (diacylglycerol acyltransferase) ps 3x (Rubisco, Lhcb6, thioredoxin H)
Stress responses (chaperones, dehydration, herbivory, nutrient limitation, pathogens, oxidative)	chap 6x (endoplasmic 2x, calreticulin, calnexin, luminal binding protein, HSP90)	chap (DNAJ protein) dehyd (dehydration-induced protein ERD15 2x) herb (vegetative storage protein) nulim (iron-stress related protein) path (TMV-response-related gene) ox (superoxid dismutase 2x, phospholipid hydroperoxide glutathione peroxidase 3x)	chap 7x (HSP83, HSP20, HSP17.6 2x, heat shock protein MTSHT, chaperone hsc70-3, HSP18) dehyd 3x (dehydration-induced protein RD22 2x, dehydrin) herb (whitefly-induced gp91-phox) nulim (autophagy8i) path (peroxidase prx14, Prf) ox (glutathione transferase 2x)
Selected genes	cytochrome P450s 6x, acid phosphatase 3x, unknown protein TC58032 3x	membrane protein YLR436c 3x, membrane related protein C5 2x, metal-transporting ATPase 4x, putative protein TC57777 4x, hypothetical protein TC65797 3x	2-oxoglutarate-dependent dioxygenase 2x, 2-oxoglutarate dehydrogenase

Figure 3: Categories of genes up-regulated in response to *M. sexta* herbivory with examples from both plant species (Both: clear boxes), *N. attenuata* (NA: darkly shaded boxes), and *S. nigrum* (SN: lightly shaded boxes; multiple clones for the same gene are indicated in parentheses).

Secondary metabolism

Little overlap between *N. attenuata* and *S. nigrum* was found in the expression of genes involved in secondary metabolism, including up-regulation of hydroperoxide lyase, an important gene in the synthesis of C6 volatile organic compounds (VOCs), spermidine synthase, which is involved in polyamine synthesis and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR2), the key enzyme in the cytosolic route to the terpenoid precursor isopentenyl pyrophosphate (IPP). In *S. nigrum*, HMGR1 is also up-regulated and it has been shown for potato that HMGR2 is mainly involved in the biosynthesis of sesquiterpenes, whereas HMGR1 produces precursors for sterol biosynthesis (Choi et al. 1994). Moreover, enzymes involved in the biosynthesis of sterols, such as cholesterol (7-dehydrocholesterol reductase), were up-regulated in *S. nigrum*. Sterol contents in *Solanum* species are tightly linked to levels of glycoalkaloids and cholesterol is thought to be a precursor of steroidal alkaloids (Bergenstrahle et al. 1996), which in turn suggests increased production of glycoalkaloids in *S. nigrum* in response to *M. sexta* feeding. Further evidence for the deployment of alkaloidal defenses in *S. nigrum* is the up-regulation of a 2-oxoglutarate-dependent dioxygenase, which is homologous to hyoscyamine 6 β -hydroxylase of *Hyoscyamus niger* (Lantin et al. 1999). Up-regulated genes of terpenoid biosynthesis include farnesyl pyrophosphate synthase and several sesquiterpene synthases, which are among the most strongly up-regulated genes in *S. nigrum*. It is known that *S. nigrum* produces a rich bouquet of VOCs in response to herbivory from flea beetles or the moth *Acherontia atropos* (Schmidt et al. 2004).

In contrast to *S. nigrum*, *N. attenuata* induces transcripts involved in the plastid-localized glyceraldehydes/pyruvate pathway (DOX synthase, DOX reductoisomerase) of IPP production, suggesting that not only sesquiterpenes but also mono- and diterpenes, are elicited in *N. attenuata* upon *M. sexta*-herbivory. This is supported by the up-regulation of linalool synthase. IPP isomerase, which is recruited by both terpenoid pathways, is specifically up-regulated in *N. attenuata*.

Also in contrast to the response in *S. nigrum*, *N. attenuata* plants elicit a strong transcriptional commitment to the production of phenol-based secondary compounds. Starting with the synthesis of shikimate (dehydroquinate synthase, shikimate kinase, 3-phosphoshikimate 1-carboxyvinyltransferase (EPSP synthase), chorismate synthase), preceding to the synthesis of prephenate (the committed step in phenylalanine and tyrosine

synthesis catalyzed by chorismate mutase), continuing with the synthesis of cinnamic acid (PAL), p-coumaric acid (cinnamate-4-hydroxylase) and p-coumaroyl-CoA (coumaric acid-CoA ligase), the genes providing the precursors for flavonoid- and phenylpropanoid biosynthesis are induced. Transcripts related to flavonoid metabolism (UDP rhamnose-anthocyanidin-3-glucoside rhamnosyltransferase, dihydroflavonol reductase) and phenylpropanoid metabolism (p-coumaroyl shikimate 3'-hydroxylases, cinnamoyl-CoA reductase) were up-regulated as well. These results are consistent with the following earlier findings in the *M. sexta* - *N. attenuata* interaction: 1. the cloning of a UDP rhamnose-anthocyanidin-3-glucoside rhamnosyltransferase by DDRT-PCR from *M. sexta*-induced *N. attenuata* plants (Voelckel and Baldwin 2003), the production of phenylpropanoid-derived compounds (caffeoyl-putrescine, chlorogenic acid) in *M. sexta*-attacked *N. attenuata* plants (Kessler and Baldwin 2004) and the production of flavonoids (quercetin, rutin) in *N. attenuata* leaf trichomes (Roda et al. 2004). Several transcripts for polyphenyl oxidases, which catalyzes the production of o-quinones, which, in turn react with insect dietary proteins and impair their digestion (e.g. Constable et al. 2000), increase in abundance specifically in *M. sexta*-attacked *N. attenuata*.

Proteinase inhibitor induction, an induced defense response well characterized in *Manduca* attacked *N. attenuata* (Zavala et al. 2004, Zavala and Baldwin 2004) was seen only in *S. nigrum*, probably because of the lack of tobacco-specific probes on the array.

Primary metabolism

Manduca attack up-regulated in both plant species genes involved in cell wall biosynthesis, such as pectin methyl esterase (PME) and extensin; genes related to lipid metabolism (acyl-CoA synthetase); and genes that play a central role in carbohydrate metabolism (e.g. glyceraldehyd 3-phosphate dehydrogenase). Interestingly, transcripts for acetyl-CoA carboxylase which catalyzes the formation of malonyl-CoA, an essential precursor of flavonoid biosynthesis, increased specifically in *N. attenuata*. Other precursors of secondary metabolism whose production is elicited in *N. attenuata* include acetyl-CoA, S-adenosylmethionine, and putrescine. Putrescine may be needed for nicotine and polyamine synthesis and S-adenosylmethionine may be used in ethylene and polyamine synthesis. The up-regulation of two different isoforms of S-adenosylmethionine decarboxylase (*secondary metabolism*) suggests that a supply of decarboxylated S-adenosylmethionine is required to

complement the supply of putrescine for higher polyamine synthesis. Additional evidence of tobacco-specific regulation in primary metabolism includes increases in xyloglucan endotransglycosylase, starch synthase, ATP citrate lyase, adenine phosphoribosyl transferase and nucleotide sugar epimerase. *S. nigrum* up-regulates genes involved in cell wall biosynthesis (cellulose synthase), amino acid metabolism and lipid synthesis. The most striking difference with the responses of primary metabolism in *N. attenuata* was the up-regulation of photosynthesis-related genes (e.g. Rubisco, light-harvesting complex b6) in caterpillar-infested *S. nigrum* plants. In general, herbivory is thought to down-regulate photosynthesis genes and, thereby, divert resources to secondary metabolism (e.g. Hermsmeier et al. 2001).

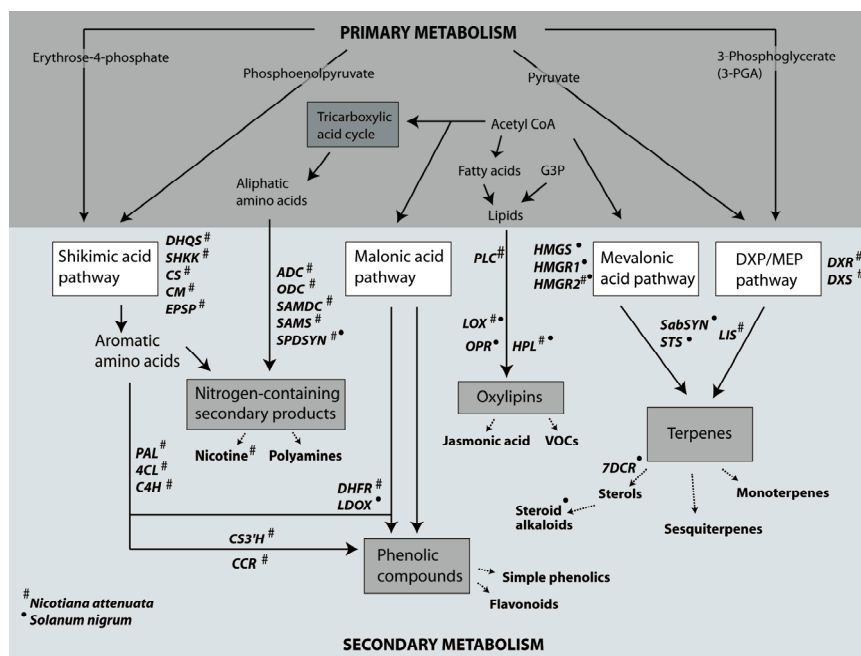


Figure 4: Simplified overview of major pathways of secondary-metabolite biosynthesis and their interrelationships with primary metabolism (modified from Gershenzon 2002). Genes found to be up-regulated are italicized and examples of Solanaceous secondary compounds are listed for some classes of secondary metabolites. Genes up-regulated in *N. attenuata* (#), *S. nigrum* (•), or both (#,•) are indicated. Abbreviations: *4CL* 4-coumarate coenzyme A ligase; *7DCR* 7-dehydrocholesterol reductase; *ADC* arginine decarboxylase; *C4H* cinnamate-4-hydrogenase; *CCR* cinnamoyl-CoA reductase; *CM* chorismate mutase; *CS* chorismate synthase; *CS3'H* *p*-coumaroyl shikimate 3'-hydroxylase; *DHFR* dihydroflavonol reductase; *DHQS* 3-dehydroquininate synthase; *DXR* 1-deoxy-D-xylulose-5-phosphate reductoisomerase; *DXS* 1-deoxy-D-xylulose-5-phosphate synthase; *EPSP* 5-enolpyruvylshikimate-3-phosphate synthase; *G3P* glycerol-3-phosphate; *HMGR* 3-hydroxy-3-methylglutaryl coenzyme A reductase; *HMGS* 3-hydroxy-3-methylglutaryl coenzyme A synthase; *HPL* hydroperoxide lyase; *LDOX* leucanthocyanidin dioxygenase; *LIS* linalool synthase; *LOX* lipoxygenase; *ODC* ornithine decarboxylase; *OPR* oxophytodienoate reductase; *PAL* phenylalanine ammonia lyase; *PLC* phospholipase C; *SabSYN* sabinene synthase; *SAMDC* S-adenosyl methionine decarboxylase; *SAMS* S-adenosyl methionine synthase; *SHKK* shikimate kinase; *SPDSYN* spermidine synthase; *STS* sesquiterpene synthase(s); *VOCs* volatile organic compounds.

Stress responses and selected genes

The stressed metabolic state of herbivore-attacked plants is reflected in the up-regulation of chaperones (HSP90, luminal binding protein, endoplasmic, etc.), dehydration induced proteins (with distinct genes elicited in both species, e.g. dehydrin in *S. nigrum*) and several cytochrome P450 monooxygenases. Among the most strongly up-regulated genes in both species were a putative acid phosphatase and the EEF53 gene. *N. attenuata* induces a vegetative storage protein reported to be herbivory-induced in *Arabidopsis* (Berger et al. 2002), a salt stress-related phospholipid-hydroperoxide glutathione peroxidase (Mittova et al. 2002), a TMV response related gene, an iron-stress related protein, a superoxid dismutase and a metal-transporting ATPase. A remarkably large number of chaperones are induced only in *S. nigrum*. Further stress-related responses in *S. nigrum* include the up-regulation of enzymes involved in oxidative stress, such as an herbivory-induced NADPH oxidase subunit (whitefly-induced gp91-phox), a peroxidase, and a glutathione transferase.

Verification of microarray data by TaqMan® real-time PCR

The results of the quantitative PCR analysis for the genes α -DOX, HPL, LOX and XTH confirmed the expression ratios obtained with microarrays in *N. attenuata* (Supplementary Table 2). The relative expression ratios of α -DOX, LOX and XTH were significantly higher (approximately two-fold) than the microarray expression ratios. Hence, the microarray data seem to even underestimate the magnitude of the gene expression differences.

*Microarray analysis of MeJA-induced responses of *S. nigrum**

The weaker response of *S. nigrum* to *M. sexta* herbivory in comparison to the response of *N. attenuata* raised the question whether *S. nigrum* was a non-responding species. To test this hypothesis, we hybridized three additional microarrays with RNA from three biological replicates of MeJA-treated plants. Of 11,243 analyzed clones on the microarray, 339 were differentially regulated ($263 > 1.5$; $76 < 0.67$) in *S. nigrum* in response to MeJA elicitation (about 3 % of the total number of spotted clones; Supplementary Table 3). In accordance with the ubiquitous role of oxilipins (JA, MeJA) in mediating plants' responses to herbivory, 72 genes are commonly up-regulated in *S. nigrum* in response to MeJA or to *M. sexta* (Figure 5). Again LAP was among the highest up-regulated genes in MeJA-elicited plants and further up-

regulated genes are involved in oxylipin biosynthesis (LOX), secondary metabolism (HMGR, FPS, PIs), synthesis of chaperones (BiP), to name just a few (Supplemental Table 3). A majority of the up-regulated genes (191) were up-regulated only in MeJA-elicited plants clearly demonstrating that *S. nigrum* is fully capable of responding to elicitation. These genes included genes involved in secondary metabolism (e.g. shikimate 5-dehydrogenase), transcription factor genes (e.g. C2H2 zinc finger protein), genes related to ubiquitin-mediated proteolysis (clone # STMDG12, STMHH55) and genes coding for several cytochrome P450s. A subset of 33 genes were commonly up-regulated in MeJA-elicited *S. nigrum* and *M. sexta*-infested *N. attenuata* plants (Figure 5B) and were dominated by genes involved in primary metabolism (carbohydrates) and secondary metabolism (phenylpropanoids, terpenoids). A similar sized (33) subset of genes were commonly up-regulated among all three treatments and were dominated by genes related to oxylipin signaling and chaperones.

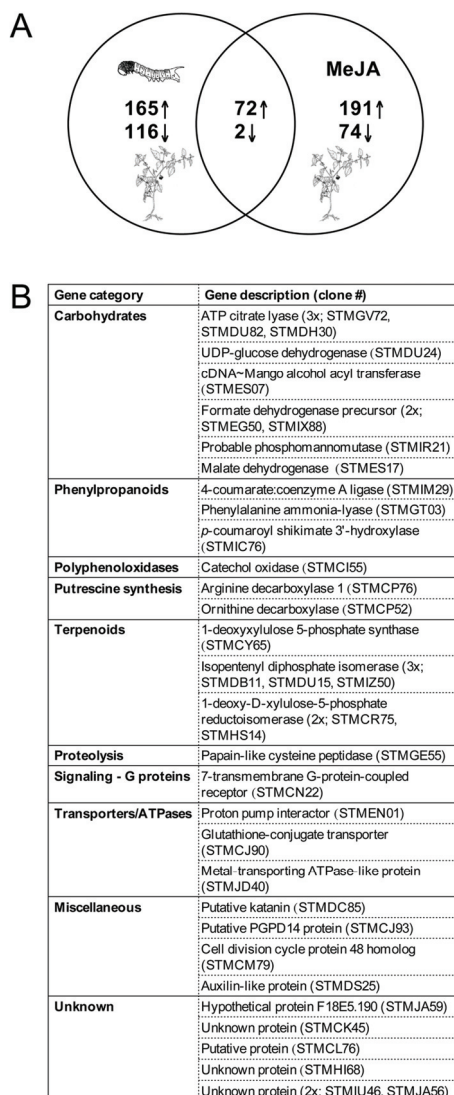


Figure 5: Transcriptional responses of *S. nigrum* to MeJA and *M. sexta*. (A) Venn diagram of the numbers of overlapping and non-overlapping significantly up-regulated (\uparrow ; $ER > 1.5$) or down-regulated (\downarrow ; $ER < 0.67$) genes in *S. nigrum* that are elicited by MeJA treatment or *M. sexta* herbivory. (B) Overlapping up-regulated genes of *M. sexta*-infested *N. attenuata* and MeJA-elicited *S. nigrum*. *S. nigrum*'s weaker response to *M. sexta* is not due to a basic unresponsiveness, because MeJA induced the expression of many genes that are up-regulated in *N. attenuata* in response to *M. sexta* herbivory, such as genes related to carbohydrate or secondary metabolism (multiple clones for the same gene are indicated in parentheses).

Discussion

Small scale microarrays are often criticized for their bias in gene selection, which makes them suitable for answering the questions for which they were designed but unsuitable for other research questions. Here we used the potato 10K-cDNA clone microarray (TIGR) to provide an unbiased comparison of the transcriptional responses of two Solanaceous species, *N. attenuata* and *S. nigrum*, to herbivory from the Solanaceous generalist, *M. sexta*. Based on the diversity of secondary metabolites produced by Solanaceous plants (e.g. Frohne and Jensen 1992) and the suite of signals that mediate plant responses to herbivory in this family (Walling 2000, Leon et al. 2001), we hypothesized that the analysis of *M. sexta*-elicited transcriptomes would yield two major results: (1) attack would elicit increases in secondary metabolism in both species, but the elicited pathways would differ, reflecting the different secondary metabolites constitutively produced by the two species and (2) signal transduction would be similar. After having found drastic differences between the two plant species and a clearly weaker transcriptional response in *S. nigrum*, we asked whether this plant species generally less responsive and tested this hypothesis with an additional microarray experiment with MeJA-elicited *S. nigrum*. We discuss our findings in the light of the outlined predictions and relate the results to what is known from other plant species. First, we evaluate what we learned about the *M. sexta*-responsive transcriptome in *N. attenuata* with a customized oligonucleotide microarray (Voelckel and Baldwin 2004) as compared to the large scale TIGR array.

The TIGR array analysis confirmed previously measured increases in the expression of jasmonate cascade genes and of genes known to be positively regulated by jasmonate signaling, such as genes involved in green leaf volatile, polyamine or phenylpropanoids synthesis. Additionally, the TIGR array analysis found many more genes representing the above mentioned branches of metabolism, including p-coumaroyl shikimate 3'-hydroxylase and cinnamoyl-CoA reductase (phenylpropanoids and their conjugates, Gang et al. 2002), SAM synthetase and spermidine synthase (polyamines), arginine- and ornithine decarboxylase (precursors for polyamines and nicotine) and several polyphenol oxidases to be induced. The TIGR array analysis also revealed the activation of almost every gene in the shikimate pathway, the plastidic route to terpenoid synthesis and additional cell wall related genes (pectin methyl esterase, extensin), to name just a few of the genes not monitored with the oligonucleotide array. Disagreements between the two analyses were mainly due to four

reasons: 1) Some genes were not present on the TIGR array (e.g. thionins); 2) differential regulation was observed in less than three biological replicates (e.g. proteinase inhibitors) and hence the gene was automatically excluded from further analysis; 3) some genes were not regulated in the TIGR array analysis (e.g. Rubisco). 4) The raw signals of the hybridized TIGR arrays were not interpretable (e.g. threonine deaminase). In general, the large scale transcriptional analysis with the TIGR array has substantially extended our understanding of the plastic responses of *N. attenuata* to *M. sexta* attack, and additionally allowed for a detailed comparison of these responses with those elicited in *S. nigrum* by this Solanaceous generalist.

Apart from the anticipated differences in alkaloid (steroidal alkaloids in *S. nigrum*) and alkaloid/polyamine precursor (putrescine in *N. attenuata*) formation, our analysis revealed the production of different defense metabolites in both species. *N. attenuata* predominantly elicited genes for the production of antinutritive polyphenol oxidases, phenylpropanoids and their precursors, and plastidic isopentenyl pyrophosphate, which is primarily channeled into mono- and diterpene synthesis (Lichtenthaler 1999). In *S. nigrum*, the transcriptional emphasis was on sesquiterpene synthesis. Both species likely increase flavonoids, polyamines and green leaf volatiles in response to attack. Analyses of the metabolome are needed to test the predictions emerging from this transcriptional analysis.

Contrary to our assumption that the two species would activate a similar suite of signaling genes, *N. attenuata* and *S. nigrum* activated different sets of signaling genes and in *N. attenuata* more signaling cascades appeared to be up-regulated. Jasmonic acid signaling was regulated in both species; calcium-based signaling, inositol-phospholipid signaling, and G-protein-mediated signaling were found to be specific to *N. attenuata*, and cytokinin signaling was only detected in *S. nigrum*. While calcium-signaling has been implicated in systemin-mediated activation of phospholipase A2 and subsequent jasmonic acid synthesis (Ryan 2000), the role of phospholipase C and heterotrimeric G proteins in defense signaling in Solanaceous plants is largely unexplored. The only prosystemin gene on the array was up-regulated in two of three replicates of *S. nigrum* but did not yield interpretable results in *N. attenuata*, consistent with the structural dissimilarities of potato and tobacco systemins (Scheer et al. 2003; Ryan 2000).

Our analysis also provided a deeper insight into the changes that accompany alterations in signaling and secondary metabolism, namely changes in carbohydrate and

amino acid metabolism, nucleic acid and protein metabolism or changes associated with other biotic or abiotic stressors. For example, both plants increased similar and dissimilar transcripts for chaperones, dehydration-related genes and oxidative stress genes. Transcript levels of enzymes involved in protein degradation / processing were increased in both species but with distinct differences. Ubiquitin-related proteolysis, which plays an important role in eliminating misfolded or abnormal proteins that probably accumulate in stressed plants, is activated only in *N. attenuata*. Furthermore, ubiquitin-dependent protein turnover influences many cellular processes by modulating levels of regulatory proteins (Hare et al. 2003) and there is increasing evidence that ubiquitin-dependent proteolysis is essential in regulating oxilipin-mediated plant responses. An important element in JA signal transduction, COI1 (CORONATINE INSENSITIVE 1), is part of an E3-type ubiquitin ligase complex and tomato mutants expressing non-functional COI1 are compromised in their resistance to two-spotted spider mites (Xie et al. 1998, Devoto et al. 2002, Li et al. 2004). Hence, ubiquitin-dependent proteolysis may play an important role in regulatory networks that mediate defense responses of *N. attenuata*.

In *S. nigrum* other genes involved in protein metabolism are induced by *M. sexta* herbivory, with the most dramatic example being a LAP gene whose expression was up-regulated 20 fold. LAPs are present in pro- and eukaryotes and plant LAPs have been intensively studied in tomato, where they are elicited by JA, wounding and pathogens and are present in floral tissues (Tu et al. 2003 and references therein). Although tomato LAPs are biochemically and physiologically well characterized, their function in plant defense responses remains elusive. Animal aminopeptidases are involved in modulation of peptide and protein activities (Barr 1991) and therefore it is tempting to speculate that a LAP cleaves precursors of polypeptide hormones that are known especially among Solanaceous plants (Ryan et al. 2002). Alternatively, wound-induced LAPs may have a more general role in the turnover of proteins that are essential components of plant defense responses (Walling et al. 1995). The fact that LAP was also highly up-regulated on the arrays of MeJA-elicited plants emphasized the importance of this gene in defense responses of *S. nigrum*.

When *S. nigrum* was treated with MeJA, a derivative of the jasmonate cascade which is largely responsible for mediating herbivore resistance, branches of secondary metabolism and signaling were activated that the previous microarray experiment had only shown for *N. attenuata*'s responses to *M. sexta* attack. These included genes involved in phenylpropanoid

and shikimate biosynthesis, the plastidic pathway of terpenoid production, genes involved in ubiquitin-mediated proteolysis and a gene related to G protein signaling. Hence, the genuinely weaker transcriptional response of *S. nigrum* to *M. sexta* herbivory can not be explained by *S. nigrum*'s inability to respond to elicitation. *N. attenuata* has likely had a longer evolutionary association with *M. sexta* and hence has had more time to evolve a strong response to *M. sexta* attack. *S. nigrum* appears not to have evolved strong responses to this herbivore, but has to others, e.g. flea beetles (Schmidt et al. 2004).

In summary, the transcriptional comparison of *M. sexta* attacked *N. attenuata* and *S. nigrum* plants did not support the existence of a Solanaceous "blueprint" for herbivore defense. Most strikingly, the differences extended beyond the activation of different alkaloid pathways and included a profound divergence in signaling pathways mediating the elicited responses. The likely difference in the length of their evolutionary associations with *M. sexta* may account for the differences in their responses.

Materials and methods

Plant and insect growth and experimental setup

Seeds of a *Nicotiana attenuata* inbred line were smoke-germinated on Phytigel as described by Krügel et al. (2002). Twelve days after germination seedlings were planted in soil into Teku pots (Waalwijk, the Netherlands), and after 12 additional days, transferred to 0.5 L pots with a peat based substrate (Klasmann Tonsubstrat, Geeste-Groß Hesepe, Germany). Seeds of a *Solanum nigrum* inbred line were germinated as described in Schmidt et al. (2004) and ten day-old seedlings were transferred to 0.5 L pots with the same substrate as for *N. attenuata*. Both plant species were grown in the glasshouse of the Max Planck Institute for Chemical Ecology (Jena, Germany) at 24-26°C (16h light; supplemental lighting by Philips Sun-T Agro 400 and 600 W sodium lights; 65% humidity).

Insect treatment

One day prior to the herbivore treatment, 24 randomly selected plants of each species were placed in glass insect cages (30cm x 30cm x 60cm, each cage accommodating 4 plants). Twelve plants were used in both herbivore and control treatment; plants were harvested

individually and later RNA from the four plants of a single cage were pooled to provide one biological replicate of the experiment. Eggs of *Manduca sexta* were obtained from a laboratory colony. On each plant of the herbivore treatment (n=12 plants for each species) ten freshly hatched caterpillars were placed on two different leaves and allowed to feed freely. After 24h of feeding, herbivores and their frass were removed and shoots and leaves of the herbivore-damaged and of non-attacked control plants were harvested, flash-frozen in liquid nitrogen, and stored at -80°C until microarray analysis.

To determine whether first instar *M. sexta* larvae consume different amounts of leaf area from the two hostplant species, we placed 2 freshly hatched caterpillars on each of another five plants per species and measured the amount of leaf material consumed after 24h. After 24h the damaged leaves were harvested, scanned (HP Scanjet 8200, 300 dpi; Hewlett Packard, Palo Alto, CA, USA), the leaf area consumed was determined by counting pixels with image analysis software (Sigma Scan Pro 5, Point Richmond, CA, USA) and the larvae were weighed. Five leaves were not included in the analysis because the caterpillars died or walked off the leaf during the experiment leaving n=8 for *N. attenuata* and n=7 for *S. nigrum*. The amount of consumed leaf area consumed did not differ significantly between the two host species (*N. attenuata* $17.0 \pm 2.9 \text{ mm}^2$; *S. nigrum* $23.6 \pm 3.0 \text{ mm}^2$; $t_{0.025, 13}=-1.375$, $p=0.192$). Larvae feeding on *N. attenuata* ($2.7 \pm 0.2 \text{ mg}$) tended to be slightly heavier than those feeding on *S. nigrum* ($2.0 \pm 0.1 \text{ mg}$).

MeJA treatment

Twelve *S. nigrum* plants were used in both control and MeJA treatment. Ten μl lanolin containing 75 μg MeJA (both Sigma, St. Louis, MO, USA) were applied to one leaf (7th leaf above cotyledons) of each plant; control plants were treated with 10 μl pure lanolin. After 24 h, the treated leaves were harvested, flash-frozen in liquid nitrogen, and stored at -80°C until RNA extraction. RNA of 4 individual plants was pooled to provide one biological replicate.

Microarray analysis

For gene expression analysis, we used two versions of the TIGR potato 10,000-clone cDNA microarray that contain 11,243 (version 10Kv1) and 11,512 (10Kv2) annotated cDNA clones spotted as duplicates on the array. Detailed information about this microarray can be found under http://www.tigr.org/tdb/potato/microarray_comp.shtml. For the transcriptional analysis

of *M. sexta*-induced responses of *N. attenuata* and *S. nigrum*, we used to array version 10Kv1; for the MeJA-elicited responses of *S. nigrum* we used array version 10Kv2. For comparisons of the datasets produced from the two versions of the microarray, we analyzed only the clones present on both versions of the array (11,243 clones). Gene expression data obtained from hybridizations of this potato microarray with a variety of Solanaceous species can be accessed through a database maintained at the TIGR website (http://www.tigr.org/tigr-scripts/sgedb/studies_SGED.pl) and thus compared and shared across different laboratories. Although Solanaceous species clearly differ in morphology, life cycle, secondary metabolism, tuber and fruit formation etc., they have similar genomes with respect to gene content and genome organization (see transcriptional analysis of several Solanaceous plants by Robin Buell, http://www.tigr.org/tigr-scripts/sgedb/search2_std.pl?study_id=35).

Plant material was ground under liquid nitrogen and total RNA was extracted with TRI Reagent™ (Sigma) according to the manufacturer's instructions. All steps of microarray processing (cDNA production, cDNA labeling, microarray hybridization, data quantification, data normalization using LOWESS) were carried out by the TIGR Expression Profiling Service according to published methods (http://www.tigr.org/tdb/potato/microarray_SOPs.shtml). The cDNAs hybridized to an individual array was produced from RNA extracted individually from the four plants of a control cage (Cy5 labeled) and the four plants of the treatment cage (Cy3 labeled). The three biological replicates of the *M. sexta*-*N. attenuata* elicitation experiment are named NA1, NA2, and NA3. SN1, SN2, and SN3 designated the corresponding replicate microarrays of the *M. sexta*-*S. nigrum* elicitation experiment; the arrays of the *S. nigrum* MeJA experiment are named SNmj1, SNmj2, and SNmj3. The raw data from the nine hybridizations including all details of the experiment are available from the TIGR Solanaceae Gene Expression Database (http://www.tigr.org/tigr-scripts/sgedb/search2_std.pl?study_id=53, experiment IDs 2450, 2455, 2460, 2465, 2470, 2475, 2928, 2929, 2930).

We analyzed the normalized data of the six microarrays with GeneSpring 6.1 (Silicon Genetics, Redwood City, CA, USA) using Hierarchical Cluster Analysis. For further analysis we calculated the mean expression ratio (ER) from the biological replicates and defined a transcript as being differentially regulated when the following criteria were fulfilled: (1) The ER was significantly different from 1 as determined by a Student's t-test ($p < 0.05$); (2) the ER exceeded the thresholds of 0.67 and 1.5 for down- and up-regulation, respectively. These

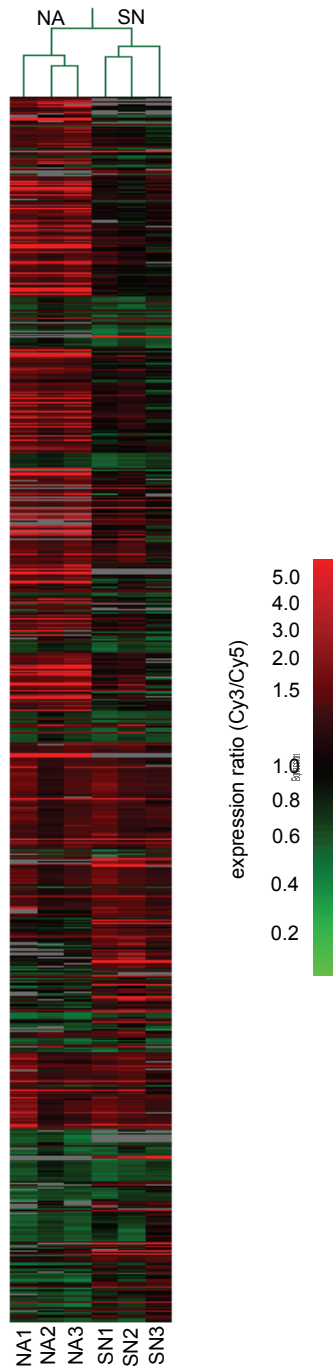
criteria had been previously tested and found to give reproducible results (Heidel and Baldwin 2004; Halitschke et al. 2003).

We confirmed the microarray expression data using TaqMan® real-time PCR (ABI PRISM® 7700 Sequence Detection System; Applied Biosystems, Foster City, CA, USA). Additionally, to test the suitability of this potato microarray for *S. nigrum* and *N. attenuata*, we chose to assay the species that is more distantly related to potato, namely *N. attenuata* (Fig. 1). We analyzed the three biological replicates of *M. sexta*-challenged *N. attenuata* using TaqMan® probes for *N. attenuata*-specific genes that are homologous to genes present on the potato array, namely α -dioxygenase (α -DOX), lipoxygenase (LOX), hydroperoxid lyase (HPL) and a xyloglucan endotransglycosylase (XTH, formerly XET; Supplementary Table 2). We calculated the relative gene expression of each sample using the comparative $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001) with ECI (sulfite reductase) as endogenous control gene, which under our experimental conditions is not regulated (B. Bubner and I.T. Baldwin, unpublished results) and the non-infested control plants as the calibrator.

Acknowledgements

We thank TIGR for generously providing the microarrays and the data acquisition; Dr. Ryan Oyama for invaluable support with the phylogenetic analysis; Jens Wurlitzer for help with the experiments; the Deutsche Forschungsgemeinschaft (BA 2132/1-1) and the Max Planck Gesellschaft for financial support.

Supplemental Data



Supplemental Figure 1: Hierarchical cluster analysis of the replicate microarrays (NA1-3 = *N. attenuata*; SN1-3 = *S. nigrum*) based on the expression ratios of the significantly regulated genes (Supplementary Table 1). The pattern of up- and down-regulated genes contained sets of similarly regulated genes, but clearly distinguished the transcriptional responses of both species to attack from the same Lepidopteran herbivore.

Manuscript III

**Systemin in black nightshade (*Solanum nigrum*). The tomato-homologous polypeptide
does not mediate direct defense responses**

Silvia Schmidt and Ian T. Baldwin*

Plant Physiology (2006) 142, 1751-1758

Max Planck Institute for Chemical Ecology
Department of Molecular Ecology
Beutenberg Campus
Hans-Knoell-Str. 8
07745 Jena, Germany

* Corresponding author:

Ian T. Baldwin
Max Planck Institute for Chemical Ecology
Department Molecular Ecology
Beutenberg Campus
Hans-Knoell-Str. 8
D-07745 Jena, Germany
Phone: ++ 49-3641-571100
Fax: ++ 49-3641-571102
E-mail: baldwin@ice.mpg.de

Abstract

We extend Ryan's seminal work on the 18-aa polypeptide, systemin, in tomato's systemic wound response to the closely related solanaceous species, *Solanum nigrum*. We compared wild-type (WT) plants to plants transformed with an inverted repeat prosystemin construct (IRSys) to silence the expression of the endogenous *S. nigrum* prosystemin gene. In WT plants elicited with wounding + oral secretions (OS) from *Manduca sexta* larvae, trypsin-proteinase inhibitors (TPIs) accumulated even though prosystemin transcripts were down-regulated. Neither reducing the endogenous systemin levels by RNAi nor complementing the plants with systemin by exogenously supplying the polypeptide through excised stems significantly increased TPI activity, indicating that systemin and TPIs are not correlated in *S. nigrum*. The performance of two herbivore species from two feeding guilds, *Manduca sexta* larvae and *Myzus persicae nicotianae*, did not differ between WT and IRSys plants, demonstrating that varying endogenous systemin levels do not alter the direct defenses of *S. nigrum*. Field experiments with WT and IRSys plants and the flea beetle *Epitrix pubescens* supported these glasshouse data. That levels of OS-elicited jasmonic acid (JA) did not differ between WT and IRSys plants suggests that systemin is unlikely to mediate jasmonate signaling in *S. nigrum* as it does in tomato. We conclude that the tomato-homologous polypeptide does not mediate direct defense responses in *S. nigrum*.

Key words: systemin, proteinase inhibitors, *Solanum nigrum*, direct defense, plant-herbivore-interaction

Introduction

Plants not only respond locally to leaf damage caused by wounding, herbivory or pathogen attack but they also induce defenses in distal, unwounded leaves. These systemic defense responses have been extensively studied in tomato (*Solanum lycopersicum*), where an 18-aa-polyptide called systemin is known to play an essential role in generating the mobile wound signal. Systemin is processed from its larger precursor, prosystemin, which is synthesized and processed in the vascular phloem parenchyma cells (Narvaez-Vasquez and Ryan, 2004). Constitutive prosystemin mRNA expression has been found throughout the plant except for the roots (McGurl et al., 1992). After leaf wounding, prosystemin mRNA is induced systemically; the highest accumulation is seen after 3 to 4 hours (McGurl et al., 1992). Like the prosystemin mRNA, proteinase inhibitor (PI) I mRNA, which encodes for a protein with antinutritional effects against several lepidopteran herbivores (Johnson et al., 1989; Ryan, 1990), accumulates systemically after wounding; it is most abundant 8 to 10 h after wounding (McGurl et al., 1992). Young tomato plants supplied with low concentrations of systemin through their cut stems accumulated PI I and II (Pearce et al., 1991). This positive correlation between systemin and PIs is supported by the work of McGurl et al. (1992), who transformed tomato plants with an antisense prosystemin construct. A transgenic line lacking prosystemin was almost completely suppressed in its systemic induction of PIs I and II. Furthermore, *Manduca sexta* larvae that fed on another transgenic plant silenced in its prosystemin expression consumed more leaf material and became three times heavier than those that fed on wild-type (WT) plants (Orozco-Cardenas et al., 1993). After wounding, transgenic plants transformed to over-express the prosystemin gene constitutively produce PI I and II proteins and accumulate more PIs in local and systemic leaves than do WT plants (McGurl et al., 1994). Grafting experiments using these over-expressers as root stocks revealed the constitutive production of PIs in wild-type scions (McGurl et al., 1994), indicating the central role of systemin in generating the systemic wound signal in tomato.

More recent grafting experiments between jasmonic acid (JA) biosynthesis mutants (called *spr2* mutants) and WT plants, or between systemin signaling mutants (called *spr1* mutants) and WT plants showed that both JA biosynthesis and the presence of systemin are required in the local, wounded leaf to produce the systemic signal and hence to induce PIs systemically. On the other hand, neither JA nor systemin is needed in the systemic, undamaged leaves of tomato plants (Howe, 2004; Schillmiller and Howe, 2005). These

findings suggest that systemin acts at or near the site of wounding by amplifying the JA-derived mobile wound signal and are consistent with the previously proposed model that places systemin at the top of the octadecanoid-based signaling pathway upstream of JA (Farmer and Ryan, 1992). Evidence that systemin levels influence the JA levels of a plant was provided by Stenzel et al. (2003), who reported a larger and more rapid rise in JA levels when leaves of prosystemin over-expressing plants were wounded compared to WT plants and less JA in plants transformed with an antisense prosystemin construct. Chen et al. (2006) also observed three-fold higher constitutive JA levels in prosystemin over-expressing plants compared to WT plants.

Using the tomato cDNA as a probe, systemin homologs have been found in three other solanaceous species (Constabel et al., 1998), including black nightshade (*Solanum nigrum* L.). *S. nigrum* is a wild relative of potato and tomato (Schmidt et al., 2005) and has been established as a model system to study plant-herbivore interactions and its underlying signaling processes. A proteinase inhibitor gene called *pin2b* was found in *S. nigrum* which is homologous to and shares 86 % sequence similarity with the tomato *piII* gene. *S. nigrum* has been shown to locally and systemically induce trypsin- proteinase inhibitor (TPI) activity after wounding (Constabel et al., 1998) as well as to respond systemically by eliciting TPI activity after methyl-jasmonate (MeJA) treatment or after attack by the flea beetle *Epitrix pubescens* (Schmidt et al., 2004).

S. nigrum systemin, which has 83 % amino acid identity to the tomato systemin with the respective prosystemins being 81 % identical, induced ten times less PI I when supplied to excised tomato plants than did tomato systemin itself or any of the other systemin homologs (Constabel et al., 1998). On the other hand *S. nigrum* plants supplied with *S. nigrum* systemin did not accumulate more proteinase inhibitors than control plants supplied with buffer despite a difference in the proteinase inhibitor transcript levels between both treatments (Constabel et al., 1998). These findings led us to hypothesize that in *S. nigrum* systemin is not mediating direct systemic defense responses as it does in tomato. To test this hypothesis, we posed the following questions: (i) Which tissues of *S. nigrum* express systemin constitutively? (ii) Does *S. nigrum* induce systemin after treatment with wounding + *M. sexta* oral secretions (OS)? (iii) Do proteinase inhibitors accumulate differently in WT *S. nigrum* plants than in plants silenced in their prosystemin expression after a wounding + OS elicitation? (iv) Does application of systemin induce proteinase inhibitor accumulation in *S. nigrum*? (v) Do

herbivores perform differently on WT plants than on plants silenced in their prosystemin expression? (vi) Do constitutive and induced JA levels differ in *S. nigrum* WT plants from plants silenced in their prosystemin expression?

Results

Spatial and temporal prosystemin transcript patterns

Prosystemin, of which at least three genes are present in *S. nigrum* (Supplemental Fig. 2A) was constitutively expressed in all reproductive and vegetative WT tissues except for the roots. The sites with the highest expression were the flower buds and the leaves, respectively (Fig. 1A + B). Interestingly low prosystemin mRNA levels were detected in black berries and stems (Fig. 1A + B). After a wounding + OS treatment, the expression of prosystemin decreased rapidly in leaves of WT plants and were lowest 30 minutes after elicitation, whereas in both lines transformed with an inverted repeat prosystemin construct (IRSys lines), the expression of prosystemin remained very low (Fig. 2 A + B).

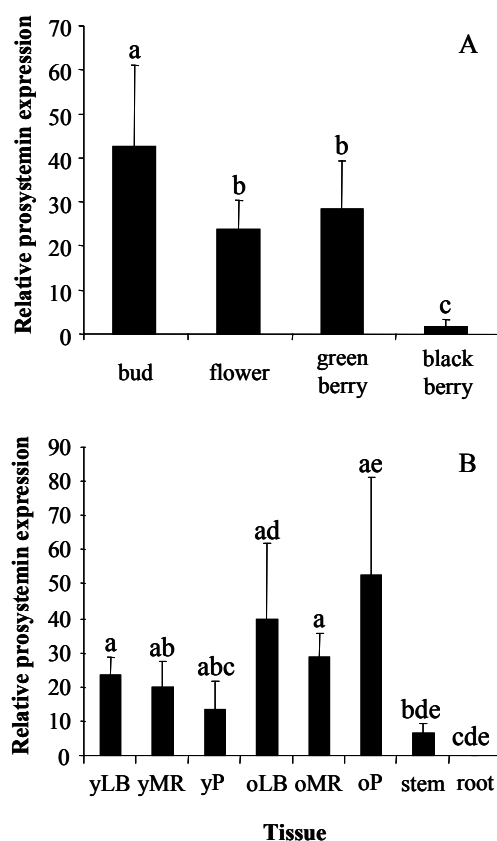


Figure 1: Constitutive prosystemin transcript levels in reproductive (A) and vegetative (B) tissues of WT plants. A: mean \pm SD of five to six pooled samples. B: mean \pm SD of four to five replicates; LB = leaf blade, MR = midrib, P = petiole, y = young, o = old. Different letters indicate significant differences

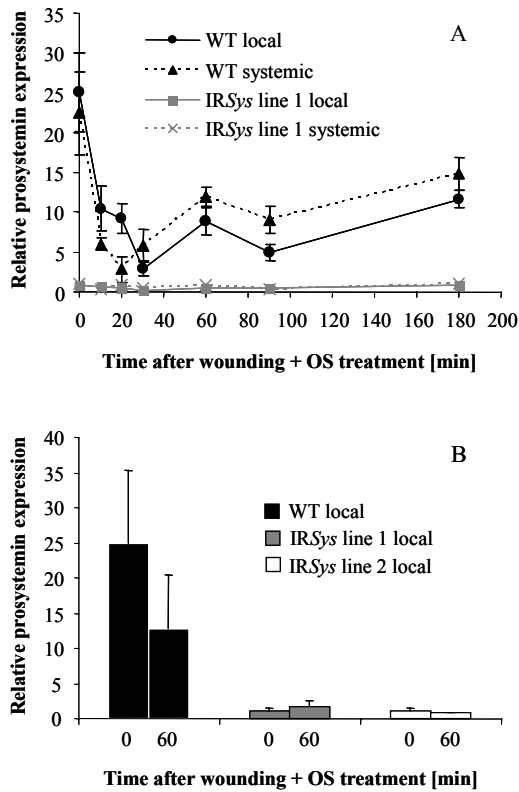


Figure 2: Prosystemin transcripts levels in (A) local and systemic leaves of WT plants and IR.Sys line 1 in the glasshouse and (B) in local leaves of WT plants and IR.Sys lines 1 and 2 in the field after elicitation with wounding + OS. Shown are the mean \pm SE (A) or SD (B) of three to five replicates.

TPI accumulation in WT and IR.Sys plants

To test whether the accumulation of TPI depends on the (pro)systemin level of a plant, the amount of TPIs was quantified in uninduced and induced WT and IR.Sys plants. Although constitutive levels in WT and transgenic plants were below the detection limit of the assay, levels increased dramatically after induction (Fig. 3). No significant difference was detected between WT and IR.Sys plants, either in local or in systemic leaves.

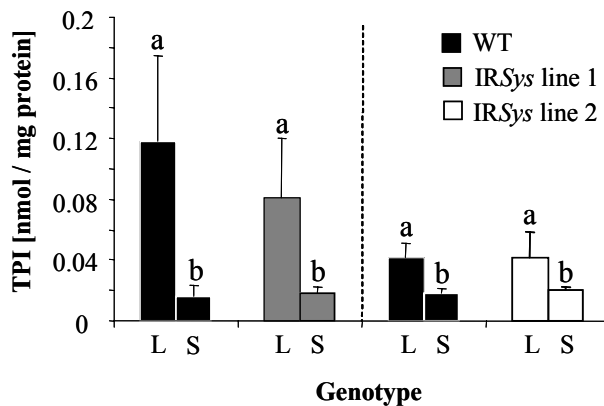


Figure 3: Trypsin-proteinase inhibitor accumulation in local (L) and systemic (S) leaves after wounding + OS treatment. Shown are the combined data of two independent experiments in which either IR.Sys line 1 or 2 was compared to WT. Constitutive levels were below the detection limit of the assay in all cases. Bars represent mean \pm SD of three to five replicates. Different letters indicate significant differences.

Influence of exogenously applied systemin on TPI levels

As reducing prosystemin mRNA levels in IR S_{ys} plants did not reduce TPI accumulation, we tried to enrich plants with systemin by applying the polypeptide through their cut stems. Applying *S. nigrum* systemin or tomato systemin to WT *S. nigrum* plants did not increase TPI levels compared to those of controls (Fig. 4A). However, the application of MeJA was clearly capable of inducing TPis (Fig. 4A). In tomato plants the application of tomato systemin significantly increased the level of TPis compared to control levels, whereas *S. nigrum* systemin did not (Fig. 4B). Using a MeJA dilution series, we demonstrated that *S. nigrum* WT plants are able to respond to an exogenously applied elicitor in a dose-dependent manner (Fig. 4A, Insert). The patterns resulting from treatment of the IR S_{ys} lines did not differ significantly from those of treated WT plants (Supplemental Fig. 3).

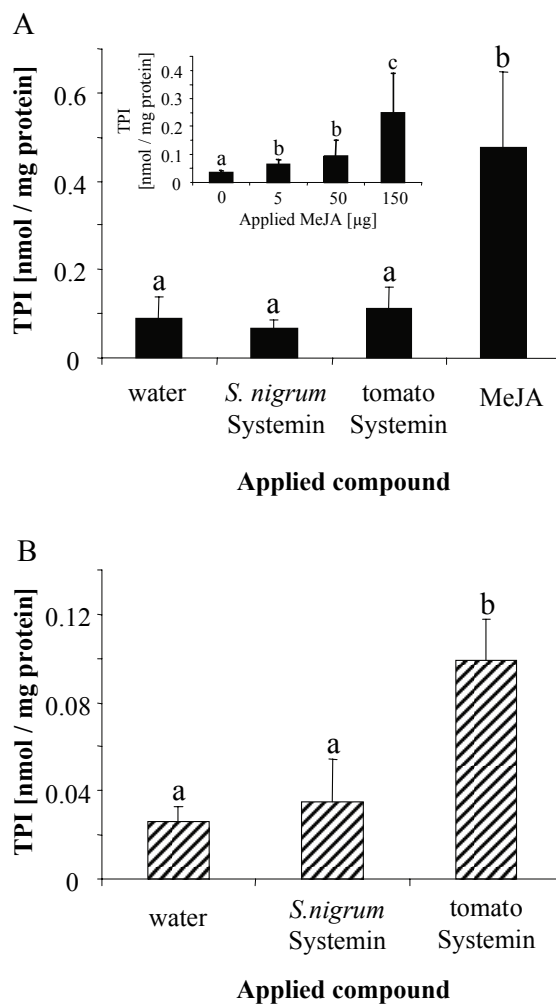


Figure 4: Trypsin-proteinase inhibitor accumulation in leaves of *S. nigrum* (A) and tomato plants (B) after application of water, *S. nigrum* systemin, tomato systemin or methyl jasmonate (MeJA) through the cut stems. Insert in (A): Dose-dependent accumulation of trypsin-proteinase inhibitors in *S. nigrum* leaves after application of different concentrations of MeJA through the cut petioles. Shown are mean \pm SD of three to five replicates. Different letters indicate significant differences.

Influence of systemin levels on herbivores of different feeding guilds

To evaluate the influence of systemin on direct defense mechanisms, we compared the performance of herbivores on WT plants and IRSys lines. Herbivores from two different feeding guilds, namely, leaf chewers (i.e. caterpillars of the tobacco hornworm *M. sexta* (Sphingidae) and the flea beetle *E. pubescens* (Chrysomilidae)) and phloem sap suckers (i.e. *Myzus persicae nicotianae* (Aphididae)), were chosen. As measures of the leaf quality of the different genotypes, we quantified the mass gain of *M. sexta*, the leaf damage caused by *E. pubescens*, and the population growth of *M. persicae nicotianae*, respectively. The measures of *M. sexta* larval mass and the *E. pubescens* assay were repeated three times. The data shown in Fig. 5 are representative for all three experiments. In none of the three herbivore species was a significant difference between WT and IRSys lines detected (Fig. 5; all $P_s > 0.1240$).

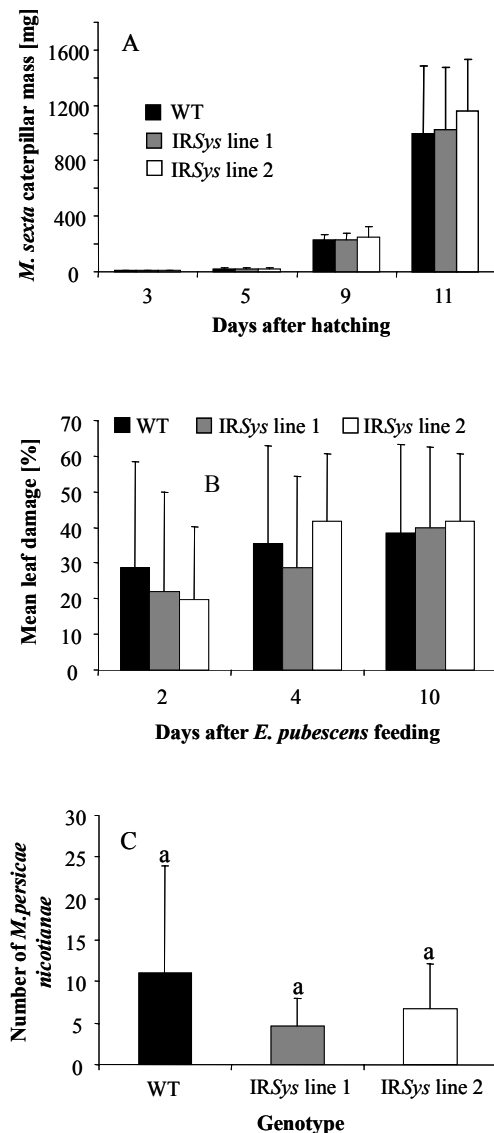


Figure 5: Herbivore performance on WT and IRSys plants. (A) Mass of *M. sexta* caterpillars reared on WT and IRSys plants. Shown are mean \pm SD of 19 to 22 larvae feeding individually on the respective genotypes (repeated measures ANOVA: $P = 0.51$). (B) Mean leaf damage caused by *E. pubescens* feeding on field-grown WT plants and IRSys lines. Shown are mean \pm SD of 15 plants per genotype (Bonferroni corrected Wilcoxon Signed Rank test for WT singly compared to each IRSys line at each day: $P > 0.025$). (C) Number of *M. persicae nicotianae* aphids after 10 days starting from one female placed on each of 15 plants of each genotype. Shown are mean \pm SD. Different letters indicate significant differences.

Influence of systemin on JA levels

To test whether systemin acts at the top of the octadecanoid pathway upstream of JA, the level of the plant hormone was quantified in the leaves of WT plants and IRSys lines treated with wounding + OS.. The time series of WT plants were characterized by two peaks 30 minutes and 3 h after elicitation (Fig. 6, Insert). The pattern was similar for IRSys lines, with the second peak more prominent than the first. Neither in uninduced nor in induced leaves were significant differences between WT and transgenic lines detected (Fig. 6). The WT time series as well as the comparison between WT plants and IRSys lines were repeated twice with similar results (only one graph is shown).

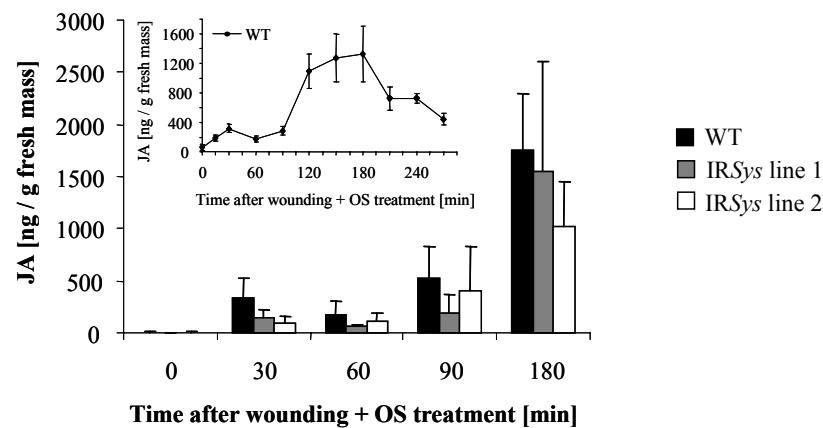


Figure 6: Jasmonic acid (JA) content in leaves of WT plants and IRSys lines after wounding + oral secretion (OS) treatment. Shown are mean \pm SD of five individual plants per timepoint and genotype (single ANOVA followed by LSD post-hoc test per timepoint: $P > 0.05$). Insert: Shown are mean \pm SE of five individual WT plants per timepoint.

Discussion

The aim of this study was to determine whether systemin's role in tomato also applies to a solanaceous species which is closely related to tomato. The question was addressed by extending Ryan's work to *S. nigrum* and testing whether systemin mediates direct systemic defense responses in this species. To compare transgenic *S. nigrum* plants silenced in their prosystemin expression to WT plants, we measured TPI and JA accumulation after wounding + OS treatment. In addition, we compared the performance of three different herbivore species on WT and IRSys plants and observed TPI levels in plants supplied with systemin.

S. nigrum harbors at least three prosystemin genes (Supplemental Fig. 2A) which are effectively silenced in both IRSys lines (Fig. 2A + B). The tissue-specific expression pattern of prosystemin in *S. nigrum* (Fig. 1A and B) seems to reflect that observed in tomato (McGurl et al., 1992) with high mRNA levels in reproductive and above-ground vegetative tissues. The small amounts of prosystemin mRNA detectable in the black berries (as opposed to the green berries) and roots suggest that systemin is unimportant in these tissues. While the levels of constitutive prosystemin transcript in *S. nigrum* match the levels reported from tomato, the picture changes after induction. In tomato, prosystemin is systemically induced after wounding (McGurl et al., 1992). In contrast, in *S. nigrum* local and systemic transcript levels rapidly decrease after OS elicitation under both glasshouse as well as field conditions (Fig. 2A and B). To demonstrate that the (pro)systemin levels have an influence on defense responses, PI levels are typically quantified as a response variable in tomato.

The ability of a transgenic tomato line, silenced in its prosystemin expression, to systemically increase PI I and II (McGurl et al., 1992) was almost completely suppressed, indicating a positive correlation between prosystemin expression and PI accumulation. Compared to tomato, the situation is different in *S. nigrum*. Dramatically reducing levels of prosystemin (in both IRSys lines; Fig. 2A and B) does not reduce TPI (Fig. 3). In addition, TPI levels in WT plants increase while prosystemin transcript levels decrease after elicitation. This demonstrates an absence of correlation between systemin and TPIs in *S. nigrum*. The observed positive correlation in tomato, however, could theoretically be due to an insertion effect of the transgene, as only the F₁-progeny of a single primary tomato transformant was tested. Working with transformed plants always carries the risk of detecting a transgene-insertion-effect rather than a gene-of-interest-effect; this risk is greatly reduced when the phenotype occurs in more than one independently transformed line. Fig. 3 shows the combined data from two independent experiments in which either IRSys line 1 or IRSys line 2 was compared to WT. Even though the absolute TPI levels differed between the two experiments, in none of the experiments did TPI levels differ significantly between transgenic and WT plants.

To increase endogenous systemin levels, plants were supplied with additional systemin by applying the polypeptide through the cut stems or petioles. In *S. nigrum* WT plants, neither *S. nigrum* systemin nor tomato systemin was able to elicit higher TPI levels compared to those of water-treated controls (Fig. 4A). The former phenomenon was reported

previously by Constabel et al. (1998), which suggests that the excision itself activated the wound response to maximal levels, making it difficult to discern whether the PI accumulation was affected by exogenously applied systemin. By using MeJA as a positive control (Fig. 4A), we demonstrated that this was not the case but that excised *S. nigrum* plants supplied with MeJA are able to significantly increase TPI levels over control levels in a dose-dependant manner (Fig. 4A, Insert). Constabel et al. (1998) hypothesized that *S. nigrum* systemin mediated defense gene induction in *S. nigrum* because systemin treatments increased PI-mRNA accumulation more than did treatment with buffer only, even though this differential transcriptional expression was not reflected on protein level. Our analysis of *S. nigrum* does not support this hypothesis. We applied tomato systemin to tomato plants, with the expected result: levels of TPIs significantly increased compared to control levels (Fig. 4B). However, applying *S. nigrum* systemin to tomato did not (Fig. 4B), which was consistent with the findings of Constabel et al. (1998), who pointed out that in tomato *S. nigrum* systemin is ten times less effective than tomato systemin in eliciting PI I. Supplying both *S. nigrum* IRSys lines with water or *S. nigrum* systemin did not lead to any significant differences compared to the WT plants (Supplemental Fig. 3), supporting the absence of a correlation between systemin and TPIs in *S. nigrum*.

To test the influence of endogenous systemin levels on *S. nigrum*'s resistance to insect attack, the performance of different herbivores on WT and IRSys plants was evaluated. Larvae of the tobacco hornworm *M. sexta*, a Solanaceae specialist, gained the same mass when reared on the different genotypes (Fig. 5A). Dramatic differences in weight gain have been reported for tomato plants over-expressing the prosystemin gene compared to WT tomato plants (Orozco-Cardenas et al., 1993). Nevertheless, data derived from these over-expressor lines need to be interpreted cautiously as the ectopic expression of prosystemin mRNA driven by an S35 promotor, produces systemin in tissues that normally do not express the gene, for example roots (McGurl et al., 1992). The flea beetle *E. pubescens*, another Solanaceae specialist, has been repeatedly observed to feed on *S. nigrum* at our field site. The mean damage inflicted by this species on the IRSys lines did not differ significantly from that done to WT plants (Fig. 5B). Finally, the population growth of *M. persicae nicotianae* did not differ between genotypes (Fig. 5C). Taken together, these results demonstrate that the quality of IRSys and WT leaves does not differ for these herbivores.

To test whether the endogenous systemin levels influence a plants' ability to produce JA, the plant hormone was quantified in both uninduced and OS elicited WT and IRSys plants. WT plants clearly responded to the treatment (Fig. 6, Insert), showing a two-peaked pattern of JA accumulation. The amount of JA did not differ significantly between WT and IRSys plants (Fig. 6), indicating little or no correlation between systemin and JA in *S. nigrum*. Given that prosystemin transcripts are down-regulated after induction (Fig. 2), systemin and JA may be negatively correlated in *S. nigrum*, but the low constitutive JA levels in both IRSys lines argue against this. These data suggest that systemin in *S. nigrum* does not act at the top of the octadecanoid-based signaling pathway upstream of JA as has been proposed for tomato (Farmer and Ryan, 1992). Current knowledge about systemin based on research in tomato appears not to apply to a closely related member of the same family.

The possibility that systemin might play completely different roles even in closely related species is supported by Boller's (2005) analysis of systemin sequences, which concluded that systemin appears to be under diversifying selection.

Materials and Methods

Plant growth

The *Solanum nigrum* L. inbred line Sn30 (Schmidt et al., 2004) was used as a WT control for all experiments. To synchronize germination, seeds of WT and transgenic plants were incubated in 5mL 1 M KNO₃ supplemented with 50 µL 0.1 M gibberellic acid (Roth, Karlsruhe, Germany) and 25 µL 0.5% (v/v) Tween-20 (Merck, Darmstadt, Germany) at 4°C overnight. Seeds were germinated in Teku pots (Waalwijk, The Netherlands) with a peat-based substrate (Klasmann Tonsubstrat, Geeste-Groß Hesepe, Germany) and transferred to 9 x 9 x 9.5 cm pots containing the same substrate after about 14 days. At all stages the plants were grown in the greenhouse (16 h light, supplemental lighting by Philips Master Sun-T PIA Agro 400 and Sun-T PIA Plus 600 W sodium lights (Turnhout, Belgium) / 23-25°C / 45-55 % humidity; 8 h dark / 19-23°C / 45-55 % humidity) of the Max Planck Institute for Chemical Ecology (Jena, Germany).

The procedure was the same for *Solanum lycopersicum* cv. Castlemart except that the seeds were soaked in water at 4°C over night.

Plants used in the two field experiments in July and August 2005 (flea beetle herbivory and prosystemin expression after wounding + OS treatment) were planted at the

field site in Dornburg (north of Jena, Germany) 24 or 21 days post-sowing after being acclimatized to outdoor conditions for three to five days. The release of transformed plants at the Dornburg field site was conducted in compliance with EU and German regulations (release application no. 6786-01–0156 (IRSys line 1) and 6786-01–0165 (IRSys line 2) as administered by the Thüringer Landesverwaltungsamt (TLVwA) and the Thüringer Landesamt für Lebensmittelsicherheit und Verbraucherschutz (TLLV).

All experiments were conducted with four- to five-week-old plants except those involving the harvesting of reproductive tissues.

Inverted repeat prosystemin lines (IRSys lines)

Transgenic *S. nigrum* lines silenced in their prosystemin expression were constructed using the silencing vector pSOL3SYS1 (Supplemental Fig. 1) which is based on the pSOL3RCA silencing vector described in detail by Bubner et al.(2006). The original RCA inverted repeat fragments were consecutively replaced by two 399 bp fragments of the *S. nigrum* prosystemin gene after being PCR amplified using the primer pairs SYS5-32 (5'-GCGGCGCCATGGTCTGTCTGCATTTTGGGAGG-3'), SYS6-31 (5'-GCGGCGCTGCAGTGG AACATGAGGAGGGAGG-3') and SYS7-32 (5'-GCGGCGCTCGAGTCTGTCTGCATTTTGGGAGG-3'), SYS8-32 (5'-GCGGCGGAGCTCGTGG AACATGAGGAGGGAGG-3'), respectively, based on the mRNA sequence published by Constabel et al. (1998). *Agrobacterium tumefaciens*-mediated transformation was conducted as described by Kruegel et al. (2002) and T₁ (transformation generation 1) -plants homozygous for the trans-gene were selected by a hygromycin resistance screen of their progeny. The progeny of homozygous T₁-plants was additionally tested to see if it harbored a single copy of the transgene. Southern blot analysis (Supplemental Fig. 2B) resulted in two independently transformed inverted repeat lines. Seeds of these lines were used for all experiments and will be made freely available to academic investigators for non-commercial research purposes. Line S03-71-13 and line S03-82-3 are referred to as IRSys line 1 and IRSys line 2.

Southern blot analysis

Genomic DNA of WT and IRSys plants was isolated from *S. nigrum* leaves using a modified cetyl trimethyl ammonium bromide (CTAB) method (Rogers and Bendich, 1994) as

described in Bubner et al. (2004). The final DNA pellet was rehydrated in 50 μ L 1 x Tris/EDTA (TE) buffer (10 mM TrisCl pH 8, 1 mM EDTA pH 8). Restriction digests were done using *Bam*HI, *Eco*RI, and *Eco*RV for the WT DNA and *Eco*RV or *Xba*I for the DNA of the two IR Δ lines. Plasmids (pSOL3SYS1) were linearized using *Bam*HI, *Xho*I or *Xba*I. Size-fractionation by 0.8% agarose gel electrophoresis was followed by Southern blotting onto a nylon membrane with a high-salt buffer (Brown, 1995). The blots were hybridized with ³²P-labeled probes specific for the prosystemin gene (primer pair SYS5-32 [5'-GCGGCGCCATGGTCTGTCTGCATTTTGGGAGG-3'] and SYS6-31 [5'-GCGGCGCTGCAGTGGAACATGAGGAGGGAGG-3']) or the hygromycin resistance gene *hptII*.

Plant treatments

To mimic herbivore feeding one leaf was wounded with a fabric pattern wheel, causing three rows of punctured wounds at each side of the midrib. The wounds were immediately supplied with the oral secretions (OS) of *M. sexta* larvae. OS were collected from third- to fourth-instar larvae hatched from eggs (Carolina Biological Supply, Burlington, NC, USA) and reared on *S. nigrum* WT plants. OS were diluted 1:1 (v/v) with deionized water prior usage. For the prosystemin expression time series, the PI assay, and the JA measurements, a fully expanded leaf of the main axis (normally one leaf at nodes six to eight) was induced as described above. After the respective time-points (for the PI assay, after 72 h) the locally treated leaf and in the case of the prosystemin time series the uninduced leaf one node above the treated leaf were harvested, flash frozen in liquid nitrogen and stored at -80°C until further processing. As the plants grew slower in the field than in the glasshouse, in the field experiment the leaf at the fourth node has been treated and harvested as described above. To measure the constitutive prosystemin expression in different plant tissues the whole plant remained untreated and RNA was extracted from old (third node) leaf blades, petioles and midribs; young (seventh node) leaf blades, petioles; and midribs; and stems, roots, buds, flowers, and green and black berries.

The PI-inducing effect of *S. nigrum* and tomato systemin was tested by excising *S. nigrum* and tomato WT plants at the base of the stem according to Pearce et al. (1993) with a scalpel and placing them into tubes containing 500 μ L water either pure or supplemented with 2.5 pmol systemin (AnaSpec, San Jose, CA, USA; according to protein sequences published

by Constabel et al., 1998) or 150 μg MeJA as a positive control. After the complete solution had been taken up, plants were transferred to water for 72 hours and the leaf at the sixth (*S. nigrum*) or the third node (tomato) was harvested, flash frozen in liquid nitrogen, and stored at -80°C until processing for TPI assay. The experiment was complemented by supplying IRSys leaves with pure water or water supplemented with 2.5 pmol systemin or 150 μg MeJA through their cut petioles. To demonstrate that excised *S. nigrum* leaves are capable of responding to exogenously applied MeJA in a dose-dependent manner, *S. nigrum* WT leaves were supplied with different concentrations of MeJA in 500 μL water through their cut petioles followed by the procedure described above.

All experiments described above were based on four to six individual plants for each treatment and / or harvest time-point. The individual reproductive tissues samples were pooled out of 15 buds, 5 flowers, 3 green berries, and 2 black berries per plant, respectively.

Herbivore experiments

M. sexta larvae were reared from eggs (obtained from the *M. sexta* colony at the Max Planck Institute for Chemical Ecology in Jena, Germany) and one neonate was placed on each of the 25 individual plants per genotype (WT and both IRSys lines). The caterpillars were weighed after 3, 5, 9, and 11 days.

Naturally occurring adult flea beetles (*E. pubescens*) were allowed to feed on 45 field grown plants planted as triplicates of the three genotypes over a period of 10 days. The damage done to the plant was recorded on days 2, 4, and 10. To quantify the damage, each individual leaf was categorized according to the following damage classes: 0 = 0% damage, 1 = 1-5% damage, 2 = 6-10% damage, 3 = 11-25% damage, 4 = 26-50% damage, 5 = 51-100% damage. Based on the damage level estimated for each individual leaf, a mean value was calculated for the entire plant. As these mean plant data are non-continuous percentage values and the damage experienced by WT plants was compared singly to the damage done to each IRSys line, thus having two analyses per recording day, Bonferroni corrected Wilcoxon Signed Rank tests have been performed.

Myzus persicae nicotianae aphids were collected on *Nicotiana attenuata* plants in our greenhouse and transferred to *S. nigrum*, where they were allowed to establish a population for about two weeks. Single females of this population have been used to infest 15 *S. nigrum*

plants of each genotype (WT and both IRSys lines) and after 10 days the population size on each plant was counted.

RNA extraction and quantitative real-time PCR

Harvested tissues were ground individually in liquid nitrogen and total RNA was isolated following a modified TRI Reagent® procedure for polysaccharide- and proteoglycan-rich sources (TIGR, 2003). Total RNA (100 ng) was reverse-transcribed into complementary DNA (cDNA) using MultiScribe™ reverse transcriptase (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions. Prosystemin mRNA expression was quantified by real-time PCR (ABI PRISM™ 7700 Sequence Detection System; Applied Biosystems, Darmstadt, Germany) using the qPCR™ core reagent kit (Eurogentec, Seraing, Belgium), a specific TaqMan primer pair, and a double fluorescent dye-labeled TaqMan probe specific for the *S. nigrum* prosystemin gene based on the sequence published by Constabel et al. (1998). The probe was designed to detect only the endogenous prosystemin gene and not the region selected for the inverted repeat construct. The relative target gene expression of each sample was determined using standard curves. cDNA for these standards has been obtained by reverse transcription of *S. nigrum* RNA extracted from a wounding + OS treated WT plant using SuperScript™ II RNaseH-Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. With each measurement TaqMan reactions of four defined cDNA dilutions (20 ng / μ L to 0.02 ng / μ L) were run using the primers and probe described above. Ct values (the cycle number C at which the PCR product triggers a certain amount of fluorescence (threshold t)) of the four standards were plotted against the respective cDNA concentration and the obtained curve was used to relatively calculate the transcript amount of the samples.

Trypsin-proteinase inhibitor assay

Harvested leaves were ground in liquid nitrogen individually and total protein was extracted using 2 mL protein extraction buffer (Jongsma et al., 1994) per mg plant tissue followed by vortexing and centrifugation as described in van Dam et al. (2001). Protein content of the samples was determined in technical triplicates using the method of Bradford (1976) with an IgG-based (Sigma) standard curve. TPI was determined by the radial immunodiffusion assay

(Jongsma et al., 1993) using a soybean trypsin inhibitor (STI, Sigma) dilution series as a standard. The final TPI activity is expressed as nmol mg^{-1} total protein.

Jasmonic acid measurements

Approximately 300 mg of harvested leaf tissue were homogenized in 1 mL ethyl acetate spiked with 200 ng mL^{-1} methanolic [$^{13}\text{C}_2$]jasmonic acid as an internal standard. After centrifugation at 13000 rpm for 20 min at 4°C , the supernatant was evaporated and the dried extract was re-dissolved in 1 mL ethyl acetate. Following another centrifugation step the supernatant was evaporated and the dried sample was re-dissolved in $500 \mu\text{L}$ 70 % (v/v) methanol. After vortexing for 5 min the sample was centrifuged for 10 min at 13000 rpm and $15 \mu\text{L}$ of the supernatant was analyzed using a Varian 1200L Triple-Quadrupol-MS.

For the high-performance liquid chromatography a Pursuit C8 column (150 mm x 2.0 mm, $3 \mu\text{m}$ particle size) was used and a gradient of water and methanol, both including 0.05 % (v/v) formic acid, was run as the mobile phase with a flow rate of 0.2 mL / min . The mass spectrometer was run in negative electro spray ionization (ESI) mode with an argon pressure of 0.279972 Pa (= 2.1 mTorr) in the collision cell. The MS was set up with a capillary voltage of -3200 V , a shield voltage of 600V and a detector voltage of 1800V . The pressure of the drying gas was 131005 Pa (= 19 psi) at 300°C , that of the nebulizing gas was 379225 Pa (= 55 psi). The most abundant and characteristic fragment ion was chosen for quantification.

The amount of JA per sample was calculated with the following formula ((peak area endogenous JA * 200 ng mL^{-1}) * peak area ISTD $^{-1}$) and related to 1 mg leaf tissue.

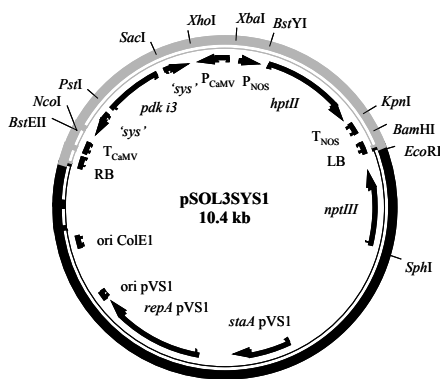
Statistics

Data were analyzed by ANOVA followed by an LSD post-hoc test. To ensure homogeneity of variances data were transformed if necessary (square root: Figure 1A, 2A; LG10: Figure 3 left, 4A, 7C, partly 8B; reciprocal: Figure 3 right, 6). In cases in which variances could not be homogenized by transformation, a Welch test followed by a Dunnett T3 test was performed (Figure 1B, 2B, 5A). The *M. sexta* caterpillar mass data were analyzed using repeated measures ANOVA. All analyses were done using the software package SPSS.

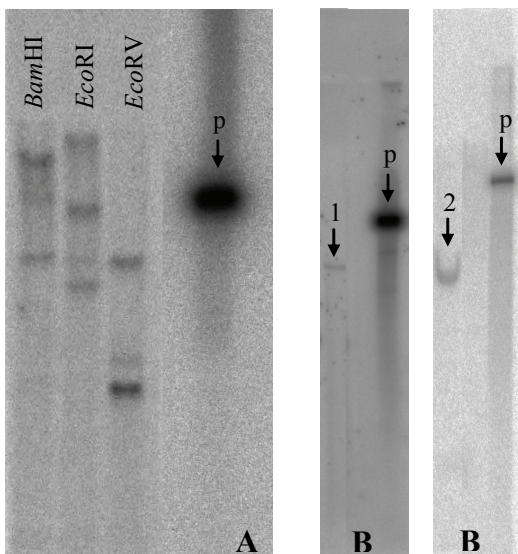
Acknowledgments

We dedicate this paper to Clarence “Bud” Ryan whose research program inspired more than three decades of research into herbivore-induced defenses. We thank K. Gase, W. Kröber, A. Wissgott, and S. Kutschbach for the transformation construct and for plant transformation, A. Berg and E. Rothe for assistance with the JA analysis, J. Kellmann for managing the onerous legal obstacles to conducting field work with genetically modified plants in Germany, G. Howe for kindly providing us *Solanum lycopersicum* seeds, and C. Kost, M. Hartl, C. Voelckel, T. Kaiser, and two anonymous reviewers for critically commenting on earlier versions of the manuscript. This work was funded by the Max-Planck Society.

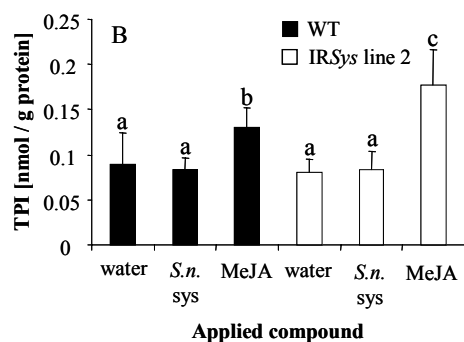
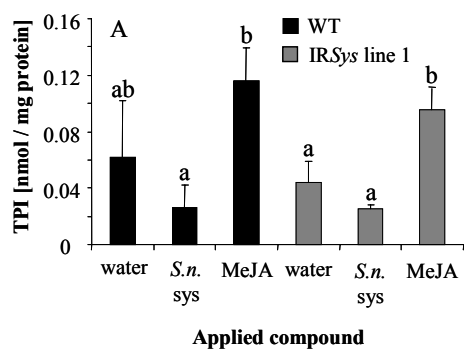
Supplemental Data



Supplemental Figure 1: Silencing vector pSOL3SYS1. The transferred T-DNA is shown in grey with RB = T-DNA right border, TCaMV = terminator cauliflower mosaic virus, *sys* = prosystemin gene fragment, *pdk i3* = spacer (intron 3 of pyruvate, orthophosphate dikinase gene), P_{CaMV} = promoter Cauliflower mosaic virus, P_{NOS} = promoter of nopaline synthase, *hptIII* = hygromycin phosphotransferase gene, T_{NOS} = promoter of nopaline synthase, LB = T-DNA left border.



Supplemental Figure 2: Southern blots of WT and both IRSys lines. (A) Genomic WT DNA has been restriction digested with *Bam*HI, *Eco*RI and *Eco*RV, pSOL3SYS1 plasmid (= p) DNA with *Bam*HI. The Blot was hybridized with a ³²P-labeled probe specific for the prosystemin gene. (B) Genomic DNA of IRSys line 1 (= 1) and 2 (= 2) has been restriction digested with *Eco*RV or *Xba*I, pSOL3SYS1 plasmid (= p) DNA with *Xho*I or *Xba*I. The blot was hybridized with a ³²P-labeled probe specific for the hygromycin phosphotransferase gene *hptIII*. In the lane between the line DNA and the plasmid DNA restriction digested wild type DNA was run giving no signal with the probe used.



Supplemental Figure 3: Trypsin-proteinase inhibitor accumulation in leaves of *S. nigrum* WT plants and both IRSys lines after application of water, *S. nigrum* systemin (*S.n.sys*) or methyl jasmonate (MeJA) through the cut petioles. Shown are mean \pm SD of four to five replicates. Different letters indicate significant differences.

Manuscript IV

**Down-regulation of systemin after herbivory is associated with increased root allocation
and competitive ability in *Solanum nigrum***

Silvia Schmidt and Ian T. Baldwin*

Submitted to *Functional Ecology*

Max Planck Institute for Chemical Ecology
Department of Molecular Ecology
Beutenberg Campus
Hans-Knoell-Str. 8
07745 Jena, Germany

* Corresponding author:

Ian T. Baldwin
Max Planck Institute for Chemical Ecology
Department Molecular Ecology
Beutenberg Campus
Hans-Knoell-Str. 8
D-07745 Jena, Germany
Phone: ++ 49-3641-571100
Fax: ++ 49-3641-571102
E-mail: baldwin@ice.mpg.de

Summary

1. After simulated *Manduca sexta* herbivory (OS-elicitation), transcripts of the systemin-precursor, prosystemin, are down-regulated in Black Nightshade (*Solanum nigrum*). Systemin is known not to mediate direct defense responses against herbivores and here we examine the hypothesis that systemin helps the plant to tolerate rather than resist herbivory.
2. Growth experiments revealed that both OS-elicited wild-type (WT) plants and transgenic plants silenced in prosystemin expression (IR S_{ys}) had significantly more root mass than untreated WTs. When OS-elicited WTs were additionally treated with synthetic systemin, root mass did not differ from that of uninduced WTs.
3. Microarray analysis of leaves revealed that the differences in root growth were associated with the regulation of transcripts involved in sugar and spermine metabolism.
4. Lifetime berry production of plants grown in pots with and without barriers was used to evaluate the fitness consequences of the systemin-associated increase in root allocation. When competition for below-ground resources was prevented by a barrier, no significant differences in berry production were found among any of the genotypes or treatments. However, when unelicited plants competed, IR S_{ys} plants produced significantly more berries than did WT plants. Berry production of OS-elicited and unelicited WT plants did not differ, but when OS-elicited WTs were additionally treated with systemin, plants produced fewer berries than did unelicited WT competitors.
6. We propose that the rapid down-regulation of systemin after herbivory is associated with increased root allocation which allows plants to more effectively compete with conspecifics and may allow plants to compensate for tissue losses during herbivory.

Key-words: competition, plant-herbivore interaction, root growth, tolerance

Introduction

Plants generally exhibit two strategies in response to herbivory: resistance and tolerance. While resistance traits directly or indirectly reduce the amount of damage a plant receives either by repelling potential herbivores or by decreasing the amount of tissue removed, tolerance traits reduce the detrimental effects of herbivore damage on plant fitness without affecting the herbivore (Tiffin 2000). Tolerance can be achieved by various mechanisms (Strauss & Agrawal 1999; Tiffin 2000): increases in photosynthetic activity (Welter 1989), compensatory growth (McNaughton 1979; Paige & Whitham 1987), bunkering reserves in protected tissues (Schwachtje *et al.* 2006), and phenological changes (Marquis 1988).

As resistance and tolerance represent opposing ways a plant can cope with attack from herbivores, trade-offs between these two strategies are assumed to occur. While some studies found a negative correlation between resistance and tolerance (Fineblum & Rausher 1995), other studies did not (Mauricio, Rausher & Burdick 1997). Leimu & Korchiva (2006) recently concluded from their meta-analysis of 31 ecological and agricultural studies that resistance and tolerance are not mutually exclusive.

Black Nightshade (*Solanum nigrum* LINNÉ), a weedy, pioneer-like plant that grows in open, disturbed areas, is likely to experience strong selection from both herbivores as well as competitors in its native habitats. After attack by the flea beetle *Expitrix pubescens* or methyl jasmonate (MeJA) elicitation, *S. nigrum* systemically induces trypsin-proteinase inhibitor (TPI) activity as a direct resistance trait (Schmidt *et al.* 2004). This response can also be elicited by a treatment involving wounding and the application of oral secretions (OS) to the wounds (Schmidt & Baldwin 2006), which mimics the responses elicited by herbivore attack and thus represents a useful way of eliciting plants in a standardized manner. The effectiveness of such OS-elicitation can be largely attributed to the fatty acid amino acid conjugates (FACs) which are present in the OS and have been shown to be responsible for all measured direct and indirect resistance responses of wild tobacco *Nicotiana attenuata* (Roda *et al.* 2004).

In addition to the induction of TPIs, another response of *S. nigrum* to simulated herbivory by OS-elicitation is the rapid and dramatic transcriptional down-regulation of prosystemin (Schmidt & Baldwin 2006), the precursor of the peptide hormone systemin. Unlike tomato (*Solanum lycopersicum* LINNÉ), in which systemin has been shown to be involved in plants' resistance to herbivory by locally amplifying a jasmonate-based mobile

wound signal (Howe 2004; Schilmiller & Howe 2005), *S. nigrum* does not rely on systemin to mediate direct resistance responses (Schmidt & Baldwin 2006). In OS-elicited WT plants, TPIs accumulated even though prosystemin transcripts were down-regulated. Furthermore, neither reducing the endogenous systemin levels by RNAi nor supplementing plants with synthetic systemin significantly increased TPI activity, indicating that systemin and TPIs are not correlated in *S. nigrum*. That levels of OS-elicited jasmonic acid (JA) did not differ between WT and IRSys plants suggests that systemin is unlikely to mediate jasmonate signaling in *S. nigrum* in the same way that it does in tomato.

However, the question remains: why is systemin down-regulated after simulated herbivory? Here we examine the hypothesis that systemin helps the plant to tolerate rather than resist herbivory. We propose that low systemin levels after herbivory are associated with increases in root mass, which in turn enhance the plants' competitive ability. In this way, the down-regulation of systemin may enable plants to compensate for the costs of induced resistance traits and perhaps as well for tissues lost to herbivores.

Material and Methods

Plant growth

Seeds of *Solanum nigrum* wild-type (WT) and an inverted-repeat prosystemin line (IRSys line) were germinated as previously described (Schmidt & Baldwin 2006) and transferred into either 1L pots containing quartz sand (particle size 0.7-1.2 mm, Euroquarz GmbH, Germany) or 2L pots containing a peat-based substrate (Klasmann Tonsubstrat, Geeste-Groß Hesepe, Germany) 16 or 17 days post-sowing. The plants were grown in the greenhouse of the Max Planck Institute for Chemical Ecology (Jena, Germany) under the light, temperature, and humidity conditions previously described (Schmidt & Baldwin 2006). Every watering event supplied all plants with 0.5 g/L combination fertilizer containing phosphate, potassium and magnesium (Euflor GmbH, Germany) and 0.5 g/L Ca(NO₃)₂ (MERCK, Germany).

Plant treatments

Singly grown WT plants planted into 1L sand pots were either left untreated or treated with wounding and 1:6 diluted oral secretions of *Manduca sexta* larvae (OS-elicitation) to mimic herbivory as described in Schmidt & Baldwin (2006). A third group of WT plants was treated with wounding, 1:6 diluted OS of *M. sexta* larvae and 2.5 pmol *S. nigrum* systemin (AnaSpec,

San Jose, CA, USA; systemin was synthesized according to the protein sequence published by Constabel, Yip & Ryan (1998) in 40 μ L water (systemin-augmented OS-elicitation). The systemin solution was applied to the puncture wounds after the oral secretions were dried to minimize the possibility that systemin would be digested by proteases possibly contained in the oral secretions. IR*Sys* plants were left untreated. The first of five consecutive treatments was performed 24 days post-sowing on the node five leaf; the respective treatment was repeated every second day on the next younger leaf. Five days after the last treatment (37 days post-sowing), the shoots and roots of all plants were harvested separately, dried for four days at 60°C in a drying oven, and weighed.

To determine whether the different systemin levels were reflected in transcriptional changes in the above-ground-tissues, plants were grown in competition in 1L sand pots in the following combinations: (1) uninduced WT versus OS-elicited WT and (2) uninduced WT versus systemin-augmented OS-elicited WT. The treatment procedure as well as the schedule was the same as that of the singly grown plants described above, except that consecutive treatments started on the node four leaf. Four days after the fifth treatment (36 days post-sowing), the plants were treated at the node nine leaf. One hour after elicitation, the treated leaf was harvested together with the corresponding leaf of the uninduced competitor. All samples were flash-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

To test for the fitness consequences of different systemin levels, plants in 2L soil pots were grown in competition in the following combinations: (1) uninduced WT versus uninduced IR*Sys* line, (2) uninduced WT versus OS-elicited WT, and (3) uninduced WT versus systemin-augmented OS-elicited WT. For all combinations, the 2L pots were either partitioned with plastic barriers to separate the below-ground space of the competing plants or the competitors shared the entire 2 L pot. The first of eight consecutive treatments was performed 23 days post-sowing beginning on the node five leaf and repeated every second day on the next younger leaf. 47 to 51 days after the last treatment (84 to 88 days post-sowing), the berries of all plants were counted individually.

To ensure that the experiments started with plants of comparable sizes, the singly grown plants belonging to the four groups as well as the competing plants growing together in one pot were matched according to their initial shoot lengths.

All experiments described above consisted of seven to ten replicate plants per treatment group.

RNA extraction and microarray procedure

Harvested leaves were ground individually in liquid nitrogen and total RNA was extracted according to a modified TRI Reagent procedure for polysaccharide- and proteoglycan-rich sources (TIGR 2003).

Equal amounts of RNA of three plants per treatment were pooled to give 400 µg RNA. After poly(A)⁺ RNA isolation the generated cDNA was fluorescently labeled with cy3 and used together with cy5-labeled cDNA originating from a similarly pooled and treated RNA sample of the respective, uninduced competition partners to hybridize a custom-made 1.4k 50mer-oligonucleotide microarray. On this microarray all clones were spotted twice in pairs, resulting in four spots per clone. The first hybridization (OS-elicited WT versus uninduced WT) was repeated twice; the second (systemin-augmented OS-elicited WT versus uninduced WT) was repeated three times. cDNA from different plants was used for all hybridizations.

The microarrays were analyzed by extracting the cy3 and cy5 spot intensities (SIs) from image files using the software AIDA (Raytest, Straubenhardt, Germany), followed by local-background (IBg) subtraction and LOWESS normalization of these SIs using the software MIDAS (Saeed *et al.* 2003). Spots below 1.5x signal-to-noise ratio ($= 2 \times \text{SI} / \text{IBg}$) were set to zero. For statistical analyses, the Bg-corrected SIs were log-transformed. Single slides were evaluated on the basis of an average treatment /control ratio > 1.5 or < -1.5 and a *P*-value (t-test of four spots per clone and exclusion of zero values) < 0.05 . When hybridizations were conducted with three biological replicates, a nested ANOVA was performed to identify significantly regulated clones as determined by an average treatment /control ratio > 1.5 or < -1.5 and a *P*-value < 0.05 . When only two hybridizations were available, clones were considered to be regulated when they were regulated in the same way on both single slides, each fulfilling the criteria of an average treatment /control ratio > 1.5 or < -1.5 and a *P*-value (t-test of four spots per clone and exclusion of zero values) < 0.05 . Clones that had a calculated treatment/control ratio of 0 and *P*-value of 1 were recalculated on the basis of their un-normalized signals. These clones were considered to be up-regulated if the cy3 value exceeded the signal-to-noise-ratio 2.5x or down-regulated if the cy5 value exceeded this threshold.

Results

Low systemin levels were associated with greater root mass

When wild-type (WT) plants were OS-elicited, a treatment previously shown to rapidly decrease the accumulation of prosystemin transcripts (Schmidt & Baldwin 2006), elicited plants accumulated significantly more root mass compared to uninduced WT plants 37 days after sowing (Fig. 1A). This difference could be negated by an additional systemin treatment; root mass in the OS-elicited WT plants supplied with synthetic systemin did not differ significantly from root mass in uninduced WT plants (Fig. 1A). The root mass of a transgenic line silenced in its prosystemin expression (IR S_{ys} line) was significantly greater than that of the uninduced WT plants (Fig. 1A).

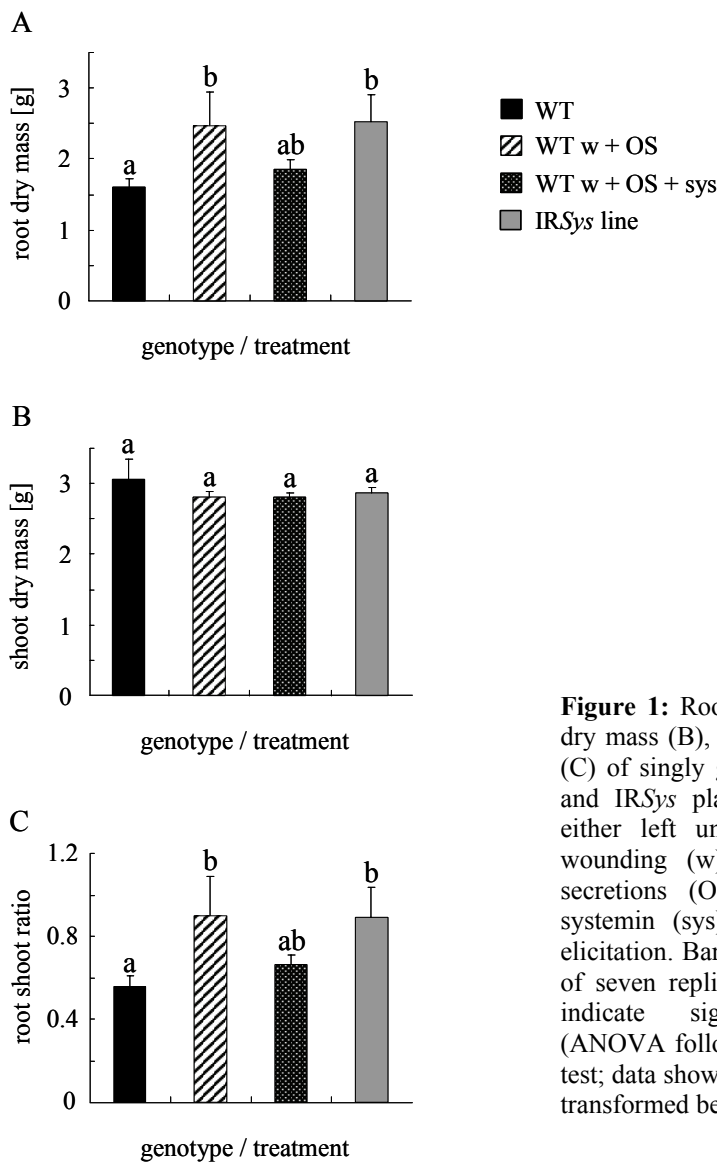


Figure 1: Root dry mass (A), shoot dry mass (B), and root to shoot ratio (C) of singly grown wild-type (WT) and IR S_{ys} plants. WT plants were either left untreated, elicited with wounding (w) and *M. sexta* oral secretions (OS) or supplied with systemin (sys) in addition to OS-elicitation. Bars represent mean \pm SE of seven replicates. Different letters indicate significant differences (ANOVA followed by LSD post-hoc test; data shown in A and C were log-transformed before analysis).

None of the treatments significantly changed the shoot mass of WT plants compared to uninduced WT plants (Fig. 1B). Similarly, no significant differences in shoot mass between WT plants and IRSys plants were observed (Fig. 1B). As differences in shoot mass were absent, the root-to-shoot (Fig. 1C) ratio reflected the different root masses.

Systemin positively affected growth-related genes

OS-elicited WT plants significantly down-regulated 86 clones, whereas systemin-augmented OS-elicited WT plants significantly down-regulated 230 clones (Fig. 2A). Both treatments commonly down-regulated 78 clones, and specifically down-regulated 8 clones in OS-elicited plants and 152 clones in systemin-augmented OS-elicited WT plants (Fig 2A). A similar pattern was found for the up-regulated clones: 95 were significantly up-regulated in OS-elicited WT plants and 218 were significantly up-regulated in systemin-augmented OS-elicited WT plants (Fig. 2B). 88 clones were up-regulated in both treatment groups; 7 and 130 clones were up-regulated in OS-elicited and systemin-augmented OS-elicited treatments, respectively. Among the 152 clones exclusively down-regulated in systemin-augmented OS-elicited WT plants when compared to uninduced WT competitors were 11 clones involved in starch metabolism or spermine metabolism (Fig. 3).

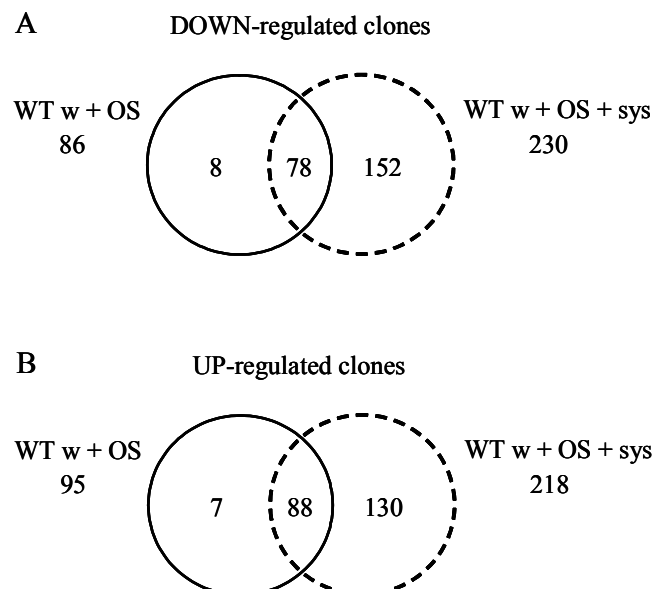


Figure 2: Number of down- (A) and up-regulated (B) clones in WT plants treated either with wounding (w) and oral secretions (OS) of *M. sexta* or with wounding and OS and systemin (sys) when compared to their uninduced WT competitors. Numbers given are based on two (WT w + OS versus uninduced WT) or three (WT w + OS + sys versus uninduced WT) microarrays, each hybridized with the cDNA of three individual plants per treatment.

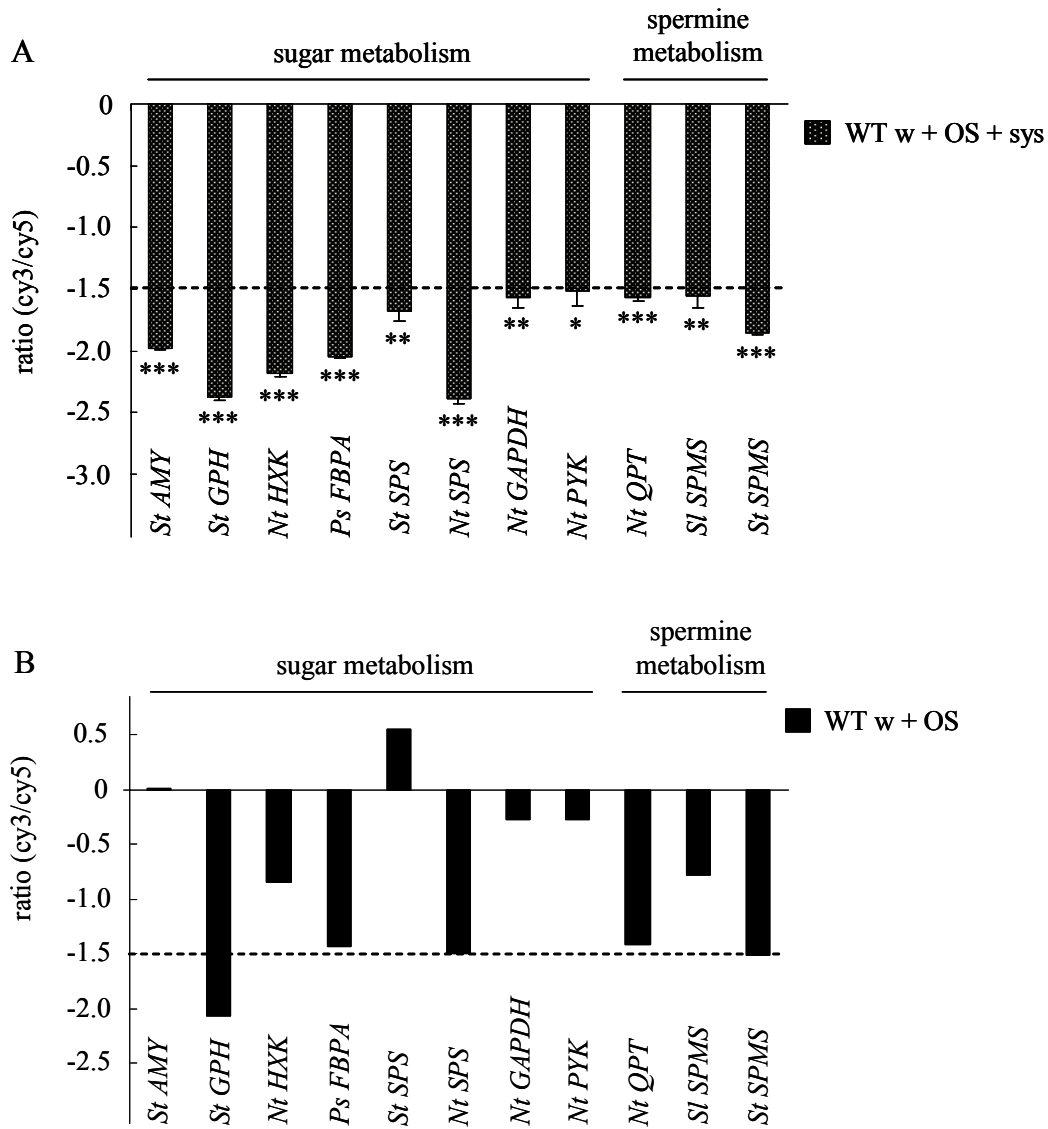


Figure 3: Regulation of growth-related clones in WT plants treated with wounding (w) and oral secretions (OS) and systemin (sys) (A) or with wounding and OS (B) when compared to their respective uninduced WT competitors. In the case of three replicates per hybridization (A), clones were referred to as significantly regulated when an average treatment /control ratio > 1.5 or < -1.5 and a P -value (nested ANOVA) < 0.05 was achieved. When only two hybridizations were available (B), clones were referred to as regulated when they were regulated in the same way on both single slides, each fulfilling the criteria of an average treatment /control ratio > 1.5 or < -1.5 and a P -value (t-test of four spots per clone) < 0.05 . Data shown are means \pm SE of the respective microarray replicates. Stars indicate significant differences (* < 0.05 , ** < 0.01 , *** < 0.001).

AMY = alpha-amylase, GPH = alpha-glucan phosphorylase H, HXK = hexokinase, FBPA = fructose-bisphosphate aldolase, SPS = sucrose-phosphate-synthase, GAPDH = glyceraldehyde-3-phosphophate dehydrogenase, PYK = pyruvate kinase, QPT = quinolinatephosphoribosyltransferase, SPMS = spermidine synthase, *St* = *Solanum tuberosum*, *Nt* = *Nicotiana tabacum*, *Ps* = *Pisum sativum*, *Sl* = *Solanum lycopersicum*.

Plants with low systemin levels showed greater fitness under competitive growth

When the below-ground space of plants grown together in one pot was separated by a plastic barrier, no significant differences in berry production were observed (Fig. 4A-C). Only OS-elicited WT plants showed a trend ($P = 0.086$) towards lower berry production compared to uninduced competitors (Fig. 4B). In the absence of a root barrier, uninduced IRSys plants produced significantly more berries compared to competing, uninduced WT plants (Fig. 4D). When OS-elicited WT plants were grown in competition with uninduced WT plants, no significant difference in berry production was observed (Fig. 4E). However, if the down-regulation of (pro)systemin in OS-elicited WT plants was counteracted by systemin supplementation, plants produced significantly fewer berries compared to their uninduced competitors (Fig. 4F).

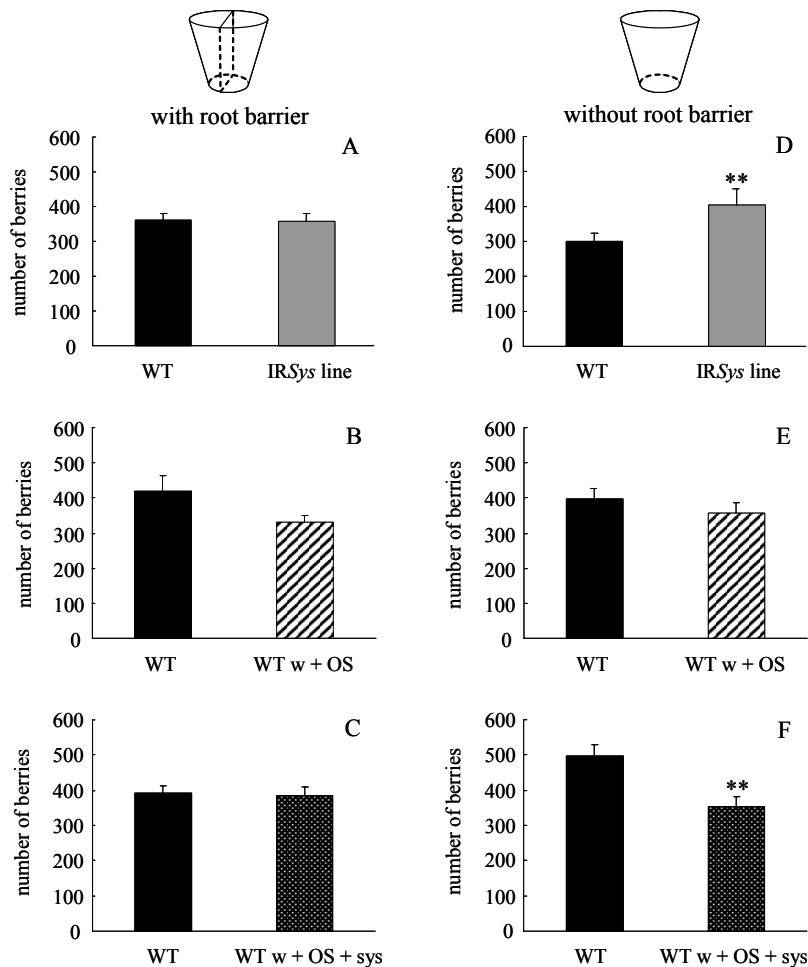


Figure 4: Number of berries produced by plants growing in competition, both competitors either being belowground separated by a solid root barrier (A-C) or having root contact (D-F). Bars represent means \pm SE of nine to ten replicates. Stars (**) indicate significant differences (paired t-test, $P < 0.01$; data shown in B were log-transformed before analysis). WT = wild-type; IRSys line = transgenic line silenced in prosystemin expression; w = wounding; OS = *M. sexta* oral secretions; sys = systemin.

Discussion

The study aimed to reveal the function of systemin in *S. nigrum* by testing the hypothesis that the rapid transcriptional down-regulation of (pro)systemin after induction enables plants to tolerate herbivory. In growth experiments, uninduced WT plants were compared with WT plants that had been either only OS-elicited or additionally supplied with systemin and with plants silenced in their prosystemin expression (IRSys line). To gain insight into what effects the observed phenotypic responses may have had on the above-ground tissues, transcriptional analyses of treated plants grown in competition with uninduced WT plants were conducted. The reproductive output of competing plants was quantified to evaluate the fitness consequences of different endogenous systemin levels.

The root mass of OS-elicited WT plants significantly exceeded the root mass of uninduced WT plants and of systemin-augmented OS-elicited WT plants (Fig.1A). These data, along with those from our previous study (Schmidt & Baldwin 2006), where we showed that prosystemin was rapidly down-regulated after induction, led us to conclude that large root masses after OS-elicitation are associated with low systemin levels. This attribution was supported by the root mass of IRSys plants, which was significantly larger than the root mass of uninduced WT plants (Fig.1A). Importantly, (pro)systemin is expressed only in the above-ground tissues, not in the roots of *S. nigrum* (Schmidt & Baldwin 2006). This fact suggests some form of shoot-root communication in which systemin affects distal plant parts.

Our findings that systemin regulation is associated with changes in root growth is consistent with the recent work of Holton *et al.* (2007). When seedlings of wild tomato (*Solanum pimpinellifolium*) were grown on systemin-containing medium, their roots were longer than those of untreated seedlings. However, the roots of cultivated tomato (*Solanum lycopersicum*) *cu3* mutants which are brassinosteroid (BR)-insensitive due to a defect in the systemin-brassinosteroid-double receptor (SR160/BRI1) were inhibited when grown on systemin-containing medium. These observations indicate the involvement of both BRI1-dependent and -independent systemin signaling in the root-growth response. Unlike in wild tomato, root length in a systemin-treated tomato line silenced in prosystemin expression was reduced, suggesting that species-specific factors influence how roots respond to systemin. That root lengths of the ethylene-insensitive tomato mutant *Never ripe* were not altered by adding systemin indicates that ethylene-perception is required for the systemin-mediated root response.

Having found that low systemin levels were associated with increased root mass, we were interested in the consequences for above-ground tissues. We assumed that the phenotypical changes in root mass would be reflected in the transcriptome of the leaves, as resources transported from the roots might be used in the shoots and vice versa. Our first assumption was that the transcriptional down-regulation of prosystemin in OS-elicited plants would result in regulation of growth-related clones relative to uninduced plants. Second, we expected this regulation to be absent in systemin-augmented OS-elicited WT plants relative to their uninduced competitors. Surprisingly, we observed the opposite, which indicated that the permanent presence of systemin positively affected gene regulation. The majority of specifically elicited clones were found in systemin-augmented OS-elicited plants (Fig. 2A and B) which had high systemin levels, like their uninduced WT competitors. In contrast, clones of OS-elicited plants were only marginally regulated (Fig. 2A and B); these plants are characterized by a dramatic down-regulation of systemin compared to the competing WT plants. The clones that were regulated in the continuously high levels of systemin were no longer regulated when systemin was down-regulated or absent.

Even though our microarray was primarily defense-related, we found eight clones involved in sugar metabolism to be down-regulated solely in systemin-augmented OS-elicited WT plants (Fig. 3). This suggests that high systemin levels after OS-elicitation along with an unchanged root mass compared to unelicited WT plants may be associated with a reduced ability to convert sugars into energy, which in turn could contribute to diminished growth. As OS-elicited plants, however, did not regulate these clones, the negative effect on their energy levels might be low, leading to unchanged growth rates.

As in sugar metabolism, clones involved in spermine metabolism were down-regulated exclusively in systemin-augmented OS-elicited WT plants (Fig. 3). Spermine, like other polyamines, is essential for growth and development, which again suggests that high systemin levels after OS-elicitation may be associated with reduced growth. The regulation of spermine metabolism in OS-elicited WT plants did not differ from that in uninduced WT plants, which would be consistent with a lack of differences in growth.

When the below-ground space of competing plants was separated by a root barrier, low systemin levels seemed not to influence plant fitness as measured by lifetime berry production (Fig. 4A-C). Only OS-elicited WT plants showed a trend towards lower fitness compared to uninduced WT plants (Fig. 4B). However, low systemin levels became

advantageous when the root barrier was removed: IRSys plants, with their large root allocations, represented potent competitors that were able to produce significantly more berries when competing with uninduced WT plants (Fig. 4D). Similarly, OS-elicited WT plants were not impaired in competitiveness and fitness (Fig. 4E) even though the OS-elicitation of *S. nigrum* is known to induce costly resistance traits such as the production of proteinase inhibitors (Constabel, Yip & Ryan 1998; Schmidt *et al.* 2004; Schmidt & Baldwin 2006). However, treating OS-elicited WTs additionally with systemin reduced their competitive abilities and caused them suffer a significant reduction in berry production when competing with uninduced WT plants (Fig. 4F).

Two explanations of these findings come to mind. First, the increased root mass which was associated with low systemin levels (Fig. 1A) may allow plants to “steal” resources from their competitors. Their competitiveness enhanced, these plants may be able to compensate for the costs of induced resistance or tissue loss due to herbivory. As IRSys plants mimic OS-elicited WT plants in terms of low systemin levels but not induced defenses, and thus had no defense costs to compensate for, their berry production increased in comparison to that of uninduced WT competitors (Fig. 4D). Consistent with the proposed “resource stealing hypothesis”, the advantage of low systemin levels and increased root masses would disappear in the presence of a root barrier. In accord with this, fitness differences between IRSys and uninduced WT plants were absent (Fig. 4A) and OS-elicited WT plants showed a trend towards lower fitness compared to uninduced WT plants (Fig. 4B) in the presence of a root barrier.

Alternatively, the increased root mass may function as a below-ground storage reserve, such as is described in Schwachtje *et al.* (2006). Plants could use such a reserve to (re)grow and hence to compensate for the costs of induced defenses or tissues lost to herbivores when the attack ceases. An elegant experiment to test this hypothesis would be to quantify photoassimilate flux to roots by supplying OS-elicited or systemin-augmented OS-elicited leaves with $^{11}\text{CO}_2$ and measuring the amount of ^{11}C in the roots.

Assuming that the increased root mass may have functioned as a below-ground storage reserve for (re)growth, the lack of fitness differences between uninduced IRSys plants and uninduced WT competitors (Fig. 4A) as well as the tendency of OS-elicited WT plants toward lower berry production compared to their uninduced competitors (Fig. 4B) in the presence of a root barrier might be a negative effect of overlapping nutrition uptake zones of

adjacent roots in the restricted rooting volume. Such observations are described by McConnaughay & Bazzaz (1992). As the number of overlapping uptake zones would have increased with increasing root mass, plants having more roots (= plants with low systemin levels) would have had a reduced net nutrient uptake per root unit; the upshot would have been the loss of the beneficial effect of a larger root mass.

Unexpectedly, the berry production of systemin-augmented OS-elicited WT plants equaled that of uninduced WT competitors when both were separated below-ground (Fig. 4C). As both treatment groups were characterized by equal root masses (Fig. 1A), other mechanisms probably independent of root mass and systemin appear to be involved in the compensatory actions taken by systemin-augmented OS-elicited WT plants in the presence of a root barrier.

The fundamental observation that large root masses which are associated with low systemin levels are beneficial for a competing plant presumably also applies to singly-grown plants (Fig. 1). As long as the extra roots do not lead to overlapping nutrient uptake zones, as in the restricted volume of a pot and increase the possibility of acquiring additional nutrients, they may be advantageous.

Even though the underlying mechanisms have not yet been clarified, the systemin-associated increase in root mass after herbivore elicitation seems to enhance the fitness of competing plants. Thus, we propose that the down-regulation of systemin helps *S. nigrum* tolerate herbivory. Still needing more detailed investigation, our data compared to those of the well-studied tomato system suggest divergent roles of systemin in different species. Thus, tolerance hypothesis presented here will hopefully stimulate research into alternative functions for this intriguing suite of peptides.

Acknowledgments

We thank Klaus Gase, Wibke Kröber, Antje Wissgott, and Susan Kutschbach for the transformation construct and plant transformation, and Silke Allmann and Wibke Kröber for accurately performing the entire microarray procedure. We are thankful for the generous help of Holger Merker, Arjen van Doorn, Dominik D. Schmidt, and Hoang Hoa Long in counting the berries. Thanks to Emily Wheeler for editorial assistance and to the Max Planck Society for funding.

4 General discussion



The present thesis demonstrates the successful establishment of *S. nigrum* as a model system by developing new tools and adopting tools that have been previously established for other solanaceous species to manipulate and quantify its responses to herbivory. Concurrently it exemplifies the great need and scientific value of using wild species to understand a plant's response to herbivory, as those responses likely change or are even lost during domestication. Field-grown *S. nigrum* plants were attacked by several herbivores belonging to different feeding guilds, which resulted in the production of proteinase inhibitors (PIs) and the emission of volatile organic compounds (manuscript I). Both traits represent induced resistance responses of plants to herbivory which are well-known from other plant systems. Furthermore, insect attack led to a drastic transcriptional reorganization (manuscripts I and II), which appeared to be quite distinct from that of another solanaceous plant, indicating that herbivore resistance is regulated via fundamentally different signaling cascades even within closely related species (manuscript II). In line with these diverging responses was the finding that systemin did not mediate direct resistance responses in *S. nigrum* as shown previously for the homologous polypeptide in tomato (manuscript III). Moreover, the regulation of systemin in *S. nigrum* was associated with enhanced root growth and improved the plant's competitive ability, suggesting that systemin might be involved in tolerance to herbivory (manuscript IV). Thus, systemin seemed to reduce the detrimental effects of herbivore damage on *S. nigrum*'s fitness without affecting the herbivore rather than reducing the preference or performance of the phytophagous insects.

4.1 Transgenic plants as powerful tools for studying plant-insect interactions

To understand plant-herbivore interactions, detailed mechanistic knowledge of a plant's responses to herbivory is required. Since these responses are regulated via complex, often interacting pathways, the challenge is to dissect these pathways and identify the ecological relevance of a particular gene or trait. A useful approach is to experimentally manipulate the gene or trait of interest by engineering a change or disrupting the DNA. A subsequent study of the resulting phenotype can provide insights into the function of the manipulated gene. This approach is generally termed reverse genetics and differs from the

forward genetic screen of classical genetics in which the researcher starts with a mutation phenotype and tries to identify the mutated gene. The process of disruption or alteration can be non-targeted and random and is achieved by transposon or chemical mutagenesis. Both techniques involve the creation of large mutagenised populations similar to those used in forward genetic screens. Alternatively, the disruption or alteration can be targeted specifically as happens in gene silencing by RNA interference (RNAi) or virus-induced gene silencing (VIGS). As the screening of large numbers of individuals to detect mutations in a given gene represents an enormous experimental effort and requires advanced high-throughput techniques (Gilchrist & Haughn 2005), the mutation approach rather is the method of choice for unraveling the genetic basis of a given mutated phenotype (i.e. forward genetics). In contrast, the use of targeted gene silencing can be regarded as the most straightforward approach to manipulate a gene or trait in order to understand its functions. Moreover, gene silencing represents a method for knocking genes down, thus allowing the study of genes which might be lethal when knocked out completely by insertion or point mutations.

Our studies on *S. nigrum* plants that are silenced in the expression of prosystemin highlight the great potential of this technique in tracking down the phenotype that resulted when a given gene was disrupted (manuscripts III and IV). The fact that this was even possible in a newly established model species for which almost no genomic sequence information was available adds further to the potential of this approach in studies on functional genomics. Furthermore, the great value of returning silenced plants to their natural habitat to study the function of the focal gene in the natural situation was demonstrated (manuscript III). Studying the gene or trait of interest not only in the greenhouse but also under field conditions is essential, as it is not possible to simulate the complexity of a field situation in an artificial environment. Thus, hypotheses tested solely in greenhouse experiments risk leading to conclusions that are not of ecological relevance.

Whereas gene silencing provides a powerful tool to manipulate a specific gene or trait to study its phenotypic consequences and its relevance in a plant's response to herbivory, microarrays are capable of providing a general overview over the transcriptional responses of a plant to a given situation such as herbivory.

4.2 The big picture provided by microarrays

Attack by leaf-chewing *M. sexta* larvae resulted in a drastic reorganization of *S. nigrum*'s transcriptome as demonstrated by means of a 10k-cDNA microarray (manuscript II). As a general and rather unspecific response, *S. nigrum* up-regulated chaperones as well as several genes related to oxidative stress and dehydration. Among the up-regulated genes which specifically targeted herbivory were those leading to the production of jasmonic acid, the central molecule mediating induced resistance responses. The main transcriptional emphasis regarding secondary metabolism was on (i) the mevalonic acid pathway leading to steroidal alkaloids and (ii) sesquiterpenoid biosynthesis; a leucine amino peptidase (LAP) gene, which appeared to be 20-fold up-regulated, was the most pronounced answer to *M. sexta* attack. In summary, this transcriptional analysis indicated that *S. nigrum* possesses an array of induced resistance mechanisms as a response to herbivory. As only little is known about *S. nigrum*'s defense responses, the predictions that can be made as a result of the identification of these genes represent a reasonable basis for further metabolomic and functional studies.

Most interestingly, *S. nigrum*'s response was both quantitatively and qualitatively distinct from that of *Nicotiana attenuata*, another solanaceous plant: *S. nigrum* was characterized by the regulation of fewer genes compared to *N. attenuata* and differences in gene regulation exceeded the anticipated differences in alkaloid biosynthesis. The comparative analysis pointed to fundamental differences in the signaling cascades and downstream genes mediating herbivore resistance even in these species belonging to the same family. In accordance with this observation were our findings that systemin is associated with tolerance to herbivory in black nightshade rather than with resistance to herbivores as has been demonstrated for tomato. These findings suggest that systemin might be under diversifying selection. However, at present it is unclear whether this resulted from the comparison between two different species or from the fact that wild and domesticated plants were compared. Thus, future studies in wild tomato genotypes that investigate the plant's response to herbivory in general and the role of systemin in particular will be necessary.

4.3 The (ongoing) evolution of systemin

Given that systemin is supposed to play a central role in the wound response of tomato by amplifying the jasmonate-based mobile wound signal, it is quite surprising that its function

seems not to be conserved among Solanaceae. Whether the divergent roles of systemin in *S. nigrum* and tomato can be attributed only to systemin or can be expanded to structural or functional changes of the systemin receptor is still an open question. Comparing the capacity of tomato and black nightshade systemin to induce proteinase inhibitors in the corresponding species and across species (Table 1) gives rise to different speculations. The observation that

	Tomato	Black Nightshade
Tomato systemin	YES	NO
Black Nightshade systemin	NO	NO

Table 1: Capacity of systemins derived from tomato and Black Nightshade to induce proteinase inhibitors (PIs) in the two species.

tomato systemin but not *S. nigrum* systemin induced proteinase inhibitors in tomato suggests that the loss of the PI-inducing ability of *S. nigrum* systemin could be attributed solely to changes in the systemin sequence which might reduce or prevent it from binding effectively to the tomato systemin receptor. To explain the absence of a PI-inducing effect of tomato systemin in *S. nigrum*, three related hypotheses are possible. First, the absence might again be due to differences in the systemin sequences which may prevent an effective binding of tomato systemin to the *S. nigrum* receptor. Second, this would imply a changed systemin receptor in *S. nigrum* as compared to tomato. Third and most intriguingly, independent of possible differences between the receptors in both species, tomato systemin could be able to effectively bind to the *S. nigrum* receptor yet activate different down-stream cascades in the two species. The activation of different cascades is suggested by the data presented in manuscript IV, which indicated systemin was associated with root growth and tolerance to herbivory. Thus, it would be fascinating to see whether tomato systemin has the same effect on *S. nigrum*'s root growth as *S. nigrum* systemin does. It might well be that systemin also effects root growth in tomato, a line of thought which is supported by the recent work of Holton et al. (2007) as discussed in manuscript IV. This argues for a dual role of systemin in tomato.

Complementing the above-mentioned hypothesis that the binding of systemin to its receptor activates different down-stream cascades, the binding of diverse ligands to the same receptor may cause the activation of different down-stream cascades. This connection is supported by the finding that the systemin receptor SR160 of wild tomato (*Lycopersicon peruvianum*) shows a high sequence similarity with the brassinosteroid receptor BRI1 from *Arabidopsis* (Scheer & Ryan 2002). If SR160 serves as a BR receptor in tomato (Yin, Wu & Chory 2002), depending on its ligand, the same receptor would activate either defense or brassinosteroid signaling within one species. It is tempting to speculate and currently under investigation in tomato whether and how systemin is involved in the brassinosteroid-mediated regulation of growth and development. Alternatively, the question arises if brassinosteroids also influence systemin-related defense responses, be it resistance as in tomato or tolerance as in *S. nigrum*.

To better understand the recent functions of systemin, it appears to be indispensable to follow the evolution of systemin and its receptor along the phylogeny of the genus *Solanum*. Future comparative molecular and functional studies between different species may disentangle the basal and derived characteristics of systemin. Beyond this, such studies may detect whether the observed functional switch between tomato systemin and *S. nigrum* systemin has occurred repeatedly during evolution. Including the proposed diploid and tetraploid ancestors of *S. nigrum*, *S. americanum* and *S. villosum* in the analysis may contribute to elucidating the effect of polyploidization on the evolution of systemin.

Both the current lack of knowledge concerning systemin's evolution as well as the emerging understanding of its recent function in *S. nigrum* bring up new and exciting questions.

4.4 Tolerance in *S. nigrum*: an interplay of extrinsic and intrinsic factors

As demonstrated in manuscript IV, the down-regulation of systemin after elicitation is associated with an increased root mass that enhances the competitive abilities of induced plants. Increased competitive abilities might in turn enable the plant to compensate for costs of induced defenses and probably also for tissue loss to herbivores. The fact that the higher allocation to roots seemed not to be advantageous when the below-ground space of the competing plants was separated by a solid root barrier suggested that extrinsic (biotic and abiotic environmental characteristics) and intrinsic factors (genetically or developmentally

determined traits) leading to tolerance in *S. nigrum* are tightly linked. As the extra roots only seemed to benefit the plant as long as they increased the possibility of acquiring additional resources, the most important extrinsic factor seemed to be the availability of soil-derived resources. The importance of a high availability of nutrients in addition to water and light in effecting tolerance has also been suggested by Maschinski & Witham (1989). Even though the role of light and nutrients has received little attention, surprisingly, nutrient availability was found to be negatively associated with tolerance in several plant systems (Gertz & Bach 1995; Mutikainen & Walls 1995; Irwin & Aarssen 1996). As one possible explanation for this phenomenon, the decrease in the root-shoot-ratio has been proposed (Olf, Vanandel & Bakker 1990), which is generally believed to be associated with reduced tolerance. Besides resource availability, low competition is thought to be positively associated with tolerance as (i) competitors might reduce soil-based resources and, (ii) the loss of apical dominance would be particularly detrimental in competitive environments where light is limited. However, in *S. nigrum* soil-borne resource availability and competition seemed to be positively associated with tolerance.

To substantiate the interpretation of these associations, a more detailed knowledge of the intrinsic factors, especially the underlying mechanisms following the down-regulation of systemin, is essential. Tests of the two hypotheses proposed in manuscript IV might shed light on the advantage of increased root mass in a competition situation and thus contribute to a better understanding of the observed phenotype. The first hypothesis, which involves nutrients stolen from the competitor with the fewest roots, could be tested in experiments with varying nutrient levels of different kinds such as nitrogen or phosphate. Furthermore, an understanding of uptake rates for different resources in plants at different life stages and in different tissues by using labeled compounds such as $K^{15}NO_3$ could help illuminate the mechanism that forms the basis of the observed phenotype. The second hypothesis, which proposes that the increased root mass functions as a below-ground storage reserve, could be elegantly tested by quantifying the photoassimilate flux to roots. This could be done by supplying OS-elicited or systemin-augmented OS-elicited leaves with $^{11}CO_2$ and measuring the amount of ^{11}C in the roots.

Given that a lack of systemin seems to help *S. nigrum*, an important question can be posed: Why did *S. nigrum* not lose systemin completely during evolution? Assuming that systemin is not a genetic load, two hypotheses to explain this phenomenon come to mind.

First, systemin may have one or more yet to be elucidated functions that support the conservation of the gene. Second, the down-regulation of systemin after induction along with the accompanying root growth might be considered a plastic response that allows the plant to react to unfavorable conditions such as herbivory. Even though constitutive high root growth would benefit the plant in the absence of herbivores, by always growing a maximum root, the plant would lose its ability to respond to the presence of herbivores.

Although we are only just starting to understand *S. nigrum*'s responses to insect herbivory in general and the role of systemin in particular, the data presented in all four manuscripts indicate that the plant possesses an array of systemin-dependent and -independent strategies to reduce the fitness-imperiling stresses caused by phytophagous insects.

4.5. Fitness safeguards in *S. nigrum*: tolerance and resistance

Tolerance and resistance represent opposing ways a plant can cope with herbivore attack, and trade-offs between these two strategies are assumed to occur. Some studies found a negative correlation between resistance and tolerance (Fineblum & Rausher 1995), whereas others did not (Mauricio, Rausher & Burdick 1997). Leimu & Korchiva (2006) recently concluded from their meta-analysis of 31 ecological and agricultural studies that tolerance and resistance are not mutually exclusive. This conclusion also seems to apply to *S. nigrum*: although we propose systemin-associated and root-growth mediated tolerance to occur in *S. nigrum* (manuscript IV), the plant exhibits in addition several putative resistance traits with which it might defend itself against herbivores (Table 2).

	direct	indirect
constitutive	trichomes	extrafloral nectaries
induced	poteinase inhibitors steroidal alkaloids	sesquiterpenes

Table 2: Overview over (putative) resistance traits in *S. nigrum*.

The leaves and stems of *S. nigrum* feature multicellular trichomes with glandular or aglandular heads (Edmonds & Chweya 1997) that likely function as a direct constitutive resistance trait to repel herbivorous insects. Whether these epidermal outgrowths actually have a defensive function such as in *Arabidopsis lyrata* (Clauss *et al.* 2006) or *Liabum mandonii* (Molina-Montenegro *et al.* 2006), and if they are potentially inducible, remains to be tested. Another poorly studied characteristic is *S. nigrum*'s secretion of extrafloral nectar (EFN) on the abaxial leaf surface. By attracting ants and wasps which benefited the nectar-producing plant by reducing the damage level, EFN has been shown to positively affect plant fitness (Bronstein 1998; Cautle & Rico-Gray 2003). As the nectar collected from field-grown *S. nigrum* plants has also been shown to attract ants (Hartl & Schmidt, personal observation), its involvement in indirect constitutive or induced resistance is likely.

As shown in manuscripts I and III, *S. nigrum* produces proteinase inhibitors as a direct induced resistance trait upon herbivore damage by the flea beetle *Expitrix pubescens*, or after induction with methyl jasmonate or treatment with wounding and oral secretions. While the defensive function of proteinase is already known from other plant systems such as tomato (Ryan 1990; Orozco-Cardenas, McGurl & Ryan 1993) and wild tobacco (Zavala *et al.* 2004), the importance of the production of proteinase inhibitors in the defense response of *S. nigrum* is currently under investigation (Hartl & Baldwin, in preparation). Importantly, it still remains to be demonstrated that the growth-inhibiting effect of proteinase inhibitors on caterpillars results in less plant damage. Complementing its repertoire of direct induced resistance, *S. nigrum* elicits steroidal alkaloids such as solasodine (Eltayeb, AlAnsari & Roddick 1997), the production of which is also reflected in its induced transcriptome (manuscript II). As steroidal alkaloids could be shown to disrupt membranes and inhibit acetylcholine esterase, a key enzyme in nerve impulse transmission, they are likely to act as defensive compounds against a broad range of organisms, from microorganisms to mammals.

Besides the up-regulation of genes involved in the production of steroidal alkaloids, a second transcriptional emphasis laid on sesquiterpenes' synthesis (manuscript II). These volatile organic compounds were shown to be induced upon herbivore damage in several plant species and are known to be involved in indirect induced defenses. Sesquiterpenes such as β -caryophyllene were also detected in the volatile blend of field-grown *S. nigrum* plants that were attacked by the Colorado potato beetle (*Leptinotarsa decemlineata*) or Deathhead

Hawk-moth (*Acherontia atropos*) (manuscript I). However, studies including predators or parasitoids which might be attracted to the volatiles emitted by *S. nigrum* have yet to be done.

Taken together, the (putative) defense responses of *S. nigrum* are diverse, ranging from systemin-associated tolerance to resistance and covering the whole spectrum from direct constitutive to indirect induced resistance. This breadth might be regarded as a response to the diverse herbivore community that feeds on *S. nigrum*: the plant has been observed to be commonly attacked by 17 generalist and a two specialist herbivores belonging to three different feeding guilds (manuscript I). In response to such a diverse herbivore community, evolution should favor a broad range of defense mechanisms. Such a pattern is commonly found in nature (Berenbaum 1985): plants are known to produce either two or more different classes of secondary compounds (Hugentobler & Renwick 1995) or, alternatively, a combination of chemical and physical defenses. Although this pattern suggests that different resistance mechanisms are not necessarily mutually exclusive, to what extent different types of defenses have evolved in response to different sets of herbivores is not yet clear. It can only be assumed that mutual exclusivity is likely when two defense mechanisms are redundant by being directed towards the same group of herbivores. In this light it is even more surprising that tolerance and resistance can coexist, because tolerance is a rather unspecific response that allows the plant to compensate for damage inflicted by a wide variety of herbivores. Tolerance might thus be redundant to other types of resistance. However, *S. nigrum* seems to have evolved several defense mechanisms to maintain its fitness after herbivore attack.

5 Summary

In order to minimize the fitness-imperiling stress caused by phytophagous insects, plants have evolved an enormous variety of defensive adaptations. These plant defenses against herbivory are generally categorized as either tolerance or resistance. Tolerance traits reduce the detrimental effects of herbivore damage on plant fitness without affecting the herbivore. In contrast, resistance traits either directly or indirectly reduce the amount of damage a plant receives by repelling potential herbivores or impairing their performance.

While tolerance mechanisms in plants remain nearly uncharacterized on the molecular level, the signals and signal pathways leading to herbivore resistance have been extensively studied. As plants are well known to show resistance responses to herbivory in not only locally attacked but also distal, undamaged leaves, the signals mediating these so-called systemic responses have received extensive attention. Among the signals proposed to be capable of transmitting information about herbivore attack from the site of wounding to the rest of the plant is the 18-aa polypeptide systemin. Systemin has long been thought to be the mobile wound signal in tomato (*Solanum lycopersicum*), but recent grafting experiments have demonstrated that both the presence of systemin and jasmonic acid (JA) biosynthesis are required in the local, damaged leaf to produce a systemic signal which subsequently induces proteinase inhibitors (PIs) in distal, unwounded leaves. Moreover, neither systemin nor JA biosynthesis seemed to be required in undamaged leaves to produce PIs. With these findings, the role of systemin in the wound response of tomato had to be reconsidered. According to the revised model, systemin plays a central role in tomato's wound response acting at or near the site of wounding by amplifying the jasmonate-derived mobile wound signal.

So far, resistance responses to insect herbivory and the underlying signal pathways have been studied almost exclusively in crop plants such as tomato. An important question thus arises: do the above-mentioned findings also apply to related but undomesticated species. The present thesis aimed to address this question by studying the defense responses of an undomesticated relative of tomato, black nightshade (*Solanum nigrum*), in general, and the role of systemin, in particular.

***S. nigrum* was attacked by a diverse herbivore community**

In two consecutive growing seasons, native and planted *S. nigrum* plants around Jena, Germany, were attacked by 17 generalist and two specialist herbivores belonging to three different feeding guilds (manuscript I). Insects were only classified as being herbivores on *S. nigrum* when adults or their larvae were repeatedly observed feeding on *S. nigrum* in both years. Thus, many species, for example, 21 species of Coleoptera were not included in this listing. The occurrence of a diverse herbivore community gave rise to the hypothesis that *S. nigrum* might respond with a similarly diverse set of defense mechanisms.

***S. nigrum* was established as a model plant system**

Molecular tools were developed and adopted from other solanaceous species to manipulate and quantify *S. nigrum*'s responses to herbivory (manuscript I). An *Agrobacterium*-based transformation system was established as a method to specifically target and silence genes of interest in order to study their roles in plants' subsequent defense responses. The use of microarrays was demonstrated to be a useful tool for identifying genes potentially involved in *S. nigrum*'s defense against herbivory. Furthermore, methods were developed to quantify PIs as a direct, induced resistance trait and volatile organic compounds (VOCs) as an indirect, induced resistance trait.

***S. nigrum* featured an array of different defense strategies**

Upon herbivore damage by the flea beetle *Expitrix pubescens*, induction with methyl jasmonate or treatment with wounding and oral secretions, *S. nigrum* produced PIs; this was considered a direct induced resistance trait (manuscripts I and III). The attack of the Colorado potato beetle (*Leptinotarsa decemlineata*) or Deathhead Hawk-moth (*Acherontia atropos*) elicited the emission of several VOCs, including sesquiterpenes in field-grown *S. nigrum* plants (manuscript I). The production of sesquiterpenes in response to herbivory also became apparent in the transcriptome of induced plants, as genes involved in the production of sesquiterpenes were highly up-regulated (manuscript II). Moreover, the expression of genes encoding proteins involved in the biosynthesis of steroidal alkaloid increased, indicating that *S. nigrum* might use these secondary metabolites to complement its direct induced resistance repertoire (manuscript II).

Systemin did not mediate direct induced resistance in *S. nigrum* (manuscript III)

Comparing wild-type (WT) plants and transgenic plants transformed with an inverted-repeat prosystemin construct (IRSys) to silence the expression of the endogenous *S. nigrum* prosystemin gene in field and glasshouse experiments revealed that amounts of PIs accumulated did not differ between WT and IRSys plants. Moreover, complementing plants with systemin by supplying the polypeptide exogenously through excised stems did not significantly increase PI activity, indicating that systemin and PIs were not correlated in *S. nigrum*. As neither the performance of three different herbivore species on these plants nor the elicited jasmonic acid levels differed between WT and IRSys plants, we concluded that the tomato-homologous polypeptide does not mediate direct defense responses in *S. nigrum*.

Down-regulation of systemin after herbivory was associated with increased root allocation and competitive ability in *S. nigrum* (manuscript IV)

Growth experiments revealed that both induced WT and uninduced IRSys plants had significantly more root mass compared to untreated WT plants. When induced WT plants were additionally treated with synthetic systemin, their root mass did not differ significantly from that of uninduced WTs. When competition for below-ground resources was prevented by a barrier, no significant differences in berry production were found among any of the genotypes or treatments. However, when uninduced plants competed, IRSys plants produced significantly more berries than did WT plants. Berry production of induced and uninduced WT plants did not differ, but when induced WTs were additionally treated with systemin, plants produced fewer berries than did unelicited WT competitors. Thus, we propose that the rapid down-regulation of systemin after herbivory was associated with increased allocation of resources to roots; this in turn allowed plants to compete more effectively with conspecifics and hence may allow plants to compensate for tissue loss during herbivory.

6 Zusammenfassung

Um den durch phytophage Insekten ausgelösten, fitnessgefährdenden Stress zu minimieren, haben Pflanzen eine Vielzahl an Verteidigungen entwickelt. Im Allgemeinen werden diese pflanzlichen Verteidigungen gegen Herbivorie in zwei Gruppen klassifiziert: Toleranz und Resistenz. Toleranzmechanismen reduzieren die nachteiligen Effekte des Fraßschadens auf die pflanzliche Fitness ohne den Herbivoren direkt zu beeinflussen. Im Gegensatz dazu reduzieren Resistenzmechanismen direkt oder indirekt den Schaden, indem sie mögliche Herbivoren abwehren oder deren Entwicklung beeinträchtigen.

Während die molekulare Basis pflanzlicher Toleranzmechanismen weitgehend unerforscht ist, wurden die in Resistenz involvierten Signale und Signalkaskaden ausgiebig untersucht. Da Pflanzen dafür bekannt sind, Resistenzmechanismen nicht nur in den lokalen, beschädigten Blättern, sondern auch in entfernt gelegenen, unbeschädigten Blättern zu aktivieren, wurde den Signalen, die diese sogenannten systemischen Antworten vermitteln, besondere Beachtung zuteil. Unter den Signalen, die in Frage kommen die Information vom Ort des Schadens in den Rest der Pflanze zu transportieren, befindet sich das aus 18 Aminosäuren bestehende Polypeptid Systemin. Systemin wurde lange Zeit für das systemische Signal in Tomate (*Solanum lycopersicum*) gehalten. Neueste Pfropfungsexperimente zeigten jedoch, dass sowohl die Anwesenheit von Systemin als auch die Biosynthese von Jasmonsäure im lokalen, geschädigten Blatt benötigt wird, um ein systemisches Signal zu erzeugen und folglich Proteinaseinhibitoren (PIs) in entfernt liegenden, unverwundeten Blättern zu induzieren. Darüber hinaus schienen weder Systemin noch die Biosynthese von Jasmonsäure in den unbeschädigten Blättern nötig zu sein, um die Produktion von PIs zu erzielen. Mit diesen Ergebnissen musste die Rolle von Systemin in der Wundantwort von Tomate neu überdacht werden. Entsprechend des überarbeiteten Modells spielt Systemin eine zentrale Rolle in der Wundantwort von Tomate, indem es an oder nahe der verwundeten Stelle agiert und das von Jasmonsäure abgeleitete, mobile Signal verstärkt.

Pflanzliche Resistanzantworten auf Herbivorie und deren zugrunde liegenden Signalkaskaden wurden fast ausschließlich in Kulturpflanzen, wie zum Beispiel Tomaten untersucht. Eine wichtige, sich daraus ergebende Frage ist, ob sich die gewonnenen Erkenntnisse auch auf verwandte, nicht domestizierte Pflanzenarten übertragen lassen. Die

vorliegende Arbeit greift diese Frage auf, indem sie die Verteidigungsmechanismen einer nicht domestizierten Verwandten von Tomate, dem Schwarzen Nachtschatten (*Solanum nigrum*) im Allgemeinen und die Rolle von Systemin im Speziellen untersucht.

***S. nigrum* wurde von einer diversen Herbivorengemeinschaft befallen**

In zwei aufeinanderfolgenden Wachstumsperioden wurden *S. nigrum* Pflanzen, die um Jena (Deutschland) entweder wild wuchsen oder ausgepflanzt wurden, von 17 Generalisten und zwei Spezialisten befallen, die drei verschiedenen Fraßgilden angehörten (Manuskript I). Nachdem Insekten nur als Herbivoren klassifiziert wurden, wenn die adulten Tiere oder ihre Larven wiederholt und in beiden Jahren fressend auf *S. nigrum* beobachtet wurden, wurden einige Arten, darunter 21 Coleopteren, nicht in die Auflistung aufgenommen. Das Auftreten einer diversen Herbivorengemeinschaft warf die Hypothese auf, dass *S. nigrum* durch eine gleichermaßen diverse Kombination an Verteidigungsmechanismen reagiert.

***S. nigrum* wurde als pflanzliches Modellsystem etabliert**

Molekulare Methoden wurden entwickelt oder von anderen Solanaceenarten übernommen, um die Antworten von *S. nigrum* auf Herbivorie zu manipulieren und zu quantifizieren (Manuskript I). Insbesondere ein auf *Agrobacterium* basierendes Transformationssystem wurde entwickelt, um relevante Gene ausschalten und deren Rolle in der pflanzlichen Abwehr untersuchen zu können. Genchips wurden dazu verwendet weitere Gene mit einer potentiellen Rolle in pflanzlicher Abwehr zu identifizieren. Darüber hinaus wurden Methoden zur Quantifizierung von PIs als direkte, induzierte Abwehr und von Duftstoffen als indirekte, induzierte Abwehr entwickelt.

***S. nigrum* weist eine Vielzahl von Verteidigungsstrategien auf**

Nachdem *S. nigrum* Pflanzen entweder von Flohkäfern (*Expitrix pubescens*) befallen oder mit Methyljasmonat beziehungsweise durch Verwundung induziert wurde, produzierten sie PIs als eine direkte, induzierte Form von Resistenz (Manuskript I und III). Der Befall von Kartoffelkäfern (*Leptinotarsa decemlineata*) oder Larven des Totenkopfschwärmers (*Acherontia atropos*) löste in im Freiland wachsenden *S. nigrum* Pflanzen die Abgabe verschiedener Duftstoffe, darunter Sesquiterpene, aus (Manuskript I). Die Produktion von Sesquiterpenen spiegelte sich auch im induzierten Transkriptom der Pflanze wieder

(Manuskript II). Neben der Hochregulierung von Genen, die in der Sesquiterpenbiosynthese von Bedeutung sind, lag der zweite transkriptionelle Schwerpunkt auf der Biosynthese von Steroidalkaloiden. Dies deutete darauf hin, dass *S. nigrum* diese Sekundärmetabolite zur Eränzung seines direkten, induzierten Verteidigungsrepertoires nutzt (Manuskript II).

Systemin vermittelte nicht die direkte, induzierte Abwehr in *S. nigrum* (Manuskript III)

Vergleichende Freiland- und Gewächshausexperimente mit Wildtyppflanzen (WT) und transgenen Pflanzen, die mit einem 'inverted repeat' Prosystemin Konstrukt transformiert wurden, um die Expression des endogenen *S. nigrum* Prosystemingens auszuschalten, ergaben, dass sich die Akkumulierung von PIs als direkte Verteidigung nicht zwischen WT- und IRSys-Pflanzen unterschied. Darüber hinaus, steigerte die exogene Applikation des Polypeptides durch die abgeschnittenen Stengel nicht die PI-Aktivität. Dies deutete darauf hin, dass Systemin und PIs in *S. nigrum* nicht korrelierten. Nachdem weder die Entwicklung dreier verschiedener Herbivoren auf, noch Mengen der induzierten Jasmonsäure in WT- und IRSys-Pflanzen unterschiedlich war, kamen wir zu dem Schluß, dass das der Tomate homologe Polypeptid nicht für die Vermittlung der direkten, induzierten Abwehr in *S. nigrum* verantwortlich ist.

Herabregulierung von Systemin nach Herbivorie ging mit einem gesteigerten Wurzelwachstum sowie einer gesteigerter Konkurrenzfähigkeit einher (Manuskript IV)

Wachstumsexperimente ergaben, dass sowohl induzierte WT-Pflanzen als auch uninduzierte IRSys-Pflanzen signifikant mehr Wurzelmasse aufwiesen als unbehandelte Pflanzen des WT. Wenn induzierte WT-Pflanzen zusätzlich mit Systemin behandelt wurden, unterschied sich die Wurzelmasse nicht von der uninduzierter WT-Pflanzen. Wenn die Konkurrenz um unterirdische Ressourcen durch eine Barriere unterbunden wurde, wurden keine signifikanten Unterschiede bezüglich der Beerenproduktion zwischen Genotypen oder Behandlungsgruppen beobachtet. In einer Konkurrenzsituation produzierten IRSys-Pflanzen signifikant mehr Beeren als WT-Pflanzen. Die Beerenproduktion induzierter und nicht induzierter WT-Pflanzen unterschied sich jedoch nicht. Wenn die induzierten WT-Pflanzen allerdings mit Systemin behandelt wurden, produzierten sie weniger Beeren als uninduzierte WT-Konkurrenten. Deshalb schlugen wir vor, dass die schnelle Herabregulierung von Systemin nach Herbivorie mit einer gesteigerten Wurzelallokation und gesteigerter

Konkurrenzfähigkeit einhergeht, die es der Pflanze erlaubt, effektiver mit Artgenossen zu konkurrieren und es ihr dadurch ermöglicht Gewebeerluste während des Herbivorenbefalls zu kompensieren.

7 References

- Applied Biosystems (1997), *ABI PRISM 7700 Sequence Detection System User Bulletin no.2*.
- Alborn, T., Turlings, T.C.J., Jones, T.H., Stenhagen, G., Loughrin, J.H. & Tumlinson, J.H. (1997) An elicitor of plant volatiles from beet armyworm oral secretion. *Science* **276**, 945-949.
- Baldwin, I.T. (1998) Jasmonate-induced responses are costly but benefit plants under attack in native populations. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 8113-8118.
- Baldwin, I.T. (1999) Inducible nicotine production in native *Nicotiana* as an example of adaptive phenotypic plasticity. *Journal of Chemical Ecology* **25**, 3-30.
- Baldwin, I.T., Kessler, A. & Halitschke, R. (2002) Volatile signaling in plant-plant-herbivore interactions: what is real? *Current Opinion in Plant Biology* **5**, 351-354.
- Barr, P.J. (1991) Mammalian Subtilisins - the Long-Sought Dibasic Processing Endoproteases. *Cell* **66**, 1-3.
- Bazzaz, F.A., Carlson, R.W. & Harper, J.L. (1979) Contribution to reproductive effort by photosynthesis of flowers and fruits. *Nature* **279**, 554-555.
- Bazzaz, F.A., Chiariello, N.R., Coley, P.D. & Pitelka, L.F. (1987) Allocating resources to reproduction and defense. *Bioscience* **37**, 58-67.
- Beaujean, A., Sangwan, R.S., Lecardonnel, A. & Sangwan-Norreel, B.S. (1998) *Agrobacterium*-mediated transformation of three economically important potato cultivars using sliced internodal explants: an efficient protocol of transformation. *Journal of Experimental Botany* **49**, 1589-1595.
- Begon, M., Harper, J.L. & Townsend, C.R. (1996) *Ecology: Individuals, Populations and Communities*. Blackwell Science, Oxford.
- Berenbaum, M.R. (1985) Brementown revisited: interactions among allelochemicals in plants. *Recent Advances in Phytochemistry* **19**, 139-169.
- Bergenstrahle, A., Borga, P. & Jonsson, M.V. (1996) Sterol composition and synthesis in potato tuber discs in relation to glycoalkaloid synthesis. *Phytochemistry* **41**, 155-161.
- Berger, B. & Baldwin, I.T. (2007) The hydroxyproline-rich glycopeptide systemin precursor *NapreproHypSys* does not play a central role in *Nicotiana attenuata*'s anti-herbivore defense response. *Plant, Cell and Environment*

- Berger, S., Mitchell-Olds, T. & Stotz, H.U. (2002) Local and differential control of vegetative storage protein expression in response to herbivore damage in *Arabidopsis thaliana*. *Physiologia Plantarum* **114**, 85-91.
- Blee, E. (2002) Impact of phyto-oxylinins in plant defense. *Trends in Plant Science* **7**, 315-321.
- Boller, T. (2005) Peptide signalling in plant development and self/non-self perception. *Current Opinion in Cell Biology* **17**, 116-122.
- Bradford, M.M. (1976) Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.
- Bronstein, J.L. (1998) The contribution of ant plant protection studies to our understanding of mutualism. *Biotropica* **30**, 150-161.
- Brown, T. (1995) Southern blotting. *Short Protocols in Molecular Biology*, pp. 2128-2132, Wiley, New York.
- Bubner, B., Gase, K. & Baldwin, I.T. (2004) Two-fold differences are the detection limit for determining transgene copy numbers in plants by real-time PCR. *Bmc Biotechnology* **4**
- 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.
- Chen, H., Jones, A.D. & Howe, G.A. (2006) Constitutive activation of the jasmonate signaling pathway enhances the production of secondary metabolites in tomato. *Febs Letters* **580**, 2540-2546.
- Chessin, M. & Zipf, A.E. (1990) Alarm systems in higher plants. *Botanical Review* **56**, 193-235.
- Choi, D., Bostock, R.M., Avdiushko, S. & Hildebrand, D.F. (1994) Lipid-derived signals that discriminate wound-responsive and pathogen-responsive isoprenoid pathways in plants - methyl jasmonate and the fungal elicitor arachidonic-acid induce different 3-hydroxy-3-methylglutaryl-coenzyme-a reductase genes and antimicrobial isoprenoids in *Solanum tuberosum* L. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 2329-2333.

- Clauss, M.J., Dietel, S., Schubert, G. & Mitchell-Olds, T. (2006) Glucosinolate and trichome defenses in a natural *Arabidopsis lyrata* population. *Journal of Chemical Ecology* **32**, 2351-2373.
- Constabel, C.P., Yip, L., Patton, J.J. & Christopher, M.E. (2000) Polyphenol oxidase from hybrid poplar. Cloning and expression in response to wounding and herbivory. *Plant Physiology* **124**, 285-295.
- Constabel, C.P., Yip, L. & Ryan, C.A. (1998) Prosystemin from potato, black nightshade, and bell pepper: primary structure and biological activity of predicted systemin polypeptides. *Plant Molecular Biology* **36**, 55-62.
- Cuautle, M. & Rico-Gray, V. (2003) The effect of wasps and ants on the reproductive success of the extrafloral nectaried plant *Turnera ulmifolia* (Turneraceae). *Functional Ecology* **17**, 417-423.
- Davey, M.R., Mulligan, B.J., Gartland, K.M.A., Peel, E., Sargent, A.W. & Morgan, A.J. (1987) Transformation of *Solanum* and *Nicotiana* species using a Ri-plasmid vector. *Journal of Experimental Botany* **38**, 1507-1516.
- de Leon, I.P., Sanz, A., Hamberg, M. & Castresana, C. (2002) Involvement of the *Arabidopsis* alpha-DOX1 fatty acid dioxygenase in protection against oxidative stress and cell death. *Plant Journal* **29**, 61-72.
- Defelice, M.S. (2003) The black nightshades, *Solanum nigrum* L. et al. - poison, poultice, and pie. *Weed Technology* **17**, 421-427.
- Devoto, A., Nieto-Rostro, M., Xie, D.X., Ellis, C., Harmston, R., Patrick, E., Davis, J., Sherratt, L., Coleman, M. & Turner, J.G. (2002) COI1 links jasmonate signalling and fertility to the SCF ubiquitin-ligase complex in *Arabidopsis*. *Plant Journal* **32**, 457-466.
- Dicke, M., van Poecke, R.M.P. & de Boer, J.G. (2003) Inducible indirect defence of plants: from mechanisms to ecological functions. *Basic and Applied Ecology* **4**, 27-42.
- Doherty, H.M., Selvendran, R.R. & Bowles, D.J. (1988) The wound response of tomato plants can be inhibited by aspirin and related hydroxybenzoic acids. *Physiological and Molecular Plant Pathology* **33**, 377-384.
- Dopke, W., Duday, S. & Matos, N. (1987) The isolation and identification of alkaloids and steroids from *Solanum nigrum* is described. *Zeitschrift Fur Chemie* **27**, 64-64.
- Edmonds, J.M. (1979) Biosystematics of *Solanum* L. section *Solanum* (*Maurella*). *The Biology and Taxonomy of the Solanaceae* (eds. J.G. Hawkes, R.N. Lester & A.D. Skelding), pp. 529-548. Academic Press, London.

- Edmonds, J.M. & Chweya, J.A. (1997) Black nightshades *Solanum nigrum* L. and related species, Italy.
- Ehrlich, P.R. & Raven, P.H. (1964) Butterflies and plants - a study in coevolution. *Evolution* **18**, 586-608.
- Eltayeb, E.A., AlAnsari, A.S. & Roddick, J.G. (1997) Changes in the steroidal alkaloid solasodine during development of *Solanum nigrum* and *Solanum incanum*. *Phytochemistry* **46**, 489-494.
- Eulgem, T., Rushton, P.J., Robatzek, S. & Somssich, I.E. (2000) The WRKY superfamily of plant transcription factors. *Trends in Plant Science* **5**, 199-206.
- Farmer, E.E., Almeras, E. & Krishnamurthy, V. (2003) Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. *Current Opinion in Plant Biology* **6**, 372-378.
- Farmer, E.E. & Ryan, C.A. (1990) Interplant communication - airborne methyl jasmonate induces synthesis of proteinase-inhibitors in plant-leaves. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 7713-7716.
- Farmer, E.E. & Ryan, C.A. (1992) Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase-inhibitors. *Plant Cell* **4**, 129-134.
- Felton, G.W., Korth, K.L., Bi, J.L., Wesley, S.V., Huhman, D.V., Mathews, M.C., Murphy, J.B., Lamb, C. & Dixon, R.A. (1999) Inverse relationship between systemic resistance of plants to microorganisms and to insect herbivory. *Current Biology* **9**, 317-320.
- Fineblum, W.L. & Rausher, M.D. (1995) Tradeoff between resistance and tolerance to herbivore damage in a morning glory. *Nature* **377**, 517-520.
- Fraenkel, G.S. (1959) Raison d'être of secondary plant substances. *Science* **129**, 1466-1470.
- Frohne, D., Jensen, U. (1992) Systematik des Pflanzenreichs. Urban & Fischer, Munich
- Gang, D.R., Beuerle, T., Ullmann, P., Werck-Reichhart, D. & Pichersky, E. (2002) Differential production of meta hydroxylated phenylpropanoids in sweet basil peltate glandular trichomes and leaves is controlled by the activities of specific acyltransferases and hydroxylases. *Plant Physiology* **130**, 1536-1544.
- Genoud, T., Santa Cruz, M.B.T. & Metraux, J.P. (2001) Numeric simulation of plant signaling networks. *Plant Physiology* **126**, 1430-1437.
- Gershenson, J. (2002) Secondary metabolism and plant defense. In: L Taiz, E. Zeiger, eds, *Plant Physiology*, Ed. 3 Sinauer Associates, Sunderland, MA, 283-308

- Gertz, A.K. & Bach, C.E. (1995) Effects of light and nutrients on tomato plant compensation for herbivory by *Manduca sexta* (Lepidoptera, Sphingidae). *Great Lakes Entomologist* **27**, 217-222.
- Gilchrist, E.J. & Haughn, G.W. (2005) TILLING without a plough: a new method with applications for reverse genetics. *Current Opinion in Plant Biology* **8**, 211-215.
- Girke, T., Todd, J., Ruuska, S., White, J., Benning, C. & Ohlrogge, J. (2000) Microarray analysis of developing *Arabidopsis* seeds. *Plant Physiology* **124**, 1570-1581.
- Givnish, T.J. (1986) Economics of biotic interactions. *On the economy of plant form and function* (ed T.J. Givnish), pp. 667-679. Cambridge University Press, Cambridge.
- Gouinguene, S.P. & Turlings, T.C.J. (2002) The effects of abiotic factors on induced volatile emissions in corn plants. *Plant Physiology* **129**, 1296-1307.
- Halitschke, R. & Baldwin, I.T. (2003) Antisense LOX expression increases herbivore performance by decreasing defense responses and inhibiting growth-related transcriptional reorganization in *Nicotiana attenuata*. *Plant Journal* **36**, 794-807.
- Halitschke, R. & Baldwin, I.T. (2004) Jasmonates and related compounds in plant-insect interactions. *Journal of Plant Growth Regulation* **23**, 238-245.
- Halitschke, R., Gase, K., Hui, D., Schmidt, D.D. & Baldwin, I.T. (2003) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. VI. Microarray analysis reveals that most herbivore-specific transcriptional changes are mediated by fatty acid-amino acid conjugates. *Plant Physiol.* **131**, 1894-1902.
- Halitschke, R., Kessler, A., Kahl, J., Lorenz, A. & Baldwin, I.T. (2000) Ecophysiological comparison of direct and indirect defenses in *Nicotiana attenuata*. *Oecologia* **124**, 408-417.
- Halitschke, R., Schittko, U., Pohnert, G., Boland, W. & Baldwin, I.T. (2001) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. III. Fatty acid-amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specific plant responses. *Plant Physiology* **125**, 711-717.
- Hamberg, M., Sanz, A. & Castresana, C. (1999) Alpha-oxidation of fatty acids in higher plants - Identification of a pathogen-inducible oxygenase (PIOX) as an alpha-dioxygenase and biosynthesis of 2-hydroperoxylinolenic acid. *Journal of Biological Chemistry* **274**, 24503-24513.

- Hare, P.D., Seo, H.S., Yang, J.Y. & Chua, N.H. (2003) Modulation of sensitivity and selectivity in plant signaling by proteasomal destabilization. *Current Opinion in Plant Biology* **6**, 453-462
- Hause, B., Hause, G., Kutter, C., Miersch, O. & Wasternack, C. (2003) Enzymes of jasmonate biosynthesis occur in tomato sieve elements. *Plant and Cell Physiology* **44**, 643-648.
- Hause, B., Stenzel, I., Miersch, O., Maucher, H., Kramell, R., Ziegler, J. & Wasternack, C. (2000) Tissue-specific oxylipin signature of tomato flowers: allene oxide cyclase is highly expressed in distinct flower organs and vascular bundles. *Plant Journal* **24**, 113-126.
- Heath, R.L., McDonald, G., Christeller, J.T., Lee, M., Bateman, K., West, J., vanHeeswijck, R. & Anderson, M.A. (1997) Proteinase inhibitors from *Nicotiana glauca* enhance plant resistance to insect pests. *Journal of Insect Physiology* **43**, 833-842.
- Heidel, A.J. & Baldwin, I.T. (2004b) Microarray analysis of salicylic acid- and jasmonic acid-signalling in responses of *Nicotiana attenuata* to attack by insects from multiple feeding guilds. *Plant Cell And Environment* **27**, 1362-1373.
- Held, M., Gase, K. & Baldwin, I.T. (2004) Microarrays in ecological research: A case study of a cDNA microarray for plant-herbivore interactions. *BMC Ecology* **4**
- Hermesmeier, D., Schittko, U. & Baldwin, I.T. (2001) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. I. Large-scale changes in the accumulation of growth- and defense-related plant mRNAs. *Plant Physiology* **125**, 683-700.
- Holton, N., Cano-Delgado, A., Harrison, K., Montoya, T., Chory, J. & Bishop, G.J. (2007) Tomato *BRASSINOSTEROID INSENSITIVE1* is required for systemin-induced root elongation in *Solanum pimpinellifolium* but is not essential for wound signaling. *Plant Cell*
- Howe, G.A. (2004) Jasmonates as signals in the wound response. *Journal of Plant Growth Regulation* **23**, 223-237.
- Huffaker, A., Pearce, G. & Ryan, C.A. (2006) An endogenous peptide signal in *Arabidopsis* activates components of the innate immune response. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 10098-10103.
- Hugentobler, U. & Renwick, J.A.A. (1995) Effects of plant nutrition on the balance of insect relevant cardenolides and glucosinolates in *Erysimum cheiranthoides*. *Oecologia* **102**, 95-101.

- Hui, D., Iqbal, J., Lehmann, K., Gase, K., Saluz, H.P. & Baldwin, I.T. (2003) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*: V. Microarray analysis and further characterization of large-scale changes in herbivore-induced mRNAs. *Plant Physiology* **131**, 1877-1893.
- Irwin, D.L. & Aarssen, L.W. (1996) Effects of nutrient level on cost and benefit of apical dominance in *Epilobium ciliatum*. *American Midland Naturalist* **136**, 14-28.
- Izaguirre, M.M., Scopel, A.L., Baldwin, I.T. & Ballare, C.L. (2003) Convergent responses to stress. Solar Ultraviolet-B radiation and *Manduca sexta* herbivory elicit overlapping transcriptional responses in field-grown plants of *Nicotiana longiflora*. *Plant Physiology* **132**, 1755-1767.
- Johnson, R., Narvaez, J., An, G.H. & Ryan, C. (1989) Expression of proteinase inhibitor-I and inhibitor-II in transgenic tobacco plants - effects on natural defense against *Manduca sexta* larvae. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 9871-9875.
- Jongsma, M.A., Bakker, P.L. & Stiekema, W.J. (1993) Quantitative determination of serine proteinase-inhibitor activity using a radial diffusion assay. *Analytical Biochemistry* **212**, 79-84.
- Jongsma, M.A., Bakker, P.L., Visser, B. & Stiekema, W.J. (1994) Trypsin-inhibitor activity in mature tobacco and tomato plants is mainly induced locally in response to insect attack, wounding and virus-infection. *Planta* **195**, 29-35.
- Jongsma, M.A. & Bolter, C. (1997) The adaptation of insects to plant protease inhibitors. *Journal of Insect Physiology* **43**, 885-895.
- Jones, C.G., Firn, R.D. (1991) On the evolution of plant secondary chemical diversity. *Philosophical Transactions of the Royal Society of London B Biological Sciences* **333**, 273-280
- Kahl, J., Siemens, D.H., Aerts, R.J., Gabler, R., Kuhnemann, F., Preston, C.A. & Baldwin, I.T. (2000) Herbivore-induced ethylene suppresses a direct defense but not a putative indirect defense against an adapted herbivore. *Planta* **210**, 336-342.
- Kane, M.D., Jatkoje, T.A., Stumpf, C.R., Lu, J., Thomas, J.D. & Madore, S.J. (2000) Assessment of the sensitivity and specificity of oligonucleotide (50mer) microarrays. *Nucleic Acids Research* **28**, 4552-4557.

- Kang, J.H., Wang, L., Giri, A. & Baldwin, I.T. (2006) Silencing threonine deaminase and *JAR4* in *Nicotiana attenuata* impairs jasmonic acid-isoleucine-mediated defenses against *Manduca sexta*. *Plant Cell* **18**, 3303-3320.
- Karban, R. & Baldwin, I.T. (1997) *Induced responses to herbivory*. The University of Chicago Press, Chicago.
- Kessler, A. & Baldwin, I.T. (2001) Defensive function of herbivore-induced plant volatile emissions in nature. *Science* **291**, 2141-2144.
- Kessler, A. & Baldwin, I.T. (2002) Plant responses to insect herbivory: The emerging molecular analysis. *Annual Review of Plant Biology* **53**, 299-328.
- Kessler, A., Halitschke, R. & Baldwin, I.T. (2004) Silencing the jasmonate cascade: induced plant defenses and insect populations. *Science* **305**, 665-668.
- Kodama, H., Nishiuchi, T., Seo, S., Ohashi, Y. & Iba, K. (2000) Possible involvement of protein phosphorylation in the wound-responsive expression of *Arabidopsis* plastid omega-3 fatty acid desaturase gene. *Plant Science* **155**, 153-160.
- Koiwa, H., Bressan, R.A. & Hasegawa, P.M. (1997) Regulation of protease inhibitors and plant defense. *Trends in Plant Science* **2**, 379-384.
- Korth, K.L. (2003) Profiling the response of plants to herbivorous insects. *Genome Biology* **4**, 221.
- Kost, C. & Heil, M. (2005) Increased availability of extrafloral nectar reduces herbivory in Lima bean plants (*Phaseolus lunatus*, Fabaceae). *Basic and Applied Ecology* **6**, 237-248.
- Krügel, T., Lim, M., Gase, K., Halitschke, R. & Baldwin, I.T. (2002) *Agrobacterium*-mediated transformation of *Nicotiana attenuata*, a model ecological expression system. *Chemoecology* **12**, 177-183.
- Kutchan, T.M. (2001) Ecological arsenal and developmental dispatcher. The paradigm of secondary metabolism. *Plant Physiology* **125**, 58-60.
- Lantin, S., O'Brien, M. & Matton, D.P. (1999) Pollination, wounding and jasmonate treatments induce the expression of a developmentally regulated pistil dioxygenase at a distance, in the ovary, in the wild potato *Solanum chacoense* Bitt. *Plant Molecular Biology* **41**, 371-386.
- Lawrence, R.J. & Pikaard, C.S. (2003) Transgene-induced RNA interference: a strategy for overcoming gene redundancy in polyploids to generate loss-of-function mutations. *Plant Journal* **36**, 114-121.

- Leimu, R. & Koricheva, J. (2006) A meta-analysis of genetic correlations between plant resistances to multiple enemies. *American Naturalist* **168**, E15-E37.
- Leon, J., Rojo, E. & Sanchez-Serrano, J.J. (2001) Wound signalling in plants. *Journal of Experimental Botany* **52**, 1-9.
- Lichtenthaler, H.K. (1999) The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **50**, 47-65.
- Livak, K.J. & Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* **25**, 402-408.
- Maffei, M., Bossi, S., Spiteller, D., Mithofer, A. & Boland, W. (2004) Effects of feeding *Spodoptera littoralis* on lima bean leaves. I. Membrane potentials, intracellular calcium variations, oral secretions, and regurgitate components. *Plant Physiology* **134**, 1752-1762.
- Maischak, H., Grigoriev, P.A., Vogel, H., Boland, W. & Mithofer, A. (2007) Oral secretions from herbivorous lepidopteran larvae exhibit ion channel-forming activities. *Febs Letters* **581**, 898-904.
- Marquis, R.J. (1988) Phenological variation in the neotropical understory shrub *Piper arieianum* - causes and consequences. *Ecology* **69**, 1552-1565.
- Maschinski, J. & Whitham, T.G. (1989) The continuum of plant-responses to herbivory - the influence of plant-association, nutrient availability, and timing. *American Naturalist* **134**, 1-19.
- Matt, P., Krapp, A., Haake, V., Mock, H.P. & Stitt, M. (2002) Decreased Rubisco activity leads to dramatic changes of nitrate metabolism, amino acid metabolism and the levels of phenylpropanoids and nicotine in tobacco antisense RBCS transformants. *Plant Journal* **30**, 663-677.
- Mattiacci, L., Dicke, M. & Posthumus, M.A. (1994) Induction of parasitoid attracting synomone in brussels sprouts plants by feeding of *Pieris brassicae* larvae - role of mechanical damage and herbivore elicitor. *Journal of Chemical Ecology* **20**, 2229-2247.
- Mattiacci, L., Dicke, M. & Posthumus, M.A. (1995) Beta-glucosidase - an elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 2036-2040.
- Mauricio, R., Bowers, M.D. & Bazzaz, F.A. (1993) Pattern of leaf damage affects fitness of the annual plant *Raphanus sativus* (Brassicaceae). *Ecology* **74**, 2066-2071.

- Mauricio, R., Rausher, M.D. & Burdick, D.S. (1997) Variation in the defense strategies of plants: are resistance and tolerance mutually exclusive? *Ecology* **78**, 1301-1311.
- McConnaughay, K.D.M. & Bazzaz, F.A. (1992) The occupation and fragmentation of space - consequences of neighboring roots. *Functional Ecology* **6**, 704-710.
- McGurl, B., Orozco-Cardenas, M., Pearce, G. & Ryan, C.A. (1994) Overexpression of the prosystemin gene in transgenic tomato plants generates a systemic signal that constitutively induces proteinase inhibitor synthesis. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 9799-9802.
- McGurl, B., Pearce, G., Orozco-Cardenas, M. & Ryan, C.A. (1992) Structure, expression, and antisense inhibition of the systemin precursor gene. *Science* **255**, 1570-1573.
- McKnight, T.D., Alexander, D.C., Babcock, M.S. & Simpson, R.B. (1986) Nucleotide-sequence and molecular evolution of 2 tomato genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase. *Gene* **48**, 23-32.
- McNaughton, S.J. (1979) Grazing as an optimization process - grass ungulate relationships in the Serengeti. *American Naturalist* **113**, 691-703.
- Mitchell-Olds, T. (2001) *Arabidopsis thaliana* and its wild relatives: a model system for ecology and evolution. *Trends in Ecology & Evolution* **16**, 693-700.
- Mithöfer, A., Wanner, G. & Boland, W. (2005) Effects of feeding *Spodoptera littoralis* on lima bean leaves. II. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission. *Plant Physiology* **137**, 1160-1168.
- Mittova, V., Tal, M., Volokita, M. & Guy, M. (2002) Salt stress induces up-regulation of an efficient chloroplast antioxidant system in the salt-tolerant wild tomato species *Lycopersicon pennellii* but not in the cultivated species. *Physiologia Plantarum* **115**, 393-400.
- Molina-Montenegro, M.A., Avila, P., Hurtado, R., Valdivia, A.I. & Gianoli, E. (2006) Leaf trichome density may explain herbivory patterns of *Actinote* sp (Lepidoptera: Acraeidae) on *Liabum mandonii* (Asteraceae) in a montane humid forest (Nor Yungas, Bolivia). *Acta Oecologica-International Journal of Ecology* **30**, 147-150.
- Mutikainen, P. & Walls, M. (1995) Growth, reproduction and defense in nettles - responses to herbivory modified by competition and fertilization. *Oecologia* **104**, 487-495.
- Narvaez-Vasquez, J. & Ryan, C.A. (2004) The cellular localization of prosystemin: a functional role for phloem parenchyma in systemic wound signaling. *Planta* **218**, 360-369.

- Olf, H., Vanandel, J. & Bakker, J.P. (1990) Biomass and shoot root allocation of 5 species from a grassland succession series at different combinations of light and nutrient supply. *Functional Ecology* **4**, 193-200.
- Orozco-Cardenas, M., McGurl, B. & Ryan, C.A. (1993) Expression of an antisense prosystemin gene in tomato plants reduces resistance toward *Manduca sexta* larvae. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 8273-8276.
- Orozco-Cardenas, M.L., Narvaez-Vasquez, J. & Ryan, C.A. (2001) Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. *Plant Cell* **13**, 179-191.
- Paige, K.N. & Whitham, T.G. (1987) Overcompensation in response to mammalian herbivory - the advantage of being eaten. *American Naturalist* **129**, 407-416.
- Paré, P.W. & Tumlinson, J.H. (1997) De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. *Plant Physiology* **114**, 1161-1167.
- Pearce, G., Johnson, S. & Ryan, C.A. (1993) Structure-activity of deleted and substituted systemin, an 18-amino acid polypeptide inducer of plant defensive genes. *Journal of Biological Chemistry* **268**, 212-216.
- Pearce, G., Moura, D.S., Stratmann, J. & Ryan, C.A. (2001) Production of multiple plant hormones from a single polyprotein precursor. *Nature* **411**, 817-820.
- Pearce, G. & Ryan, C.A. (2003) Systemic signaling in tomato plants for defense against herbivores - isolation and characterization of three novel defense-signaling glycopeptide hormones coded in a single precursor gene. *Journal of Biological Chemistry* **278**, 30044-30050.
- Pearce, G., Siems, W.F., Bhattacharya, R., Chen, Y.-C. & Ryan, C.A. (2007) Three hydroxyproline-rich glycopeptides derived from a single petunia polyprotein precursor activate *defensin I*, a pathogen defense response gene. *The Journal of Biological Chemistry* **282**, 17777-17784.
- Pearce, G., Strydom, D., Johnson, S. & Ryan, C.A. (1991) A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. *Science* **253**, 895-898.
- Pena-Cortez, H., Sanchez-Serrano, J.J., Mertens, R., Willmitzer, L. & Prat, S. (1989) Abscisic-acid is involved in the wound-induced expression of the proteinase inhibitor-II gene in potato and tomato. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 9851-9855.

- Portis, A.R. (1995) The regulation of rubisco by rubisco activase. *Journal of Experimental Botany* **46**, 1285-1291.
- Quick, W.P., Schurr, U., Fichtner, K., Schulze, E.D., Rodermel, S.R., Bogorad, L. & Stitt, M. (1991) The impact of decreased Rubisco on photosynthesis, growth, allocation and storage in tobacco plants which have been transformed with antisense Rbcs. *Plant Journal* **1**, 51-58.
- Rahbe, Y., Deraison, C., Bonade-Bottino, M., Girard, C., Nardon, C. & Jouanin, L. (2003) Effects of the cysteine protease inhibitor oryzacystatin (OC-I) on different aphids and reduced performance of *Myzus persicae* on OC-I expressing transgenic oilseed rape. *Plant Science* **164**, 441-450.
- Reichhardt, M. & Rogers, S. (1994) Preparation of plant DNA using CTAB. *Current Protocols in Molecular Biology* (eds. F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidmann, J.A. Smith & K. Struhl), pp. 2.3.3-2.3.7. John Wiley & Sons, New York.
- Reymond, P. & Farmer, E.E. (1998) Jasmonate and salicylate as global signals for defense gene expression. *Current Opinion in Plant Biology* **1**, 404-411.
- Ridout, C.L., Price, K.R., Coxon, D.T. & Fenwick, G.R. (1989) Glycoalkaloids from *Solanum nigrum* L, Alpha-Solamargine and Alpha-Solasonine. *Pharmazie* **44**, 732-733.
- Robinson, S.P. & Portis, A.R. (1989) Adenosine-triphosphate hydrolysis by purified rubisco activase. *Archives of Biochemistry and Biophysics* **268**, 93-99.
- Roda, A., Halitschke, R., Steppuhn, A. & Baldwin, I.T. (2004) Individual variability in herbivore-specific elicitors from the plant's perspective. *Molecular Ecology* **13**, 2421-2433.
- Roda, A.L., Oldham, N.J., Svatos, A. & Baldwin, I.T. (2003) Allometric analysis of the induced flavonols on the leaf surface of wild tobacco (*Nicotiana attenuata*). *Phytochemistry* **62**, 527-536.
- Rogers, S. & Bendich, A. (1994) *Extraction of total cellular DNA from plants, algae and fungi*. 2nd edn. Kluwer Academic Publishers, Dordrecht/Boston/London.
- Rosenheim, J.A., Wilhoit, L.R., Goodell, P.B., GraftonCardwell, E.E. & Leigh, T.F. (1997) Plant compensation, natural biological control, and herbivory by *Aphis gossypii* on pre-reproductive cotton: the anatomy of a non-pest. *Entomologia Experimentalis Et Applicata* **85**, 45-63.

- Rosenthal, J.P. & Kotanen, P.M. (1994) Terrestrial plant tolerance to herbivory. *Trends in Ecology & Evolution* **9**, 145-148.
- Ryan, C.A. (1987) Oligosaccharide signaling in plants. *Annual Review of Cell Biology* **3**, 295-317.
- Ryan, C.A. (1990) Protease inhibitors in plants - genes for improving defenses against insects and pathogens. *Annual Review of Phytopathology* **28**, 425-449.
- Ryan, C.A. (2000) The systemin signaling pathway: differential activation of plant defensive genes. *Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology* **1477**, 112-121.
- Ryan, C.A. & Pearce, G. (2003) Systemins: a functionally defined family of peptide signal that regulate defensive genes in Solanaceae species. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 14577-14580.
- Sadras, V.O. (1996) Cotton compensatory growth after loss of reproductive organs as affected by availability of resources and duration of recovery period. *Oecologia* **106**, 432-439.
- Saeed, A.E., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., Sturn, A., Snuffin, M., Rezansev, A., Popov, D., Ryltsov, A., Kostukovich, E., Borisovsky, I., Liu, Z., Vinsavich, A., Trush, V. & Quackenbush, J. (2003) TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* **34**, 374.
- Sanz, A., Moreno, J.I. & Castresana, C. (1998) PIOX, a new pathogen-induced oxygenase with homology to animal cyclooxygenase. *Plant Cell* **10**, 1523-1537.
- Scheer, J.M., Pearce, G. & Ryan, C.A. (2003) Generation of systemin signaling in tobacco by transformation with the tomato systemin receptor kinase gene. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 10114-10117.
- Scheer, J.M. & Ryan, C.A. (1999) A 160-kD systemin receptor on the surface of *Lycopersicon peruvianum* suspension-cultured cells. *Plant Cell* **11**, 1525-1535.
- Scheer, J.M. & Ryan, C.A. (2002) The systemin receptor SR160 from *Lycopersicon peruvianum* is a member of the LRR receptor kinase family. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 9585-9590.
- Schilmiller, A.L. & Howe, G.A. (2005) Systemic signaling in the wound response. *Current Opinion in Plant Biology* **8**, 369-377.
- Schittko, U., Hermsmeier, D. & Baldwin, I.T. (2001) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host

- Nicotiana attenuata*. II. Accumulation of plant mRNAs in response to insect-derived cues. *Plant Physiology* **125**, 701-710.
- Schmelz, E.A., Alborn, H.T., Engelberth, J. & Tumlinson, J.H. (2003) Nitrogen deficiency increases volicitin-induced volatile emission, jasmonic acid accumulation, and ethylene sensitivity in maize. *Plant Physiology* **133**, 295-306.
- Schmidt, D.D., Kessler, A., Kessler, D., Schmidt, S., Lim, M., Gase, K. & Baldwin, I.T. (2004) *Solanum nigrum*: A model ecological expression system and its tools. *Molecular Ecology* **13**, 981-995.
- Schmidt, D.D., Voelckel, C., Hartl, M., Schmidt, S. & Baldwin, I.T. (2005) Specificity in ecological interactions. Attack from the same lepidopteran herbivore results in species-specific transcriptional responses in two solanaceous host plants. *Plant Physiology* **138**, 1763-1773.
- Schmidt, S. & Baldwin, I.T. (2006) Systemin in *Solanum nigrum*. The tomato-homologous polypeptide does not mediate direct defense responses. *Plant Physiology* **142**, 1751-1758.
- Schwachtje, J., Minchin, P.E.H., Jahnke, S., van Dongen, J.T., Schittko, U. & Baldwin, I.T. (2006) SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 12935-12940.
- Simms, E.L. & Rausher, M.D. (1992) Quantitative Genetics. *Ecology and Evolution of Plant Resistance* (eds. R.S. Fritz & E.L. Simms), pp. 42-68. University of Chicago Press, Chicago.
- Singh, K.B., Foley, R.C. & Onate-Sanchez, L. (2002) Transcription factors in plant defense and stress responses. *Current Opinion in Plant Biology* **5**, 430-436.
- Stenzel, I., Hause, B., Maucher, H., Pitzschke, A., Miersch, O., Ziegler, J., Ryan, C.A. & Wasternack, C. (2003) Allene oxide cyclase dependence of the wound response and vascular bundle-specific generation of jasmonates in tomato - amplification in wound signalling. *Plant Journal* **33**, 577-589.
- Strauss, S.Y. & Agrawal, A.A. (1999) The ecology and evolution of plant tolerance to herbivory. *Trends in Ecology & Evolution* **14**, 179-185.
- Strauss, S.Y., Watson, W. & Allen, M.T. (2003) Predictors of male and female tolerance to insect herbivory in *Raphanus raphanistrum*. *Ecology* **84**, 2074-2082.
- Strong, D.R., Lawton, J.H. & Southwood, R. (1984) *Insects on Plants*. Blackwell Scientific Publications, Oxford.

- Thaler, J.S. (1999) Induced resistance in agricultural crops: effects of jasmonic acid on herbivory and yield in tomato plants. *Environmental Entomology* **28**, 30-37.
- Thaler, J.S., Fidantsef, A.L. & Bostock, R.M. (2002) Antagonism between jasmonate- and salicylate-mediated induced plant resistance: effects of concentration and timing of elicitation on defense-related proteins, herbivore, and pathogen performance in tomato. *Journal of Chemical Ecology* **28**, 1131-1159.
- Thaler, J.S., Stout, M.J., Karban, R. & Duffey, S.S. (1996) Exogenous jasmonates simulate insect wounding in tomato plants (*Lycopersicon esculentum*) in the laboratory and field. *Journal of Chemical Ecology* **22**, 1767-1781.
- Tiffin, P. (2000) Mechanisms of tolerance to herbivore damage: what do we know? *Evolutionary Ecology* **14**, 523-536.
- TIGR (2003) RNA Isolation using Trizol.
URL http://www.tigr.org/tdb/potato/images/SGED_SOP_3.1.1.pdf
- Tornero, P., Gadea, J., Conejero, V. & Vera, P. (1997) Two PR-1 genes from tomato are differentially regulated and reveal a novel mode of expression for a pathogenesis-related gene during the hypersensitive response and development. *Molecular Plant-Microbe Interactions* **10**, 624-634.
- Tu, C.J., Park, S.Y. & Walling, L.L. (2003) Isolation and characterization of the neutral leucine aminopeptidase (LapN) of tomato. *Plant Physiology* **132**, 243-255.
- Turlings, T.C.J., Tumlinson, J.H. & Lewis, W.J. (1990) Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* **250**, 1251-1253.
- Van Dam, N.M., Horn, M., Mares, M. & Baldwin, I.T. (2001) Ontogeny constrains systemic protease inhibitor response in *Nicotiana attenuata*. *Journal of Chemical Ecology* **27**, 547-568.
- Van der Meijden, E., Wijn, M. & Verkaar, H.J. (1988) Defense and regrowth, alternative plant strategies in the struggle against herbivores. *Oikos* **51**, 355-363.
- Van Poecke, R.M.P., Posthumus, M.A. & Dicke, M. (2001) Herbivore-induced volatile production by *Arabidopsis thaliana* leads to attraction of the parasitoid *Cotesia rubecula*: chemical, behavioral, and gene-expression analysis. *Journal of Chemical Ecology* **27**, 1911-1928.
- Voelckel, C. & Baldwin, I.T. (2003) Detecting herbivore-specific transcriptional responses in plants with multiple DDRT-PCR and subtractive library procedures. *Physiologia Plantarum* **118**, 240-252.

- Voelckel, C. & Baldwin, I.T. (2004) Herbivore-induced plant vaccination. Part II. Array-studies reveal the transience of herbivore-specific transcriptional imprints and a distinct imprint from stress combinations. *Plant Journal* **38**, 650-663.
- Walker-Simmons, M., Hadwiger, L. & Ryan, C.A. (1983) Chitosans and pectic polysaccharides both induce the accumulation of the anti-fungal phytoalexin pisatin in pea pods and antinutrient proteinase-inhibitors in tomato leaves. *Biochemical and Biophysical Research Communications* **110**, 194-199.
- Walling, L.L. (2000) The myriad plant responses to herbivores. *Journal of Plant Growth Regulation* **19**, 195-216.
- Walling, L.L., Pautot, V., Gu, Y.Q., Chao, W.S. & Holzer, F.M. (1995) Leucine Aminopeptidase - a complex array of proteins induced during the plant defense response. *Journal of Cellular Biochemistry*, 137-137.
- Wang, L., Halitschke, R., Kang, J.H., Berg, A., Harnisch, F. & Baldwin, I.T. (2007) Independently silencing two JAR family members impairs levels of trypsin proteinase inhibitors but not nicotine. *Planta* **226**, 159-167.
- Waterhouse, P.M. & Helliwell, C.A. (2003) Exploring plant genomes by RNA-induced gene silencing. *Nature Reviews Genetics* **4**, 29-38.
- Wei, Z.M., Kamada, H. & Harada, H. (1986) Transformation of *Solanum nigrum* L protoplasts by *Agrobacterium rhizogenes*. *Plant Cell Reports* **5**, 93-96.
- Welter, S.C. (1989) Arthropod impact on plant gas exchange. *Insect-Plant Interactions* (ed E.A. Bernays), pp. 135-150. CRC Press, Boca Raton, FL.
- Winz, R.A. & Baldwin, I.T. (2001) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. IV. Insect-induced ethylene reduces jasmonate- induced nicotine accumulation by regulating putrescine N- methyltransferase transcripts. *Plant Physiology* **125**, 2189-2202.
- Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M. & Turner, J.G. (1998) COI1: An *Arabidopsis* gene required for jasmonate-regulated defense and fertility. *Science* **280**, 1091-1094.
- Xu, D.P., Xue, Q.Z., McElroy, D., Mawal, Y., Hilder, V.A. & Wu, R. (1996) Constitutive expression of a cowpea trypsin inhibitor gene, CpTi, in transgenic rice plants confers resistance to two major rice insect pests. *Molecular Breeding* **2**, 167-173.

- Xu, Z.F., Qi, W.Q., Ouyang, X.Z., Yeung, E. & Chye, M.L. (2001) A proteinase inhibitor II of *Solanum americanum* is expressed in phloem. *Plant Molecular Biology* **47**, 727-738.
- Yin, Y.H., Wu, D.Y. & Chory, J. (2002) Plant receptor kinases: systemin receptor identified. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 9090-9092.
- Zavala, J.A., Patankar, A.G., Gase, K. & Baldwin, I.T. (2004a) Constitutive and inducible protease inhibitor production incurs large fitness costs in *Nicotiana attenuata*. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 1607- 1612
- Zavala, J.A., Patankar, A.G., Gase, K., Hui, D. & Baldwin, I.T. (2004b) Manipulation of endogenous trypsin proteinase inhibitor production in *Nicotiana attenuata* demonstrates their function as antiherbivore defenses. *Plant Physiology* **134**, 1181-1190.
- Zhang, N., Kallis, R.P., Ewy, R.G. & Portis, A.R. (2002) Light modulation of Rubisco in *Arabidopsis* requires a capacity for redox regulation of the larger Rubisco activase isoform. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 3330-3334.
- Zhu, L., Hu, N., Zhai, W. & Li, X. (1989) The nucleotide sequence of the chloroplast *psbA* gene from *Solanum nigrum* atrazine resistant biotype and its relevant analysis. *I Chuan Hsueh Pao* **16**, 381-388.
- Zimmerli, L., Jakab, G., Metraux, J.-P. & Mauch-Mani, B. (2000) Potentiation of pathogen-specific defense mechanisms in *Arabidopsis* by beta -aminobutyric acid. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 12920-12925.
- Zimmermann, S., Ehrhardt, T., Plesch, G. & Muller-Rober, B. (1999) Ion channels in plant signaling. *Cellular and Molecular Life Sciences* **55**, 183-203.

8 Acknowledgments

This thesis could not have been completed successfully without the direct and indirect help of numerous people. Especially considering that all the help I got did not need to be induced but was, rather, constitutively expressed, I want to express my warm gratitude to all people who supported me.

First, I would like to thank my doctoral advisor **Ian T. Baldwin** for giving me the opportunity to work on this exciting project, for always finding time for stimulating discussions, and for challenging and supporting me.

I am deeply grateful to **Christian Kost** for his endless love and for always standing by me and believing in me. Many thanks also for all your encouraging words, your patience and practical help during all stages of my PhD.

Special thanks I owe to the ‘better half’ of the early *Solanum*-group **Markus Hartl** who accompanied me as a friend and incredibly patient fellow worker along the whole way. Many thanks for all the inspiring discussions, invaluable suggestions and great ideas.

For their help in converting an acre in Dornburg into an experimental field plot and making fieldwork an enjoyable event, even when the weather was bad, I would like to thank **Markus Hartl, Holger Merker, Benjamin Tschida, Frank Walther, and Doreen Weimer**. Sincere thanks are given to **Markus Hartl, Tobias Kaiser** and **Rayko Halitschke** for an unexpected and unforgettable field season in Utah.

I am especially thankful to **Andreas Weber, Andreas Schünzel, Ralf Fornteil, Elke Müller, Tamara Krügel** and the rest of the greenhouse team for always making way for *Solanum nigrum* even when the greenhouse was crowded, as well as for raising and taking care of countless plants.

To the vibrant and fluctuating ‘coffee-round’ composed of **Markus Hartl, Caroline C. von Dahl, Claudia Voelckel, Rayko Halitschke, Anja Paschold, Merijn Kant, Silke Allmann, Beatrice Berger, Markus Benderoth, and Arjen van Doorn** I would like to express my gratefulness for stimulating scientific discussions and especially for never getting tired of philosophizing about the role of systemin.

Many thanks to **Rayko Halitschke, Dominik D. Schmidt** and **Ben Bubner** for their help in getting me started with my work on plant-insect interactions in general and on *Solanum nigrum* in special.

I am deeply grateful to my parents **Inge** and **Walter Schmidt** for their unquestioning support, their love, and for never doubting me and my path of life.

Special thanks to my ‘weekend family’ **Tobias Kaiser, Dorothea Keudel** and **Mateusz Jochym** for always having an open door, for distracting me from work and giving me the feeling of not being alone.

By critically commenting on earlier versions of this thesis **Christian Kost, Tobias Kaiser, and Emily Wheeler** substantially contributed to its final form - many thanks for this.

I would like to thank **Jan Kellmann, Karin Groten, Klaus Gase, and Thomas Hahn** for their help in finding an appropriate field site and managing the lawful affairs to work with genetically modified plants in Germany.

The financial support of the **Max Planck Society** is gratefully acknowledged.

9 Eigenständigkeitserklärung

Entsprechend der Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena (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 Verwendung der angegebenen Hilfsmittel, persönlichen Mitteilungen, Quellen und Literatur angefertigt. Personen, die an der experimentellen Durchführung, Auswertung des Datenmaterials oder bei der Verfassung der Manuskripte beteiligt waren, sind am Beginn der Arbeit (2 Thesis outline –List of manuscripts and authors’ contribution) oder am Ende jedes Manuskripts (Acknowledgments) angegeben. Ich habe weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte unmittelbar oder mittelbar geldwerte Leistungen von mir für Arbeiten erhalten, die in Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen. Ich habe die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht. Ferner habe ich nicht versucht, diese Arbeit oder eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung bei einer anderen Hochschule als Dissertation einzureichen.

Silvia Schmidt

10 Curriculum vitae

Personal data

Name	Silvia Schmidt
Date of birth	17.06.1977
Place of birth	Neunkirchen, Germany
Home address	Volkerstal 48, 66539 Furpach
Marital status	unmarried
Email	sischmidt@ice.mpg.de

Scientific career

07/2003 - present	Max Planck Institute for Chemical Ecology, PhD student Department of Molecular Ecology Advisor: Prof. Dr. Ian T. Baldwin Primary research focus: Responses of Black Nightshade (<i>Solanum nigrum</i>) to insect herbivory with a special focus on the 18-amino acid polypeptide systemin.
04/2003 - 07/2003	Martin-Luther-University Halle, Scientific co-worker Department of Herpetology and Biodiversity Advisor: PD Dr. habil. Wolf-Rüdiger Grosse Cooperation partner: Hochharz National Park Primary research focus: Adaptions of the common lizard <i>Lacerta vivipara</i> to different altitudes at the Brocken in the Hochharz National Park.
02/2002 - 03/2003	University of Würzburg, Scientific co-worker Department of Animal Ecology and Tropical Biology Advisor: Prof. Dr. Karl E. Linsenmair Primary research focus: Mechanisms underlying the switch between two different life history strategies of the reed frog <i>Hyperolius nitidulus</i> in the Ivory Coast.

09/1996 - 02/2002 University of Kaiserslautern, Diploma student
Department of Ecology
Advisor: PD Dr. Jürgen Kusch & Prof. Dr. Ulrich Sinsch
Cooperation partner: University Koblenz-Landau
Primary research focus: Factors determining the reproductive success of mating males of the common toad *Bufo arenarum* in Argentina.

Education

1993 – 1996 Gymnasium am Krebsberg, Neunkirchen (Abitur)
1987 – 1993 Kreisrealschule Neunkirchen-Wellesweiler (Mittlere Reife)
1983 – 1987 Grund -und Hauptschule Furpach

Field work

2006 Utah, USA (2 weeks)
2002 Comoé National Park, Ivory Coast (3 months)
2000 Rio Cuarto, Argentina (2 months)
2000 French Guiana (2 months)
1999 Costa Rica (2 weeks)

Publications

Schmidt D. D., Kessler A., Kessler D., **Schmidt S.**, Lim M., Gase K. & Baldwin I. T. *Solanum nigrum*: A model ecological expression system and its tools. *Molecular Ecology* 13: 981- 995

Schmidt D. D., Voelckel C., Hartl M., **Schmidt S.** & Baldwin I. T. Specificity in ecological interactions. Attack from the same lepidopteran herbivore results in species-specific transcriptional responses in two solanaceous host plants. *Plant Physiology* 138: 1763- 1773

Schmidt S. & Baldwin I.T. Systemin in *Solanum nigrum*. The tomato-homologous polypeptide does not mediate direct defense responses, *Plant Physiology* 142 (4): 1751- 1758

Schmidt S. & Baldwin I.T. Down-regulation of systemin after herbivory is associated with increased root allocation and competitive ability in *Solanum nigrum*, submitted to *Functional Ecology*

Conference contributions and attendances

- 2007 **Schmidt S.** & Baldwin I. T. Systemin in *Solanum nigrum*: Safeguard after herbivory (talk), 13th Symposium on Insect-Plant Relationships, Uppsala, Sweden.
- 2005 **Schmidt S.** & Baldwin I. T. Systemin in *Solanum nigrum*: a little conductor of a big orchestra (poster), 2nd Solanaceae Genome Workshop, Ischia, Italy.
- 2003 Summer school “Functional genomics: theory and hands-on data analysis”, Utrecht, The Netherlands.
- 2001 Wirth R., Kost C. & **Schmidt S.** Costs of trail construction and maintenance may affect the pattern of herbivory in leaf-cutting-ants (poster), Proceedings of the Society for Ecology, Volume 31, Basel, Switzerland.

Silvia Schmidt