From plant defense to development:

Serine protease inhibitors and their multiple functions

in *Solanum nigrum*

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
Mag. rer. nat. Markus Hartl

geboren am 16.2.1978
in Wien, Österreich

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Datum der öffentlichenVerteidigung: 10. März 2010

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1. INTRODUCTION

> *"…if we wished in imagination to give the plant the power of increasing in number, we should have to give it some advantage over its competitors, or over the animals which preyed on it."*
>
> Charles Darwin, The Origin of Species, Chapter III

1.1 PLANTS AND INSECT HERBIVORES: AN EVOLUTIONARY PERSPECTIVE

In any organism today, we see the result of an evolutionary process which started when the first forms of life emerged. Ever since Darwin formulated the main driving forces of this process in his theory of evolution, biologists have tried to understand how these have sculpted the breathtaking complexity and diversity of life. Certainly abiotic factors – namely, the chemical and physical features of a habitat – define the fundamental conditions for life. Within these limits, organisms have adapted to many different conditions and have been able to populate even the most adverse habitats. However, adaptation to abiotic conditions also strongly depends on the interaction with other organisms, which in their sum represent the biotic factors. Competition for resources, within or among species, and predation create strong selection pressures and determine a genotype's contribution to the gene-pool of the next generation (in an ideal population without genetic drift or other random factors). If, for example, an organism becomes capable of synthesizing a chemical which is toxic to its most common predator, it will indeed increase in number in future generations. However, any such advantage will be only temporary because this new trait creates strong selection pressure on predators (and competitors) and will lead to counter-strategies such as detoxification mechanisms.

After Darwin published his ideas on evolution, it took about a century for these mechanisms of adaptation and co-adaptation to become integrated in a general concept that explained the diversity of the two most abundant and diverse terrestrial macroscopic life forms on earth: plants and herbivorous insects. Gottfried S. Fraenkel (1959) suggested that the stunning diversity of plants' secondary metabolites – non-nutritious compounds whose function had long remained elusive (Kutchan, 2001) – was the consequence of "reciprocal adaptive evolution" because these compounds mediate plant-insect interactions as repellents, toxins, or attractants[1]. On the basis of Fraenkel's work, Ehrlich and Raven

[1] Fraenkel himself mentioned that 70 years earlier, Ernst Stahl, a professor of botany at the University of Jena, investigated the protection of plants against snails and came to similar conclusions: *"Schon lange ist man gewohnt, viele Erscheinungen der Gestaltung, sowohl der Vegetations- als der Fortpflanzungsorgane, aus den Beziehungen zwischen Pflanzen und Tieren zu begreifen, und niemand wird in unserem speziellen Fall daran zweifeln, daß die äußeren mechanischen Schutzmittel der Pflanzen im Kampfe dieser mit der Tierwelt erworben worden sind. Die große Mannigfaltigkeit der mechanischen Schutzmittel erscheint uns nicht mehr sinnlos, sondern ebenso begreiflich wie die Mannigfaltigkeit in den Bildungen der Blumen. So werden auch die großen Verschiedenheiten in der Beschaffenheit der Exkrete und mithin der Stoffwechselprozesse unserem Verständnis*

expanded the idea in a comparative analysis of butterflies, their host plants, and the taxon-specific secondary substances present in these plants (Ehrlich and Raven, 1964). In their milestone paper, the authors argue that the coevolution between plants and phytophagous insects was responsible not only for the diversification of plant secondary metabolites but also for the diversification of angiosperms and insect herbivores. Considering that 45% of the estimated 4 to 6 million insect species present today are herbivores and have coevolved with more than 300,000 plant species during the last 350 million years (Novotny et al., 2002; Zheng and Dicke, 2008), we can barely imagine how complex their interactions really are. Moreover, this complexity is further increased by other types of interactions, such as pollination and seed dispersal.

Ehrlich and Raven's work inspired research on plant-herbivore interaction and chemical ecology in the decades that followed (reviewed in Berenbaum and Zangerl, 2008). However, their macro-evolutionary focus on plant defenses was experimentally difficult to grasp and is only now, through recent advances in high-throughput sequencing and metabolomic screening, being addressed (Futuyma and Agrawal, 2009). Thus, researchers have focused primarily on the microevolutionary, or ecological, dimension of the interactions, trying to explain which and how chemicals defend plants, what the costs and trade-offs of these defenses are, and if selection pressures could be observed on the population level (Futuyma and Agrawal, 2009). We have learned that plants produce a plethora of secondary metabolites which serve either as constitutive or as induced defenses – the latter being formed only when the plant is under attack[2]. In addition to these direct defenses, plants have evolved alternative strategies to counter herbivore attack. Indirect defenses involve attracting the natural enemies of the herbivores through the release of chemical cues or the provision of a food source by the plant (Kessler and Baldwin, 2002). Tolerance mechanisms allow the plants to recover from herbivore attack and, ideally, to compensate for the negative effects of herbivory on fitness, which may be facilitated, for example, through shifts in resource allocation (Strauss and Agrawal, 1999; Schwachtje et al., 2006).

How plants perceive herbivore damage and how they regulate induced responses has been studied intensively. Signaling pathways are known to be activated by external cues, e.g. elicitors in insect saliva, which trigger signaling cascades and eventually lead to numerous defense responses (Howe and Jander, 2008). At the core of these cascades are various plant hormones, especially jasmonic acid, ethylene, and salicylic acid, which form a complex, interdependent and not yet fully understood signaling network that fine-tunes defense responses against not only herbivores but also pathogens (Koornneef and Pieterse, 2008; Diezel et al., 2009). Although the arsenals of plant taxa differ substantially in the nature and characteristics of their direct and indirect defenses, it has been

näher gerückt sein, wenn wir die Exkrete als Schutzmittel betrachten, welche im Kampf mit der Tierwelt erworben worden sind. Die die Pflanzen umgebende Tierwelt ist nicht bloß auf die Gestaltung, sondern auch auf den Chemismus der Pflanzen von tiefgreifendem Einfluß gewesen." Stahl, E. (1888). Pflanzen und Schnecken - eine biologische Studie über die Schutzmittel der Pflanzen gegen Schneckenfrass. Jenaische Zeitschrift für Naturwissenschaft und Medizin. Bd. XXII. N.F. XV. Gustav Fischer Verlag, Jena.

[2] Several hypotheses have been put forward to explain the distribution patterns of constitutive and induced defenses [reviewed in: Stamp, N. (2003). Out of the quagmire of plant defense hypotheses. Q. Rev. Biol. 78, 23 - 55.].

suggested that signaling cascades are conserved across taxa (Mitchell-Olds, 2001). Thus the model plant *Arabidopsis thaliana* has been thought to provide a blueprint for the signaling pathways that mediate ecological interactions in higher plants. In Manuscript I we aimed to characterize potential differences in transcriptional responses to herbivory within one plant family to confirm whether such a defense-related blueprint really exists. We compared the transcriptional response of two solanaceous wild species, *Solanum nigrum* and *Nicotiana attenuata,* to the attack of the well-characterized natural herbivore *Manduca sexta* using a 10k cDNA potato microarray. This largely unbiased approach allowed us not only to pick up differences between the two species but also to identify the defense-related gene families that served as ideal candidates for answering the subsequent questions.

1.2 How plant defenses diversify

Clearly, the coevolution of plants and (herbivorous) insects has contributed to chemical and species diversity. However, although we understand why there are secondary metabolites in plants, why there are so many different ones remains unknown. This problem was recently identified as a major "challenge for the 21st century" in this area of research by M. Berenbaum and A. Zangerl (2008). What are the evolutionary forces that lead to genetic and chemical novelties? How have different defense strategies and connected biosynthetic pathways evolved in different plant families?

To answer these questions, it might be worthwhile to develop a holistic view of an organism's genetic and metabolic composition. Although classic gene-for-gene coevolution certainly shaped the interaction between organisms and their (living) environment, we cannot assume that all interactions are similarly one-dimensional. Through the application of transcriptomic, proteomic, and metabolomic approaches, we have learned that plants respond to herbivory with large-scale reconfigurations of their metabolism, including many genes, proteins, and metabolites which were previously believed to be important only for primary metabolism (Schwachtje and Baldwin, 2008). This functional overlap of primary and secondary metabolism suggests that any given trait (of a gene, protein, or metabolite) might serve more than one function or trigger reactions in more than one interacting partner. For example, flavonoids play multiple roles in plants, serving as UV protectants and regulating pollen development, but their colors can also attract pollinators to flowers (Koes et al., 1994). Similarly, proteins can have several functions, facilitated through alternative posttranslational processing, the formation of different multimeric complexes, differential cellular location, or variable substrate and product specificities. On the transcriptional level, several processing mechanisms allow different mRNAs to be generated from the same strand of DNA (Kazan, 2003; Licatalosi and Darnell, 2010), making the one-gene-one-protein-one-function paradigm partially obsolete.

The functional plasticity of genes and their products is not well understood, even in the simplest self-replicating organisms. In a recent attempt to model the bacterium *Mycoplasma pneumoniae*, which has a reduced genome of 816 kbp, it was found that the observed protein-protein interactions

correlated poorly with genome organization and transcriptional patterns. Despite its small genome, *M. pneumoniae* displayed a highly dynamic transcriptome, novel homo- and heteromultimeric, multifunctional protein complexes, and regulatory networks and metabolic pathways which were more eukaryote-like than previously assumed (Güell et al., 2009; Kühner et al., 2009; Yus et al., 2009). The reduction in genome size obviously went hand in hand with a diversification of functions and regulatory mechanisms, which in turn would compensate for the genetic loss. Although this study greatly improved our understanding of prokaryotic cell biology, it also showed how difficult modeling more complex organisms is when multifunctional genes and proteins are taken into account. Without an understanding of all the components of a system, their functions, and their regulatory mechanisms on all organizational levels, the potential that systems biology offers, namely, the ability to model a complete organism, will be hard to realize.

Thus, we can understand an organism as a genetic, protein, and metabolic network which, as a whole, underlies selection pressures. However, the multivariate nature of these selection pressures on the interdependent networks creates evolutionary conflicts: one gene/protein/metabolite may need to be functionally conserved for one purpose, but optimized for another. For example, if the expression of a plant gene that is important for a particular physiological function becomes beneficial when the plant is under herbivore attack, it will be under two conflicting evolutionary selection pressures: stabilizing selection, to retain the physiological function, and positive selection, to counter new adaptations of the herbivorous attacker in a continuing coevolutionary arms race.

There are several evolutionary tricks for escaping this adaptive conflict: structural, through gene duplication and neo-functionalization or alternative reading frames, and regulational, through alternative splicing, tissue-specific or signal-dependent gene activation, smRNAs or post-translational processing (Kazan, 2003; Wang and Brendel, 2006; Baerenfaller et al., 2008; Flagel and Wendel, 2009; Ruiz-Ferrer and Voinnet, 2009). Although these mechanisms are known, how they contribute to functional diversification in the context of ecological interactions is still poorly understood. Given that the coevolution of plants and their herbivores is highly dynamic and has produced uncountable chemicals and species, we regarded the area of plant defenses as ideal for studying the functional diversification of genes and their products. As a consequence, we aimed to identify a gene family with several similar members which are simultaneously involved in plant physiology and defense.

1.3 PLANT PROTEASE INHIBITORS

Proteinaceous protease inhibitors (PIs) have been found in all domains of life and have been intensively studied for their potential to control proteolysis, a key process in all living cells (Birk, 2003). They occur ubiquitously in plants, particularly in seeds and storage organs, and in some plant families with exceptional diversity (e.g. Fabaceae, Solanaceae, Cucurbitaceae). Up to now, 18 different families of plant PIs have been found with activities against almost all major families of

proteases (Rawlings et al., 2008). M. Kunitz described the fundamental concepts of protease interactions with inhibitors which he had isolated from soybean (Kunitz, 1945). These studies attracted a lot of attention from nutritional scientists, but the investigation of plant PI functions from the standpoint of plant physiology or ecology remained restricted to protein storage and germination processes (Birk, 2003).

Although it was realized that PIs in seeds could also protect the seeds against predators, their importance for plant defense became more apparent when Green and Ryan (1972) found that tomato plants produced PIs in leaves after herbivore attack. Since then, numerous PIs have been isolated, characterized, and tested for their ability to defend plants against herbivorous insects as well as pathogenic microorganisms. There is no doubt that plant PIs can affect herbivore growth and survival (Jongsma and Bolter, 1997; Zavala et al., 2004b; Steppuhn and Baldwin, 2007); however, PI expression does not always function as a defense. Some herbivore-inducible PIs were not found to have any defensive function and in some cases were even found to be detrimental due to counter-adaptations by insects (Zhu-Salzman et al., 2008). Moreover, how well the PIs actually protect plants against herbivores in their natural environment is an open question. As most studies used herbivore-feeding assays with transgenic crop plants ectopically over-expressing PIs or with artificial diet supplemented with isolated PIs, it is not really clear how natural herbivores respond to the native PIs of a particular plant species. To our knowledge, only one field study using *Nicotiana attenuata* silenced in the expression of a PI with high activity in response to trypsin has addressed this question so far (Steppuhn et al., 2008). This publication suggested that the silenced PI is indeed an effective defense but only under specific circumstances and against particular insect species. It is likely that the defensive function of PIs depends on synergy with other defense chemicals (Steppuhn and Baldwin, 2007) or with indirect defenses, such as the release of volatiles to attract the predators of herbivores. It has been suggested that PIs buy a plant more time to recruit these predators by slowing down the growth of the herbivores, but this hypothesis has never been tested (Zavala et al., 2004b).

The focus on plant defenses, motivated by the hope that transgenic crop plants with increased herbivore resistance could be engineered, distracted from the idea that herbivore-inducible PIs regulate processes *in planta*. Recent reports have shown that plants altered in the expression of herbivore-inducible PIs display changes in growth rate (Zavala et al., 2004a; Xie et al., 2007), flower morphology and seed development (Sin et al., 2006), protein turnover (Martinez et al., 2009), phloem structure (Xie et al., 2007), and nectar protein composition (Bezzi et al., submitted). However, the proteins interacting with PIs in the plant remain unknown.

The multiple roles of PIs in defense and in growth and development make them an ideal model for studying how genes diversify to resolve an adaptive conflict. The dynamic nature of plant herbivore interactions and their immediate fitness consequences should promote the fast evolution of new defensive PIs, while PIs important for physiological functions *in planta* should be conserved. To

study the functional diversity of PIs in plants, we had to select an appropriate model system which expressed a variety of herbivore-inducible PIs.

1.4 Developing model systems and tools

For our purposes, we chose a plant species which had not undergone domestication and had thus retained its coevolutionary signature from adaptating to a diverse herbivore community. In addition, the plant had to express a variety of PIs, and genetic, biochemical, and ecological information and tools had to be available to allow experimental manipulation. Schmidt et al. (2004) established *Solanum nigrum* L. (Solanaceae, Figure 1) as an ecological expression system which suited our needs ideally. *S. nigrum* is an annual herbaceous plant with worldwide distribution (Edmonds and Chweya, 1997). A close relative of potato and tomato, it offers a number of genetic tools and databases and hosts a large community of herbivorous insects from different feeding guilds (Schmidt et al., 2004).

Figure 1. Young *S. nigrum* on the field site near Dornburg, Jena, Germany.

When attacked by herbivorous insects, *S. nigrum* responds by accumulating serine PIs[3] (SPI), as other solanaceous species do (Ryan, 1990; Schmidt et al., 2004). Schmidt et al. (2004) identified one SPI gene of the potato-inhibitor-II (PI-II) family in *S. nigrum,* but we expected that other SPI genes of the same or another family are also expressed in this species. Apparently, an ancestral PI-II gene, which most likely occurs throughout the Magnoliophyta, was duplicated early in the evolution of Solanaceae; this led to diversification and further duplication events, and eventually resulted in the great diversity of PIs observed in this family today (Barta et al., 2002; Christeller, 2005). A variety of SPI genes was found to be expressed in tomato, potato, or *Capsicum annuum* (Barta et al., 2002; Kong and Ranganathan, 2008; Tamhane et al., 2009), and we were expecting a similar situation in *S. nigrum*. Some of these SPIs were found to affect herbivore performance in laboratory experiments, and recently herbivore-inducible SPI genes were also shown to be involved in seed development in *Solanum americanum* (Sin et al., 2006), a close relative of *S. nigrum*. Due to the possible multiple

[3] Serine proteases are characterized by a serine residue in the catalytic triad of the active site, which acts as a nucleophile during proteolytic cleavage. These proteases include many important digestive enzymes of insects and mammals, such as trypsins and chymotrypsins, as well as enzymes of other families, as, for example, subtilases. Serine proteases are also the largest group of proteases in plants, but instead of trypsins and chymotrypsins, plants primarily express proteases of the classes S8 (subtilases), S9, S10, and S33. The majority of plant PIs found so far specifically inhibit members of the serine proteases, cysteine proteases, or aspartic proteases. [van der Hoorn, R.A.L. (2008). Plant proteases: from phenotypes to molecular mechanisms. Annu. Rev. Plant Biol. 59**,** 191-223].

roles of SPIs and their presumable diversity in the genus *Solanum,* we decided to concentrate our study on the identification and functional characterization of all SPIs of *S. nigrum*. Additionally, the PI-II family has interesting structural features, including sequence repeats, multimeric proteins, and alternative processing, which enhance their adaptive potential and which will be discussed in detail in Manuscript IV.

To identify the unknown SPIs of *S. nigrum,* we had to optimize and apply different techniques, including transcript profiling with microarrays (Manuscripts I and IV), protein biochemistry to profile PI activity quantitatively and qualitatively (Manuscript IV), and a new approach based on MALDI-TOF-MS which allows proteolytic activity to be detected in the presence of SPIs and which could be used to screen for novel PIs in samples from various origins (Manuscript III).

Kessler et al. (2004) demonstrated the power of using stable transformants of wild species, silenced in the expression of a native gene, to study the importance of a particular gene for survival and fitness in the natural environment. As a stable transformation technique was already established for *S. nigrum* (Schmidt et al., 2004), we aimed to use the same approach in field experiments to investigate the importance of SPIs for plant defense. However, creating stable transformants of *S. nigrum* is a lengthy process and given that we first had to identify all the native SPIs, we needed to find a faster way to silence gene expression.

Virus-induced gene silencing (VIGS) has recently emerged as the method of choice to rapidly, although transiently, silence genes, particularly in non-model plant species (Burch-Smith et al., 2004). A vector system based on the tobacco rattle virus (TRV) was found to be very effective in Solanaceae and was even reported to work in *S. nigrum* (Brigneti et al., 2004). However, we found the procedure did not work in our genotype of *S. nigrum*. A closer taxonomic examination revealed that the species used by Brigneti et al. was *Solanum nigrescens,* which explained the difficulties in adapting the method for *S. nigrum.* In Manuscript II, we describe the successful optimization of the TRV-based VIGS-system for *S. nigrum* and how we applied it to demonstrate the influence of leucine-aminopeptidase, a defense-related gene which is highly up-regulated after herbivory, on herbivore performance.

1.5 AIMS OF THIS THESIS

We have outlined how the coevolution of plants and herbivores could have influenced genetic, metabolic, and, eventually, species diversity. Although the general mechanisms seem to be clear, we know few details about how this diversity evolved and continues to evolve. Is there a commonly regulated response in plants to plant-herbivore interactions? How do new defenses evolve, how could they influence other processes and how do plants balance potential trade-offs between them? To answer these questions in all their breadth will certainly keep the whole field busy for a few more decades – especially because it will also be necessary to investigate these questions from the

herbivores' perspective and then, finally, integrate both viewpoints into one coevolutionary scenario. However, in this thesis we have tried to address some aspects of these problems, mainly from the plant's perspective.

We focus on the following questions:

1. What are the similarities and differences in the transcriptional responses of the two related wild plant species, *S. nigrum* and *N. attenuata,* after herbivory by the same insect herbivore? Is there a common blueprint of defense signaling which allows predictions across plant families? (Manuscript I)
2. Which protease inhibitor families are regulated in *S. nigrum* after herbivore attack? (Manuscripts I and IV)
3. Which serine protease inhibitors are expressed in *S. nigrum*? (Manuscripts I and IV)
4. What role do these serine protease inhibitors play in the defense against natural herbivores and in growth and development? (Manuscript IV)
5. Do different SPIs of the same gene family show signs of functional diversification or synergistic action? (Manuscript IV)

To answer these questions, we developed the following tools:

1. a VIGS-method for *S. nigrum* which facilitates the silencing of single or multiple genes to study their relevance for the defense against herbivores. (Manuscript II)
2. a rapid screen for proteolytic and PI activity using MALDI-TOF-MS (Manuscript III) or gel-based activity profiling techniques. (Manuscript IV)

2. MANUSCRIPT OVERVIEW AND AUTHORS' CONTRIBUTIONS

Manuscript I

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 Manuscript I we compared the transcriptional responses of *Nicotiana attenuata* with those of *Solanum nigrum* when both were attacked by the solanaceous generalist herbivore, *Manduca sexta*. Both species showed distinct transcriptional patterns, with only 10% overlap in significantly regulated genes. This suggests that blueprints for commonly regulated responses to plant-herbivore interactions appear unlikely and that evolution constantly fine-tunes even central mechanisms of the defense signaling system.

Authors' contributions:

D. Schmidt and C. Voelckel planned and performed the experiments with *M. sexta*, analyzed the data and wrote the manuscript. M. Hartl and S. Schmidt performed the experiments with methyljasmonate in *S. nigrum*, wrote the respective parts in Materials and Methods and critically commented and edited the manuscript. I.T. Baldwin supervised the project and edited the manuscript.

Manuscript II

Optimized virus-induced gene silencing in *Solanum nigrum* reveals the defensive function of leucine aminopeptidase against herbivores and the shortcomings of empty vector controls

Markus Hartl, Holger Merker, Dominik D. Schmidt and Ian T. Baldwin

New Phytologist (2008) 179(2): 356-365

This manuscript describes the first application and optimization of VIGS in *S. nigrum*, which allowed efficient gene silencing. The development of an appropriate control vector and the optimization of the infiltration technique constitute substantial improvements to the method which are of general importance for all TRV-based VIGS approaches. The silencing of LAP as a proof of principle experimentally revealed for the first time the long-postulated involvement of this enzyme in direct defense against herbivores.

Authors' contributions:

M. Hartl planned and performed the experiments and wrote the manuscript. H. Merker performed experiments, contributed to writing the Materials and Methods, and edited the manuscript. D.D. Schmidt and I.T. Baldwin were involved in the planning of the experiments and edited the manuscript.

Manuscript III

A dual fluorescent/MALDI chip platform for analyzing enzymatic activity and for protein profiling.

Vincentius Halim, Alexander Muck, Markus Hartl, Alfredo J. Ibáñez,
Ashok P. Giri, Florian Erfurth, Ian T. Baldwin, and Aleš Svatoš

Proteomics (2009) 9: 171-181

Manuscript III describes a dual fluorescence/MALDI-TOF platform which permits rapid screening for proteolytic enzyme activity and the corresponding inhibitors. It combines high sensitivity with low sample consumption, facilitates the quantitation of proteolytic activity and simultaneously yields qualitative information in terms of molecular masses of substrate peptides and SPIs, remaining after digestion.

Authors' contributions:

V. Halim and A. Muck developed and carried out all work related to the MALDI-TOF-MS measurements and wrote the manuscript. M. Hartl characterized the SPI-silenced lines, performed the plant experiments, prepared the raw samples from plant tissue, summarized this part of the work for the manuscript, oversaw the statistical analysis, and edited the manuscript. The remaining authors participated in the experimental planning and edited the manuscript.

Manuscript IV

Between defense and development:
functionally diversified serine protease inhibitors play multiple roles in *Solanum nigrum*.

Markus Hartl, Ashok P. Giri, Harleen Kaur, and Ian T. Baldwin

submitted to *The Plant Cell* (December 9, 2010)

In this manuscript we examine *S. nigrum's* complete SPI profile, comprising four different active inhibitors, to understand their functional specialization in an ecological context. Transcript and activity characterization revealed tissue-specific insect-elicited accumulation patterns, which indicated that the different SPIs are likely to have functionally diversified. In field and lab experiments with SPI-silenced plants we discovered that SPIs have strongly herbivore-specific defensive properties, with opposite effects on closely related species. In contrast to observations in other species, developmental phenotypes resulting from the silencing of SPIs were only weak or not apparent in *S. nigrum*, suggesting that SPIs' developmental functions are not fully conserved in the genus *Solanum*.

Authors' contributions:
M. Hartl planned and performed the experiments. A.P. Giri introduced the gel-x-ray contact print method to our lab and participated in the planning of related experiments. H. Kaur processed the samples and analyzed the data for Figures 2B, 2C and 2D and edited the manuscript. I.T. Baldwin supervised the project, participated in the planning of the experiments and edited the manuscript.

3. MANUSCRIPT I

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

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4. MANUSCRIPT II

Optimized virus-induced gene silencing in *Solanum nigrum* reveals the defensive function of leucine aminopeptidase against herbivores and the shortcomings of empty vector controls

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5. MANUSCRIPT III

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Vincentius Halim, Alexander Muck, Markus Hartl, Alfredo J. Ibáñez,
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Proteomics (2009) 9: 171-181

6. MANUSCRIPT IV

Between defense and development: functionally diversified serine protease inhibitors play multiple roles in *Solanum nigrum*.

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Estimated number of printed pages: 21.4

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ABSTRACT

Solanaceaeous taxa produce diverse serine-proteinase-inhibitors (SPIs), known anti-digestive defenses that might also control endogenous plant proteases. How plants balance the conflicting requirements of functional stability for regulation of growth and development with the requirements for diversification to thwart the counter-adaptations of attackers is unknown. Gene duplication, neofunctionalization, or sub-localization are possible scenarios, but not well studied with respect to defense and development. We examine *Solanum nigrum's* complete SPI profile, comprising four different active inhibitors, of which the most abundant proved to be novel, to understand their functional specialization in an ecological context. Transcript and activity characterization revealed tissue-specific insect-elicited accumulation patterns. Stable and transient gene silencing of all SPIs revealed different specificities for target proteinases: defense-related inhibitors had high specificity for trypsin- and chymotrypsin, while more developmentally-related inhibitors were highly active against subtilisin. Field- and lab-experiments found strongly herbivore-specific defensive properties, with opposite effects on closely related species. Developmental phenotypes resulting from the silencing of SPIs were not apparent, suggesting that SPIs' developmental functions are not fully conserved in the genus *Solanum*. In summary this study frames the multiple functions and diversification of SPIs in *S. nigrum* in light of the conflicting priorities between defense and development.

INTRODUCTION

Directed and tightly controlled proteolysis is a key cellular process in all domains of life. More than 800 proteases from 60 families in *Arabidopsis* and more than 600 in rice highlight the importance of proteolytic processes in plants (van der Hoorn, 2008). Such proteolytic machinery requires tight control to prevent the unwanted degradation of proteins. Protease inhibitors (PIs) represent one possible way to achieve such control and there is evidence that plant PIs protect specific tissues, act as storage proteins and regulate the activity of proteases and direct their release (Ryan, 1973; Mosolov and Valueva, 2005). While these multiple potential physiological functions of PIs *in planta* have been discussed, the vast majority of research on the function of PIs has been on their roles in a plant's interactions with other organisms.

Green and Ryan's (1972) ground-breaking discovery of the wound-inducible production of PIs that inhibit digestive herbivore gut proteases inspired the field of plant-insect interactions and became a classic example of induced plant defenses. Since then numerous PIs have been isolated, characterized, and tested for their potential to control herbivorous insects as well as pathogenic microorganisms, research largely motivated by the hope to engineer transgenic crop plants with increased herbivore resistance (reviewed by: Ryan, 1990; Jongsma and Bolter, 1997; Mosolov and Valueva, 2005). After four decades of research there is no doubt that plant PIs are able to negatively affect herbivore growth and survival and act as plant defenses (Jongsma and Bolter, 1997; Zavala et

al., 2004b; Steppuhn and Baldwin, 2007). However, PI expression does not always function as a defense, increasing plant resistance. There are examples of herbivore-inducible PIs with no defensive function or even the opposite effect due to the different counter-adaptations by insects (Zhu-Salzman et al., 2008). Several studies demonstrated that some insects respond to PIs by a constitutive or induced production of PI-insensitive proteases (Jongsma et al., 1995; Bown et al., 1997; Bayés et al., 2005; Bayés et al., 2006) or by proteolytically inactivating the PIs to prevent binding to sensitive proteases (Girard et al., 1998; Giri et al., 1998; Zhu-Salzman et al., 2003). For many insects, the ingestion of PI-containing tissues elicits behavioral and physiological counter responses that increase the amount of damage they inflict on plants: sub-lethal PI levels stimulate feeding and induce a general overproduction of proteolytic enzymes. Such compensatory feeding responses can negate the detrimental effects of PIs and sometimes result in an even greater loss of plant biomass as compared to plants not expressing PIs (De Leo et al., 1998; Winterer and Bergelson, 2001; Abdeen et al., 2005; Steppuhn and Baldwin, 2007).

As a consequence the development of transgenic plants expressing PIs that increase crop plant resistance to herbivores proved difficult (Gatehouse, 2008). Recent approaches using novel inhibitors, combinations of several inhibitors, or co-expression with other synergistic defense compounds might be more successful (Christou et al., 2006; Mosolov and Valueva, 2008). The limited success of transgenic crops expressing PIs also made evident that we need to understand the underlying mechanisms and interactions of multiple PIs with other defenses in more detail. Due to the focus on agricultural importance, most studies use herbivore feeding assays with transgenic crop plants ectopically over-expressing PIs or on artificial diet supplemented with isolated PIs. There are only a few publications that altered the expression of endogenous PIs in un-domesticated native plants to study their relevance for plant defense in an ecological context (Zavala et al., 2004a; Zavala et al., 2004b; Sin et al., 2006; Steppuhn and Baldwin, 2007; Steppuhn et al., 2008). To our knowledge just one of them included field-experiments to investigate the importance of PIs for defense against the natural herbivore community (Steppuhn et al., 2008). In this study, the diverse natural herbivore community revealed that PIs are indeed effective defenses but only under specific circumstances and against particular insect species. For example in the wild tobacco, *Nicotiana attenuata,* PIs are effective against *Spodoptera exigua* only in combination with nicotine (Steppuhn and Baldwin, 2007). It is also very likely that the defensive function of PIs against other members of the native herbivore community requires coordinated expression with indirect defenses, such as the emission of volatile "alarm calls" that attract the predators of the herbivores (Kessler and Baldwin, 2001). PI expression is frequently not lethal to the herbivores, but slows their growth and thereby extends the time that herbivores might be susceptible to predators. Moreover, these studies highlighted another aspect which had been neglected: the *in planta* physiological functions of defense-related PIs. Plants altered in the expression of endogenous PIs display changes in growth rate (Zavala et al., 2004a; Xie et al., 2007), flower morphology and seed development (Sin et al., 2006), phloem structure (Xie et al., 2007), and

nectar protein composition (Bezzi et al., submitted). These alterations in fitness-relevant traits showed for the first time that members of the potato inhibitor type II family (PI-II), formerly associated with defense, clearly have other physiological functions. Multiple functions have also been suggested for other PI classes: e.g. cystein PIs in barley interact with endogenous cystein proteases to regulate protein turnover during germination, but are also supposed to defend the plant against herbivores and pathogens (Martinez et al., 2009). Philippe et al. (2009) found 31 different Kunitz PIs with tissue-specific and induction-dependent expression patterns in a genomic analysis of poplar which also suggests multiple functions of this PI family.

Genes which influence plant development and growth but which are also important for plant defense represent an interesting evolutionary dilemma. The conservation of the physiological function through stabilizing selection conflicts with the positive selection pressure resulting from the co-evolutionary arms race that plants have with their herbivorous and microbial attackers. Thus it is unlikely that a single PI gene which is essential for growth and development simultaneously represents an important and evolutionary stable defense mechanism. However, if such a gene was duplicated we can hypothesize that the different selection pressures favor sub- and neofunctionalization to escape from this adaptive conflict (Conant and Wolfe, 2008; Flagel and Wendel, 2009). Interestingly, the PI-II family of serine protease inhibitors (SPI), classified as I20 in the MEROPS database (Rawlings et al., 2008), might be an ideal example of this evolutionary process. It is distributed throughout the Magnoliophyta mostly as a single copy, e.g. in Arabidopsis, maize, rice and poplar, suggesting a common ancestral gene (Barta et al., 2002; Finn et al., 2008, Pfam domain: PF02428). The function of PI-II homologues in these species is unknown, although *Arabidopsis* expression profiles suggest a possible role in flower development and defense against pathogens (Genevestigator, gene ID: AT1G72060, Hruz et al., 2008). In contrast, approximately 90 % of the known PI-II members found in the Solanaceae to date, display a stunning diversity of 78 different protein sequences comprising 288 domain forms in 30 species (Finn et al., 2008). Obviously the ancestral gene was duplicated early in the evolution of the Solanaceae (Barta et al., 2002; Kong and Ranganathan, 2008), laying the foundation for gene diversification in this family.

In addition, the proteins of the PI-II class acquired remarkable structural features that enhanced their adaptive potential even further. PI-II genes contain one to several sequence repeats (up to eight in *Nicotiana glutinosa*), which code for one precursor. Through circular permutation and intramolecular domain swapping many of these pre-proteins form a circular bracelet in which the structural domains are shifted and do not overlap with the sequence repeats (Lee et al., 1999; Schirra and Craik, 2005). Proteolytic cleavage of these precursors at specific linker sites results in several single or multi-domain PIs with one active site per domain, and the processing can be altered when plants are elicited by insect specific elicitors (Horn et al., 2005). Thus a single PI-II gene can code for several inhibitors with different specificities for the serine proteases trypsin, chymotrypsin, and subtilisin (Atkinson et al., 1993; Beekwilder et al., 2000; Tamhane et al., 2007; Wang et al., 2007).

The hypervariability due to positive Darwinian selection, especially at the active sites and linker regions, is a common feature of PIs and of PI-II inhibitors in particular (Christeller, 2005; Schirra and Craik, 2005). The underlying high selection pressure illustrates the importance of these genes for plant fitness and points towards co-evolutionary processes typical for plant-herbivore or plant-pathogen interactions. Therefore gene duplication, sequence repeats, and certain structural features of the PI-II family may have enabled solanaceous plants to retain specific PIs that function in the regulation of growth and development and to simultaneously allow evolutionary forces shape the defensive function of other specific PIs.

As a consequence of these considerations, the PI-II family represents an ideal model to study functional gene diversification under opposing selection pressures in plants. Clearly the evolutionary context of the process makes it imperative to study undomesticated plants species that have not been shaped by breeding for particular traits (Kant and Baldwin, 2007). In our group we have established the black nightshade, *Solanum nigrum* L., as an ecological model system which allows us to use molecular tools to study gene function in the context of the natural habitat (Schmidt et al., 2004). Schmidt et al. (2004; 2006b) have shown that *S. nigrum* responds typically to wounding and herbivory with the production of SPIs. They identified one member of the PI-II class called *SnPIN2b*, a homolog of a gene first isolated from *Solanum americanum* (Xu et al., 2001). Most likely additional homologs of PI-II exist in *S. nigrum*, as they do in many other species of the genus *Solanum*. Additionally *S. nigrum* is hexaploid (Edmonds and Chweya, 1997; Schmidt et al., 2004) and we can assume that this has multiplied the number of PI-II genes and further increased the chances of their functional diversification.

Here we identify all SPIs present in *S. nigrum*, profile their tissue-specific expression, and investigate their substrate specificity. Furthermore we manipulated SPI gene expression, using RNA interference mediated gene-silencing, and evaluated the consequences of PI-silencing on plant defense, growth and development. Another goal of this analysis was to investigate if SPIs in *S. nigrum* fulfill different functions, complement each other as sub-functionalized copies, or if their function generally overlaps. However, it is beyond scope of this study to identify the function of each single gene in full detail on a mechanistic level. As such this is an attempt to analyze a network of a functional group of PIs in terms of their degree of involvement in different processes. The analysis provides an understanding of the importance of SPIs for defense, especially in natural systems, and it indicates where and which substrates they might bind to in mediating their *in planta* effects on physiological processes.

RESULTS

Transcripts of PI-I and PI-II inhibitors are up-regulated after insect herbivory and methyl jasmonate elicitation

We chose a transcriptomic approach to estimate which PIs account for the increase in PI activity after herbivory in *S. nigrum*. Datasets from two microarray platforms were used to profile seven different PI families. First, we re-analyzed data from a potato 10k cDNA array (from The Institute for Genomic Research, TIGR, now J. Craig Venter Institute) published by Schmidt et al. (2005). This study compared gene expression patterns in the two plant species *N. attenuata* and *S. nigrum* after herbivory by larvae of the solanaceous specialist *Manduca sexta* L. (Sphingidae) or after treatment with methyl jasmonate (MeJA), which was used as a positive control to induce defense responses mediated by jasmonic acid (JA). We selected all clones annotated as PIs and grouped them according to families. Figure 1 (left panel) shows a heat map of the mean gene expression from three independent microarray replicates, each hybridized with pooled and differentially labeled cDNA from three plants. PIs of the potato inhibitor type I family (PI-I) were significantly up-regulated in both treatments and one clone of a metallocarboxypeptidase inhibitor (MCPI) showed a significant down-regulation after herbivory by *M. sexta*. In addition to these clear signals there were no general patterns of regulation although two clones of the PI-II class (STMCC57 and STMCX74, see Supplementary Table S1) showed an up-regulation after treatment with MeJA although statistically not significant.

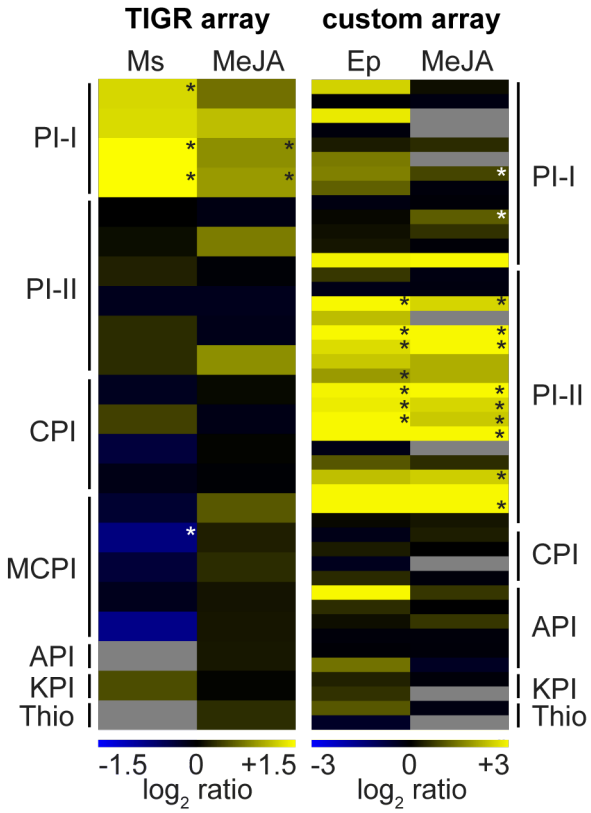

Figure 1. Differential PI transcript accumulation in *S. nigrum* after herbivore challenge and methyl jasmonate treatment. Cy3/Cy5 labeled cDNA of treated and untreated plants was hybridized to cDNA microarrays from TIGR (now JCVI) or to custom 50-mer oligo-microarrays. The heat maps show the mean logarithmic transcript ratios of all clones which were annotated as PIs on both array types, grouped by inhibitor families. Asterisks indicate genes that are significantly > 1.5-fold up- or < 0.67-fold down-regulated over three micro-array replicates ($p < 0.05$; for further details see "Materials and Methods"). Ms, Ep: plants attacked by *Manduca sexta* larvae or by *Epitrix pubescens*; MeJA: methyl jasmonate; PI-I: potato inhibitor I; PI-II: potato inhibitor II; CPI: cysteine PI; MCPI: metallocarboxypeptidase inhibitor; API: aspartate PI; KPI: Kunitz PI; Thio: thionin-like PI; gray bars: signal too low.

The second microarray contains 1421 50-mer oligonucleotides representing herbivory-related genes selected from differential experiments and public databases – including a large number of PI genes from different solanaceous species. We hybridized cDNA from leaves of field-grown plants which were either attacked by the flea-beetle *Epitrix pubescens* KOCH (Chrysomelidae) or which had been treated with an insecticide and remained un-attacked. Additionally we used the same mRNA from control and MeJA-elicited leaf material that we had used for the three TIGR microarray replicates to hybridize another three replicates of the custom array for means of comparison. Both treatments showed a strong up-regulation of PI-II transcripts while the regulation pattern of the PI-I class was much less pronounced, with only two oligos indicating significant up-regulation in the MeJA treatment (Fig. 1, right panel). One homologue of PI-I that we cloned from *S. nigrum* and which is also represented on the microarray shows high up-regulation (around 8-fold in both treatments; ID 1404, see Supplementary Table S2) but did not meet the criteria of significance due to high variation among the replicates.

SPIs are expressed in all aboveground tissues but differ strongly in abundance and inducibility

Based on the microarray data and previous studies (Schmidt et al., 2004; Schmidt and Baldwin, 2006a), we assumed that PI-I and PI-II-class inhibitors of *S. nigrum* respond to leaf-chewing herbivores. To get a better understanding of this response and the tissue-specific localization of SPIs, we measured trypsin inhibitory activity in extracts from various tissues, after different treatments, and at several time-points after elicitation (Fig. 2). Trypsin PI (TPI) accumulation is slightly enhanced after mechanical wounding of leaves but this response is strongly amplified when caterpillar feeding is mimicked through the application of regurgitates from *M. sexta* larvae to wounds (W+R). A treatment with MeJA, which directly triggers the JA-mediated responses, dramatically increased this accumulation. In contrast, salicylic acid and ethylene, two other phytohormones related to plant defense, did not increase TPI accumulation when applied to wounds (Fig. 2A).

Constitutive and MeJA-inducible TPI activity strongly varied across tissues. It was found in all above-ground parts of the plant but was not detectable in roots. Vegetative organs contained relatively small amounts of constitutive TPIs but responded to MeJA treatment with up to ten-fold increases in TPI activity (Fig. 2B). Generative organs were not or only weakly responsive to MeJA but flowers displayed the highest TPI activity per gram fresh mass of all tissues examined. After fertilization these high constitutive levels decreased and by the time the berries had ripened, the levels were as low as those of constitutive vegetative organs.

The accumulation of TPIs after herbivore damage is a late defense response. Figures 2C and 2D show the temporal dynamics of TPI accumulation in local and systemic leaves after a single elicitation by wounding plus *M. sexta* regurgitates (W+R) or by MeJA. In both treatments TPI levels increase in the local leaf after 12 to 24 h, reaching a maximum after three days in the case of W+R or rising continuously for 7 days in the case of MeJA-elicited leaves. Systemic un-elicited leaves on

elicited plants respond approximately in the same time-frame but show a markedly lower maximum. The TPI levels in un-induced control plants were additionally recorded at day 3 and 7 but remained at the initial level throughout the experiment.

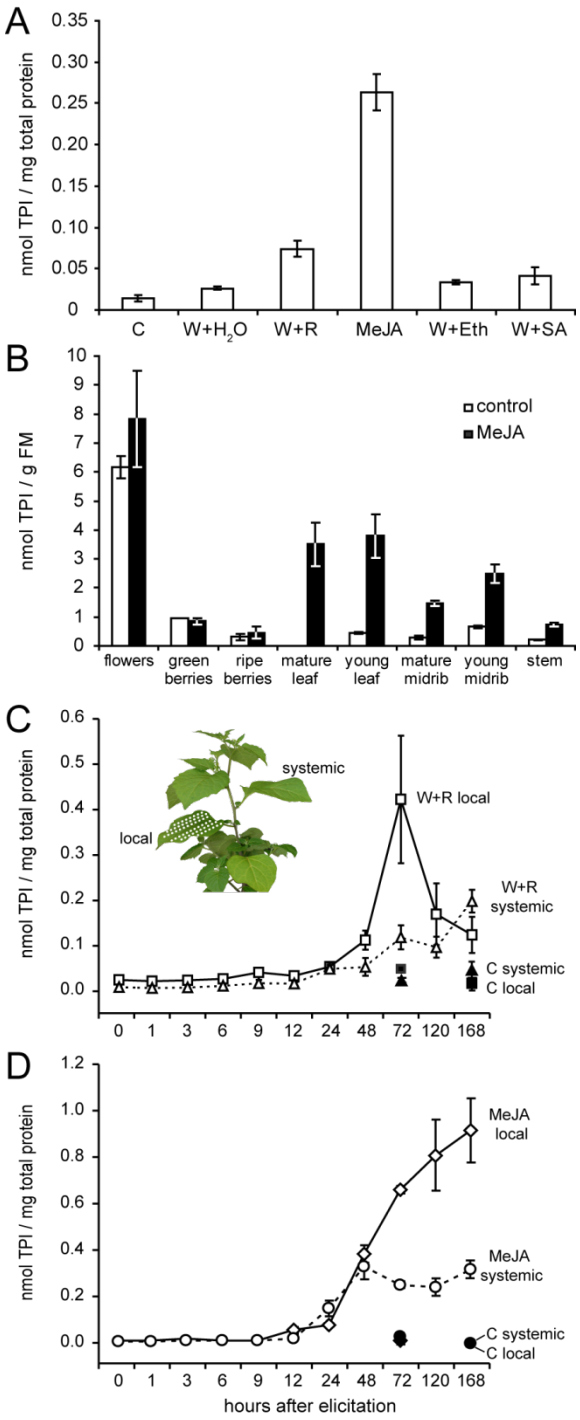

Figure 2. Inducibility, tissue specificity, and dynamics of trypsin PI (TPI) accumulation in *S. nigrum*. A, mean ± SE TPI concentration in leaves 48 h after different elicitor treatments (n = 4), (C, control; W, mechanical wounds treated with : H_2O, water; R, regurgitate of *M. sexta* larvae; Eth, ethephone; SA, salicylic acid; MeJA, methyl jasmonate in lanolin paste). B, mean ± SE TPI concentration in different aboveground tissues of control and MeJA-treated plants 48 h after induction (n = 3, pooled from 9 plants; FM = fresh mass). C, D, mean ± SE TPI accumulation in local and systemic leaves after a single induction with either W+R (C) or MeJA (D), (n = 5). Un-treated control samples were taken after 0 h, 3 d, and 7 d. The inset shows the position of the locally elicited leaf and the harvested systemic leaf, one node above the local.

Identifying SPI genes in S. nigrum

In a cDNA library screen Schmidt et al. (2004) found the PI-II homolog *SnPIN2b* (Genbank: AY422686) using probes based on genes from *S. americanum* cloned by Xu et al. (2001). We suspected that another homolog, similar to *SaPIN2a* from *S. americanum*, could also be present in *S. nigrum* and succeeded in identifying one clone by PCR with cDNA and gDNA as templates. To avoid confusion of these PI genes with the well established *PIN* auxin transporters we will from here on refer to the new gene as *S. nigrum Serine-Protease-Inhibitor-2a* (*SnSPI2a*) and to *SnPIN2b* as *SnSPI2b*. The new clone *SnSPI2a* shares 96% nucleotide sequence similarity with *SaPIN2a* and 78% with *SnSPI2b*.

We also obtained two fragments of a PI-I gene through PCR-based cloning using genomic DNA as template. They share 98% similarity in nucleotide sequence which translates into two amino acid changes. Both gene sequences are 84% identical to a PI-I inhibitor from potato and we refer to these two genes as *SnSPI1a* and *SnSPI1b*. In a Southern blot we assessed the number of genes similar to *SnSPI1*, *SnSPI2a*, or *SPI2b* in *S. nigrum* using gene-specific probes and three different enzymes for digestion (Supplementary Fig. S1). Although the high similarity between *SnSPI2a* and *SnSPI2b* produced cross-signals which complicated the analysis, we estimate that all three SPI genes are present with three to four copies in the *S. nigrum* genome.

Silencing SPI2a and SPI2b has no effect on flowers and growth and marginally affects seed development

To study the function of these genes, we stably transformed *S. nigrum* plants with inverted-repeat (ir) RNAi constructs to silence the expression of either both *SPI2* genes alone (ir*SPI2a+b*) or additionally the two *SPI1* genes (ir*SPI1/2a+b*). For both constructs we selected two independently transformed lines, each one containing a single T-DNA insertion (Supplementary Fig. S2). Figure 3A shows the mRNA levels of all three gene groups in wild-type (WT) plants and in the silenced lines 24 h after elicitation with MeJA. Both ir-constructs contained a fragment of *SnSPI2b* which reduced transcript levels to approximately 0.1% of WT levels. The high similarity of *SnSPI2b* to *SnSPI2a* resulted in a simultaneous reduction of *SnSPI2a* transcripts to 9-14% in all genotypes. Lines transformed with the double RNAi construct ir*SnSPI1/2a+b* were additionally silenced in *SnSPI1* transcripts at levels of 16 and 31%. Sin et al. (2006) have reported an increase in flower size and an 80% seed abortion after silencing homologs of *SnSPI2a* and *SnSPI2b* with similar efficiency in *S. americanum*. However, in *S. nigrum* we did not observe any effect on flower size and found that only 0.7 to 2.8 % of the seeds were aborted or defective (Supplementary Fig. S3). Zavala et al. (2004a) and Xie et al. (2007) have reported that silencing or the ectopic overexpression of SPI genes can affect plant growth. In comparative growth experiments we found no differences in plant height between WT and SPI-silenced plants (Supplementary Fig. S3). The lack of clear phenotypes suggested that the investigated SPIs have either no developmental function or that their loss is compensated by other SPIs in *S. nigrum*.

SPI2a and SPI2b are strong inhibitors of subtilisin but account for only one third of total trypsin inhibition

We determined the inhibitory activity of leaf extracts from all genotypes against trypsin, chymoptrypsin, and subtilisin to find out how well the gene silencing translated into reduced PI activity and to assess the substrate specificity of the different SPIs, indicating functional specialization (Fig. 3B). Although the silencing reduced mRNA levels of the SPI genes efficiently, we found that both constructs (ir*SPI2a+b* and ir*SPI1/2a+b*) retained two thirds of their activity against trypsin and chymotrypsin, suggesting that another, yet unknown, SPI was likely present in the extracts.

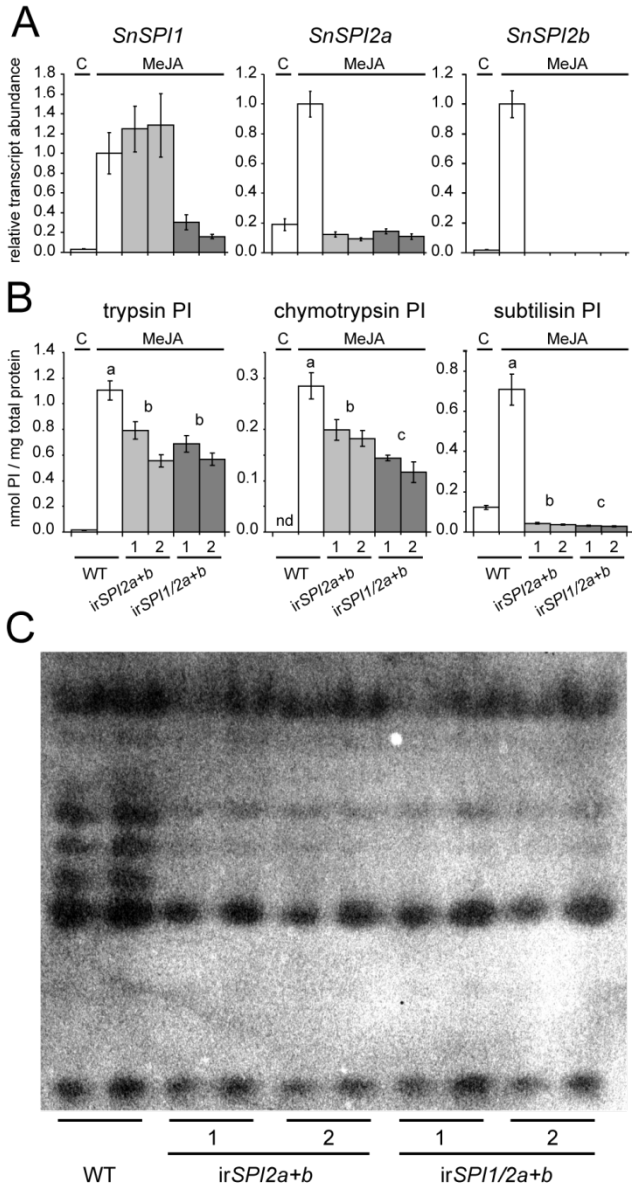

Figure 3. Silencing efficiency and remaining SPI activity in plants silenced in the expression of *SnSPI1*, *SnSPI2a*, and *SnSPI2b*. A, mean ± SE relative transcript abundance of *SnSPI1*, *SnSPI2a*, and *SnSPI2b* in leaves of wild-type (WT) plants and of transgenic lines expressing an inverted-repeat construct specific for *SnSPI2a* and *SnSPI2b* (ir*SPI2a+b*) or a construct specific for *SnSPI1*, *SnSPI2a*, and *SnSPI2b* (ir*SPI1/2a+b*), (n = 7; C: control; MeJA: MeJA treatment for 24 h). B, mean ± SE inhibitory activity against trypsin, chymotrypsin, and subtilisin in leaves of WT plants and of SPI-silenced lines (C: control; MeJA: MeJA-treated for three days). Different letters indicate a significant difference (nested ANOVA, lines nested within genotypes, TPI: $F_{2,30} = 21.95$, $p < 0.001$, ChyPI: $F_{2,29} = 21.42$ $p < 0.001$, SubPI: $F_{2,30} = 349.82$ $p < 0.001$, followed by a Scheffé *post-hoc* test, $P < 0.05$, WT control plants were excluded from the analysis). C, Profiles of active TPI proteins present in MeJA-induced leaves of WT and SPI-silenced plants, visualized with GXCP after 12% native PAGE. Each genotype is represented by two pools of two biological replicates.

ir*SPI1/2a+b* lines showed slightly but significantly lower inhibitory activity against chymotrypsin, indicating a specificity of the *SnSPI1* genes. The most striking effect though was observed when measuring activity against subtilisin. All lines showed a drastic reduction of subtilisin-PI activity, mainly caused by the silencing of *SnSPI2a* and *SnSPI2b* (~ 96% reduction in ir*SPI2a+b*) with a small contribution by *SnSPI1* genes (~ 97% reduction in ir*SPI1/2a+b*).

The high remaining activity against trypsin and chymotrypsin in the silenced lines demanded a qualitative analysis of SPIs. We used gel-x-ray-film contact prints (GXCP) after native polyacrylamide gel electrophoresis (PAGE) to obtain profiles of active inhibitors. Four proteins were indeed missing or strongly reduced in all four SPI-silenced lines (Fig. 3C). While these missing bands bands with high activity were not affected by the RNAi constructs. These proteins explained the majority of remaining activity of the extracts against trypsin, however their identity remained unknown.

SnSPI2c, a new isoform of PI-II inhibitors, is the main inhibitor of trypsin and chymotrypsin

Although the activity on x-ray film was remarkably high, we could not detect the corresponding PI proteins after staining the gels with Coomassie Blue. This disconnect suggested that these very active PIs occurred at low abundance and demanded further purification. We started with 50 g of flower material because the PIs displayed high constitutive expression in these tissues. The active peptides, like many other PIs, are heat-stable which allowed them to be concentrated by incubating the extract for 30 min at 60°C. After dialysis and freeze-drying, we ultra-filtrated the protein solution at a 30 kD molecular weight cut-off and checked for remaining activity in both fractions by native PAGE followed by GXCP. The active inhibitors were present in both but the filtrate contained less contamination from other proteins and was used for further purification steps.

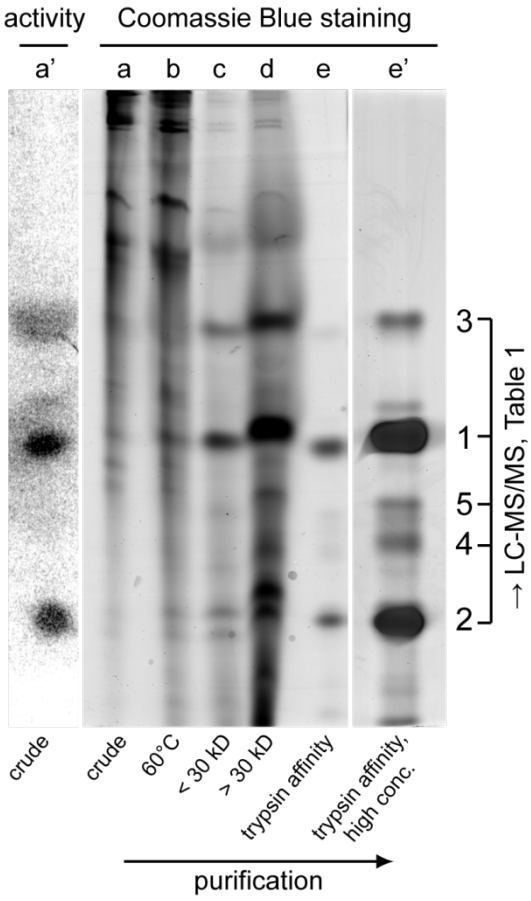

Figure 4. Native PAGE (12%) representing the purification of SnSPI2c visualized with GXCP for PI activity or Coomassie Blue stain. GXCP: lane a', crude extract. Coomassie stain, lanes: a, crude extract, same as in a'; b, heat-treated crude extract; c, ultrafiltrate (< 30 kD molecular weight cut-off); d, ultrafiltration concentrate (> 30 kD molecular weight cut-off); e, eluate of c, after trypsin affinity chromatography; e' same as e but larger amount loaded for subsequent *de-novo* sequencing (numbered bands were excised and sequenced on LC-MS/MS, see Table I); protein amounts loaded: a' and a-d 40 µg, e-f 1 µg, and e' 20 µg.

Table I. Peptide sequences of protein bands in Figure 4 (lane e') as determined by LC-MS/MS *de novo* sequencing.

Peptide	Peptide mass[a]	Peptide (partial) sequence[b]	band no.				
			1	2	3	4	5
A	2375.02	(X)XXXYFSNDGTFL[c]CEGESEY	x				x
B	1321.58	(SD?)CTNCCAGKK	x	x	x	x	x
C	1683.72	ECDTR[d]L[c]DYGL[c]CPVS		x			
D	1022.50	L[c]DYGL[c]CPVS		x			x
E	992.52	L[c]AYGL[c]CPL[c]S	x		x	x	x
F	978.52	L[c]AYGL[c]CPVS					x

[a]Molecular mass of the uncharged peptide.
[b]Given from N-terminus to C-terminus.
[c]Leucine and isoleucine are not distinguishable.
[d]Missed cleavage site (R).

After purification by trypsin-affinity chromatography, we separated the eluate on native PAGE and visualized the bands by Coomassie-staining and GXCP (Figure 4). The active PI bands were excised and subjected to MS/MS *de-novo* sequencing. We obtained six different peptides (Table I) which were all similar to a protein from the PI-II family in tomato (Uniprot accession: Q43710) containing three repeats.

Using primers designed on this sequence from tomato, we cloned several fragments of a PI-II inhibitor gene from *S. nigrum* by PCR with cDNA and gDNA as templates. At least two different genes with high similarity to each other were found, one coding for two and one for three domains, sharing 97% amino acid sequence similarity with each other and 60% or 64% similarity with *SnSPI2a* and *SnSPI2b*, respectively (for a sequence alignment see Supplementary Fig. 4). We refer to these new genes as *SnSPI2c-R2* and *SnSPI2c-R3*. The high similarity between the two *SnSPI2c* sequences facilitated the design of a construct for virus-induced gene silencing (VIGS), which transiently reduced the transcripts of both genes simultaneously. Figure 5A shows the active peptides present in flowers and MeJA-induced leaves from WT plants and stably PI-silenced lines (ir*SPI2a+b* and ir*SPI1/2a+b*) after silencing both *SnSPI2c* genes with the VIGS vector v*SPI2c* or after treatment with a control VIGS vector (CV). While the silencing in leaves was much more efficient than that observed in flowers, the results clearly demonstrated that the VIGS vector v*SPI2c* reduced the three most abundant peptides in both tissues, confirming their identity as *SnSPI2c*. In leaves the efficient silencing also demonstrated that the four peptides remaining after silencing *SnSPI2c* in WT plants were *SnSPI2a* and *SnSPI2b*. VIGS silencing of *SnSPI2c* in the background of the two stably SPI-silenced lines resulted in a complete absence of active peptides. When quantifying SPI activity in leaf extracts it became evident that *SnSPI2c* does not or only weakly bind to subtilisin but that it is a strong inhibitor of trypsin and chymotrypsin (Fig. 5B) – exactly opposite specificity of *SnSPI2a* and *SnSPI2b*.

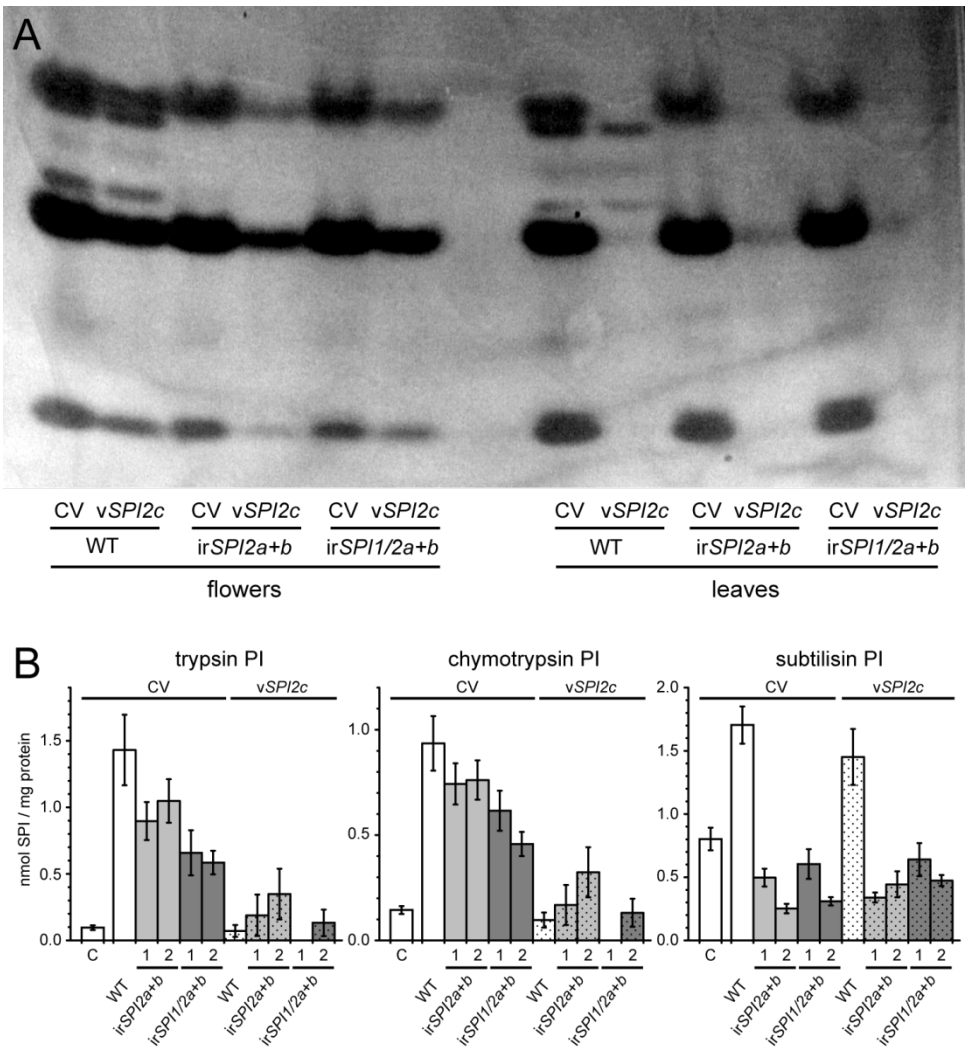

Figure 5. Virus-induced gene silencing of *SnSPI2c* in WT plants and in four independently stably transformed lines, silenced either for *SnSPI2a* and *SnSPI2b* (ir*SPI2a+b*) or additionally for *SnSPI1* (ir*SPI1/2a+b*). Each genotype was either inoculated with tobacco-rattle-virus containing a control vector (CV) or a vector harboring a fragment of *SnSPI2c* for gene silencing (v*SPI2c*). A, profiles of active TPI proteins present in flowers and leaves of WT plants and of two SPI-silenced lines (ir*SPI2a+b* line 1 and ir*SPI1/2a+b* line 1) after VIGS and treatment with MeJA. The active TPIs were visualized with GXCP after 12% native PAGE. Each lane represents a pool of three biological replicates. B, mean ± SE inhibitory activity against trypsin, chymotrypsin, and subtilisin in leaves of WT and SPI-silenced plants (n = 9; C, control; MeJA, 3 d after MeJA treatment).

SPI accumulation after simulated herbivory – not all SPIs respond equally

Using quantitative real-time PCR (QPCR) we assessed the transcriptional contribution of the different groups of SPI genes to the W+R induced SPI activity measured in Fig. 2. In local leaves there was a strong up-regulation of all four gene-types reaching a maximum around 12 h after induction (Fig. 6A). In terms of absolute transcript amounts, two groups could be distinguished: at the peak of highest accumulation, *SnSPI2b*- and *SnSPI2c*-like transcripts were about 30 or 100 times more abundant than *SnSPI2a* or *SnSPI1*, respectively. In systemic leaves, the induction of *SnSPI1*-, *SnSPI2a*-, and

SnSPI2b-like genes was attenuated, with the latter two barely responding to the treatment. In contrast, *SnSPI2c* genes attained transcript levels in systemic untreated leaves comparable to the high levels observed in locally treated leaves. Transcript levels in untreated leaves did not vary significantly throughout the experiment and thus were not included in the graph.

Similarly we analyzed the tissue specific expression of the SPI genes. In general, the highest transcript levels of all four gene groups were found in flowers, with *SnSPI2c* being by far the most abundant (Fig. 6B). MeJA-treatment of flowers affected SPI gene expression only slightly, as we had observed for TPI activity. After fertilization the high levels in flowers drop significantly with just

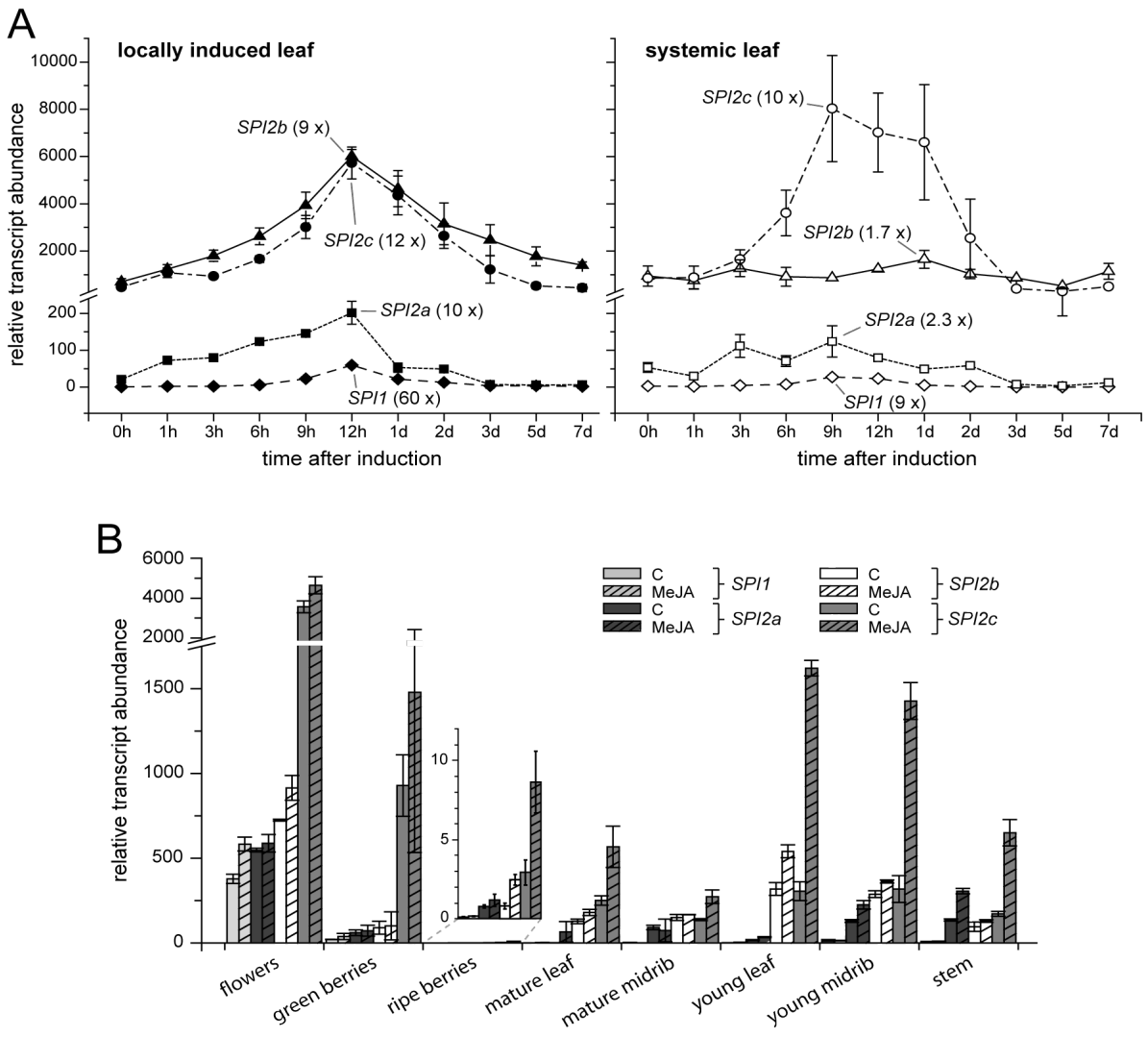

Figure 6. Transcript levels of SPI-genes as determined by QRT-PCR. A, time-series of the mean ± SE relative transcript abundance of *SnSPI1*, *SnSPI2a*, *SnSPI2b*, and *SnSPI2c* in locally elicited leaves (left) and systemic untreated (right) leaves of WT plants after mechanical wounding and application of *M. sexta* regurgitant. Numbers in parentheses show the maximum fold-change in transcript levels compared to untreated leaves at time-point 0 h (n = 4, per time-point). B, mean ± SE relative transcript abundance of *SnSPI1*, *SnSPI2a*, *SnSPI2b*, and *SnSPI2c* in different aboveground tissues of control and MeJA-treated plants 48 h after induction (n = 3). Inset: magnified plot of the data from ripe berries.

SnSPI2c being strongly expressed in unripe fruits and almost no expression is observed in ripe fruits. In vegetative tissues, *SnSPI2c* and *SnSPI2b* are the main SPI transcripts. Particularly in the leaf lamina these two forms are predominant and strongly MeJA-inducible, with higher levels in younger than in older leaves. On the other hand, transcripts of *SnSPI2a*, although also present in the lamina, were particularly abundant in leaf mid-ribs and stems. Except for flowers, *SnSPI1* transcripts were found in much lower amounts in all other tissues but displayed a preferential expression in midribs and stems (ca. 4- to 8-fold higher when compared to leaf lamina), similar to *SnSPI2a*.

SnSPI2a and SnSPI2b have mild defensive properties against natural herbivorous insects

The genotype Sn30 of *S. nigrum*, which we used in all experiments, originates from a collection from a native population growing near Jena, Germany. To investigate the importance of SPIs for defense against the herbivores in the plant's natural habitat, we obtained permission to conduct field-experiments with transgenic plants at a field site near Dornburg, close to Jena. We expected herbivores in the natural habitat of the plant to be more adapted to *S. nigrum* and we were also interested in how less adapted generalist herbivores might respond to plants with different PI levels. *S. nigrum* has been recently introduced to the USA (Edmonds and Chweya, 1997) and we assumed the herbivore community to be less adapted to the defenses of *S. nigrum* in this new habitat. Thus we also obtained permission for and performed release experiments on our field station at the Lytle Ranch Preserve, Utah, in the Great Basin Desert of the southwestern US.

At the native site in Germany, we conducted experiments during summer in the years from 2004 to 2006. The restrictions of the release permit did not allow the transgenic plants to grow to the flowering stage and as a consequence experiments at the Dornburg field site were restricted to approximately three weeks after planting during which flower buds had to be removed regularly. Consistent with a previous study (Schmidt et al., 2004), we identified the flea-beetle *E. pubescens* to be the main leaf-chewing herbivore. Corresponding to its life-cycle, it occurred only for a few weeks in June and July during which it is able to cause heavy damage. In the 2005 season, we observed the largest population of *E. pubescens*, which heavily infested the planted *S. nigrum* population. During the following years, we found only a few individuals which did not cause sufficient leaf loss to obtain reliable data. Thus we present the data from the 2005 season, in which we had permission to release lines silenced in the expression of *SnSPI2a* and *SnSPI2b* (ir*SPI2a+b*). The flea-beetle damage occurred during two experiments, planted on June 6 and July 15. We planted WT plants and the SPI-silenced line (ir*SPI2a+b*, line 1) in pairs and monitored the percent loss of total leaf area. SPI-silenced lines tended to be more damaged by flea-beetles, although the differences in damage levels were not statistically significant (Fig. 7A). Earlier in the season, we also encountered large numbers of the black bean aphid *Aphis fabae* (Aphididae) feeding on *S. nigrum* but their numbers did not differ between WT and SPI-silenced plants (data not shown).

The field season in 2006 in Utah was characterized by a generally low abundance of herbivores. Especially *M. sexta*, a typical herbivore on the native wild tobacco *N. attenuata*, hardly occurred during this season. To generate an herbivore density that resembles that of a more typical field season, we placed an equal number of WT and ir*SPI2a+b* plants into mesh tents and allowed field-collected herbivores to feed on them for four days. The plants were either treated with MeJA in lanolin paste or just with lanolin to amplify possible differences due to variations in SPI concentration. Indeed MeJA-induced WT plants were significantly less damaged when compared to SPI-silenced plants (Fig. 7B). Un-induced plants of both genotypes did not differ but showed higher damage levels than the MeJA-induced WT plants.

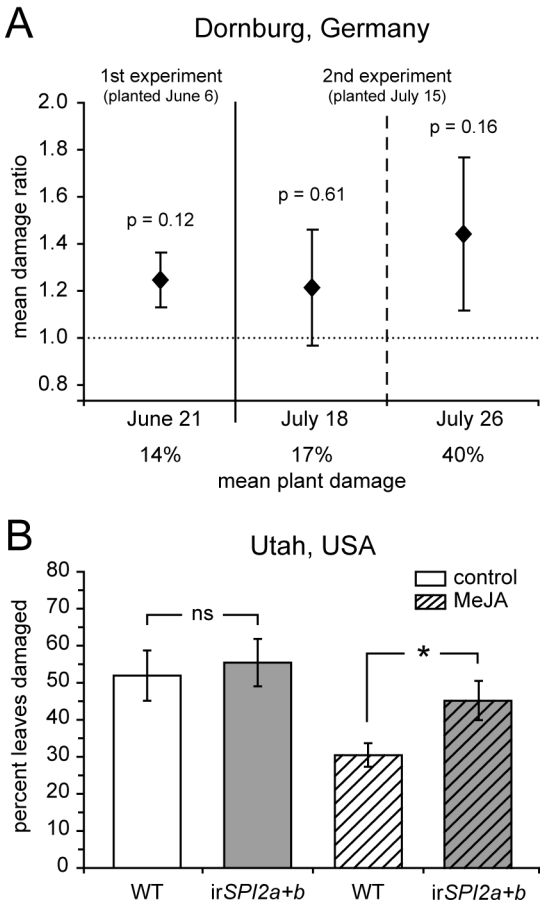

Figure 7. Natural herbivore damage on WT and a stably transformed line silenced for *SnSPI2a*, and *SnSPI2b* (ir*SPI2a+b*). A, mean ± SE herbivore damage ratio (% damage of line / % damage of WT) of WT and ir*SPI2a+b* (line 1) plants grown in pairs under field-conditions. The two consecutive experiments were carried out in 2005 on a field-site near Dornburg, Germany. Damage was estimated twice in the second experiment. The dotted line indicates a ratio of 1 (when both genotypes were damaged to the same extent). P-values were calculated from a paired t-test after arcsine-transformation: June 21, t_{18} = 1.659; July 18, t_{18} = 0.513; July 26, t_{15} = 1.485. B, mean ± SE percentage of herbivore-damaged leaves per plant, WT or ir*SPI2a+b* (line 1), which were either untreated or MeJA-induced. In June 2006 potted plants were grown outdoors at the Lytle Preserve field station in Utah, USA. Both plant genotypes were transferred to mesh tents in which field-collected generalist insect herbivores were allowed to feed for five days. On the large plants overall percent damage levels were low (WT = 1.8 ± 0.4, ir*SPI2a+b* = 3.0 ± 0.5), thus the percentage of damaged leaves was assumed a reliable measure of herbivore damage. The two genotypes were compared separately for control and MeJA-elicited plants with t-tests (C: t_6 = -1.43; MeJA: t_{12} = -2.66; ns: no significant difference; asterisk: $p < 0.05$).

SnSPI2a and SnSPI2b reduce the growth of a generalist but not of a specialist herbivore

To investigate the responses of generalist and specialist herbivores in more detail, we used the stably transformed lines to study the performance of *M. sexta* and the beet armyworm *Spodoptera exigua* HÜBNER (Noctuidae) in laboratory experiments. Both, *M. sexta* and a *Spodoptera sp.*, most likely *S.*

exigua, were observed to occur and feed on *S. nigrum* in Utah (personal observation). *M. sexta* is a solanaceous specialist and to some extent adapted to PI-II inhibitors (Zavala et al., 2008). *S. exigua* is considered a generalist with a very wide host range that includes solanaceous crop plants (Brown and Dewhurst, 1975). Nevertheless there are reports that *Spodoptera spp*. are able to compensate an SPI-rich diet by differential regulation or *de-novo* synthesis of proteases (Jongsma et al., 1995; Brioschi et al., 2007). Unfortunately a closer examination of the interaction with the flea-beetle *E. pubescens* was not possible because its detailed life-cycle is unknown and stable laboratory cultures could not be established.

M. sexta larvae were reared on excised leaves from WT and SPI-silenced plants (ir*SPI2a+b* and ir*SPI1/2a+b*), which had been pre-induced with MeJA or were left untreated. Silencing of SPI had no effect on larval mass (Fig. 8A). Induction with MeJA, however, caused a significant decrease in caterpillar mass, irrespective of the plant genotype (for statistics see Supplementary Table S3). To understand this response in more detail, we measured the consumed leaf area four and five days after hatching and related it to larval mass on day four, as a measure of consumption (Fig. 8B), or to the mass gain within 24 h of feeding, as a measure of the food conversion efficiency (Fig. 8C). The consumption was significantly reduced by MeJA-treatment but the plant genotype and thus the absence of the silenced PIs did not have any effect (for statistical analysis, see Supplementary Table S4). *M. sexta* was able to convert the ingested food efficiently, regardless of plant genotype or treatment. The high but opposite variation of ir*SPI2a+b* line 1 and ir*SPI1/2a+b* line 1 between control and MeJA-induced tissue accounted for a significant genotype*treatment interaction that is not supported by the other lines. To summarize, MeJA induces defenses which decrease the consumption rate of *M. sexta* but which are independent of *SnSPI1*, *SnSPI2a*, and *SnSPI2b*.

In contrast, *S. exigua* larvae showed a significant mass increase when fed on un-induced tissue of SPI-silenced lines (Fig. 8D, for statistical analysis, see Supplementary Table S3). This difference in performance was also reflected in the mortality rate: larvae on WT plants showed a higher mortality (38%) than larvae feeding on SPI-silenced lines (ir*SPI2a+b* line 1: 14%; ir*SPI1/2a+b* line1: 10%). On MeJA-induced tissue, the difference between genotypes was lost with the larvae performing generally poorly and displaying high mortality (~ 50% on all genotypes). An analysis of consumption and food conversion efficiency between days 10 and 12 was complicated by the higher mortality of caterpillars feeding on WT and MeJA-induced plants. The resulting high variation did not allow meaningful conclusions to explain the differences between plant genotypes more precisely. Nevertheless, the MeJA-treatment demonstrates that the plants are able to produce defenses which restrict caterpillar growth and these elicited defenses masked the effects of the silenced SPIs.

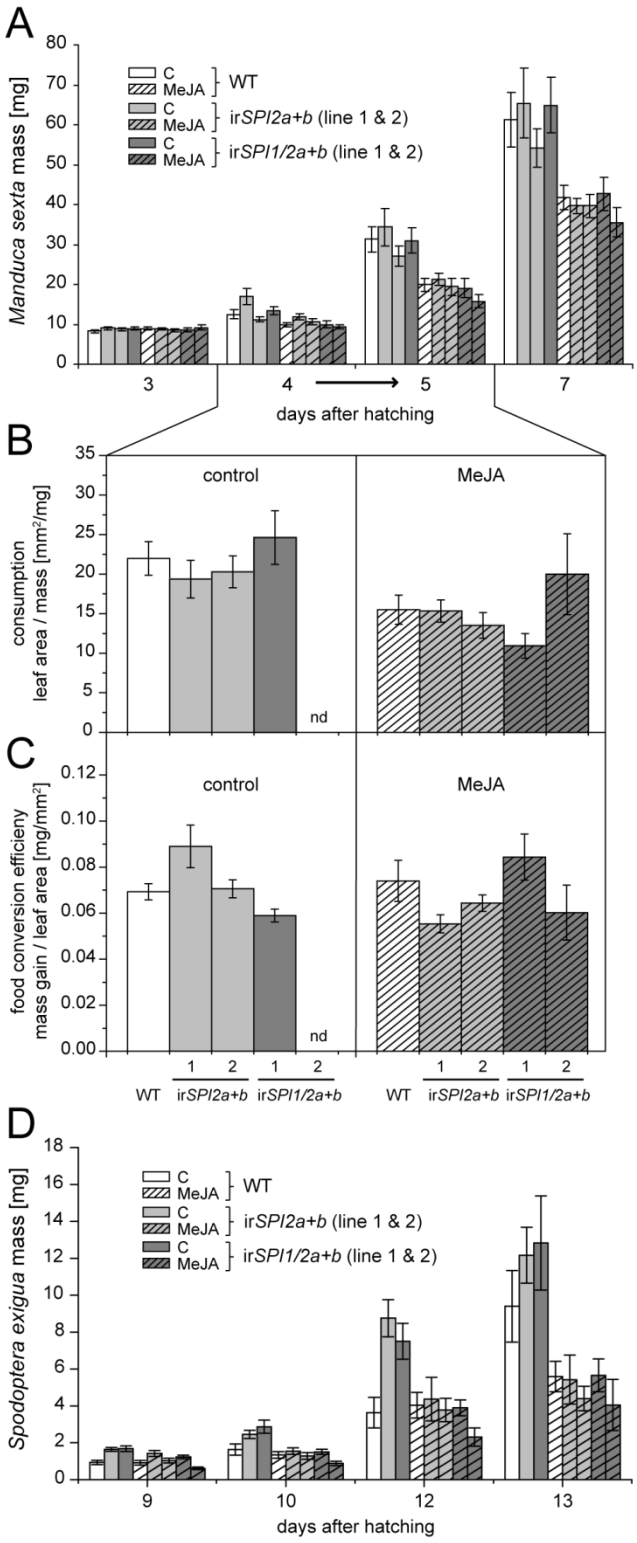

Figure 8. Performance of *M. sexta* (A) and *S. exigua* (D) larvae feeding on excised leaf discs from WT plants and from four independently stably transformed lines silenced either for *SnSPI2a* and *SnSPI2b* (ir*SPI2a+b*) or additionally for *SnSPI1* (ir*SPI1/2a+b*). The plants were either control-treated (C) or induced with MeJA. A, D, mean ± SE larval mass over time (*M. sexta*, n ≈ 17; *S. exigua*, n = 21). A repeated measures ANOVA of larval mass (over all days) with 'genotype', 'line' (nested within 'genotype'), and 'treatment' as factors revealed 'day', 'day * treatment' (within subjects) and 'treatment' (between subjects) as significant effects for *M. sexta*. *S. exigua* mass depended significantly on the same factors but additionally 'genotype' had a significant effect in a reduced model (see Supplementary Table S3). B, mean ± SE leaf consumption by *M. sexta*, measured as leaf area relative to larval mass before the feeding period of 24 h from days 4 to 5. A general linear mixed-effects model with 'genotype', 'line' (random, nested within 'genotype'), and 'treatment' as factors indicated a clear treatment-effect ($p < 0.01$), a possible effect of the line within genotypes ($p = 0.05$), but no genotype- and thus no SPI-effect on consumption by *M. sexta* (for details see Supplementary Table S4). C, mean ± SE food conversion efficiency of *M. sexta*, measured as mass gain relative to consumed leaf area during 24 h from days 4 to 5. A statistical model, similar to the one in B, indicates no effect of genotype or treatment but of a 'genotype*treatment' interaction ($p < 0.01$, see Supplementary Table S4, nd: not determined).

SPI2c has no effect on the specialist, Manduca sexta, and elicits compensatory feeding in generalist, Spodoptera littoralis

We have shown that *SnSPI2c* is the inhibitor which accounts for two thirds of inducible activity against trypsin and chymotrypsin. Thus the missing response of *M. sexta* in plants silenced for the other three SPIs could have simply been due to *SnSPI2c* still being expressed in the stable lines. To test whether *SnSPI2c* functions as a defense against *M. sexta* we used VIGS to reduce *SnSPI2c* expression in WT plants and in the stably SPI-silenced lines. Freshly hatched larvae were allowed to feed on excised leaves and on the same day all plants were induced with MeJA to simulate induction of plant defenses through continuous feeding. To make this experiment with 300 larvae more manageable, we raised them for the first three days in communal boxes (10 to 15 caterpillars per box, three boxes per genotype and VIGS-vector) before they were transferred to individual containers. When we fitted a linear mixed-effects model, which accounted for possible box effects, to the mass data on day 9, we could not find any significant effects of silencing *SnSPI2c* or the co-silencing of all SPIs on *M. sexta* larval mass (Fig. 9A, for statistics see Supplementary Table S5). We repeated the experiment another two times with similar results and conclude that larval mass gain of *M. sexta* is not influenced by the SPIs of *S. nigrum*.

A similar approach with *S. exigua* was hindered by the repeatedly high mortality rates and poor larval performance. Although these attempts indicated that silencing *SnSPI2c* decreased mortality and improved the growth rate of *S. exigua*, it was clear that another approach was necessary. We had previously learned that the related generalist *S. littoralis* performs better on *S. nigrum* than does *S. exigua*. Like *S. nigrum* and *S. exigua*, *S. littoralis* originates from Africa, Europe and Asia with an even larger recorded plant host range that includes *S. nigrum* (Brown and Dewhurst, 1975; Martins et al., 2005). In spite of its relatedness to *S. exigua, S. littoralis* displayed a completely different response, revealing another aspect of how SPIs affect herbivore performance. We conducted the experiment in the same way as with *M. sexta*, feeding caterpillars for 3 days on un-induced and later on MeJA-induced leaf tissue. The silencing of *SnSPI2c* significantly reduced larval mass (Fig. 9B, for statistics see Supplementary Table S6). The effect was already discernable at the start of data collection, six days after hatching. We repeated the experiment with WT plants and monitored larval mass and consumed leaf area starting on day zero. Four days after hatching, the mass of larvae on CV plants increased, continuously until day 6 (Fig. 9C). Conversion efficiency did not show differences between control and *SnSPI2c*-silenced plants (Fig. 9D) but the increase in mass correlated with higher consumption on CV plants between day 2 and 4 (Fig. 9E). This suggests that the presence of *SnSPI2c* stimulated feeding but did not influence the digestibility of the material. After day 4, the conversion efficiency doubled for larvae feeding on plants treated with both VIGS constructs while their consumption halved, suggesting a major readjustment of larvae's digestive physiology, which masked any effects of *SnSPI2c* silencing.

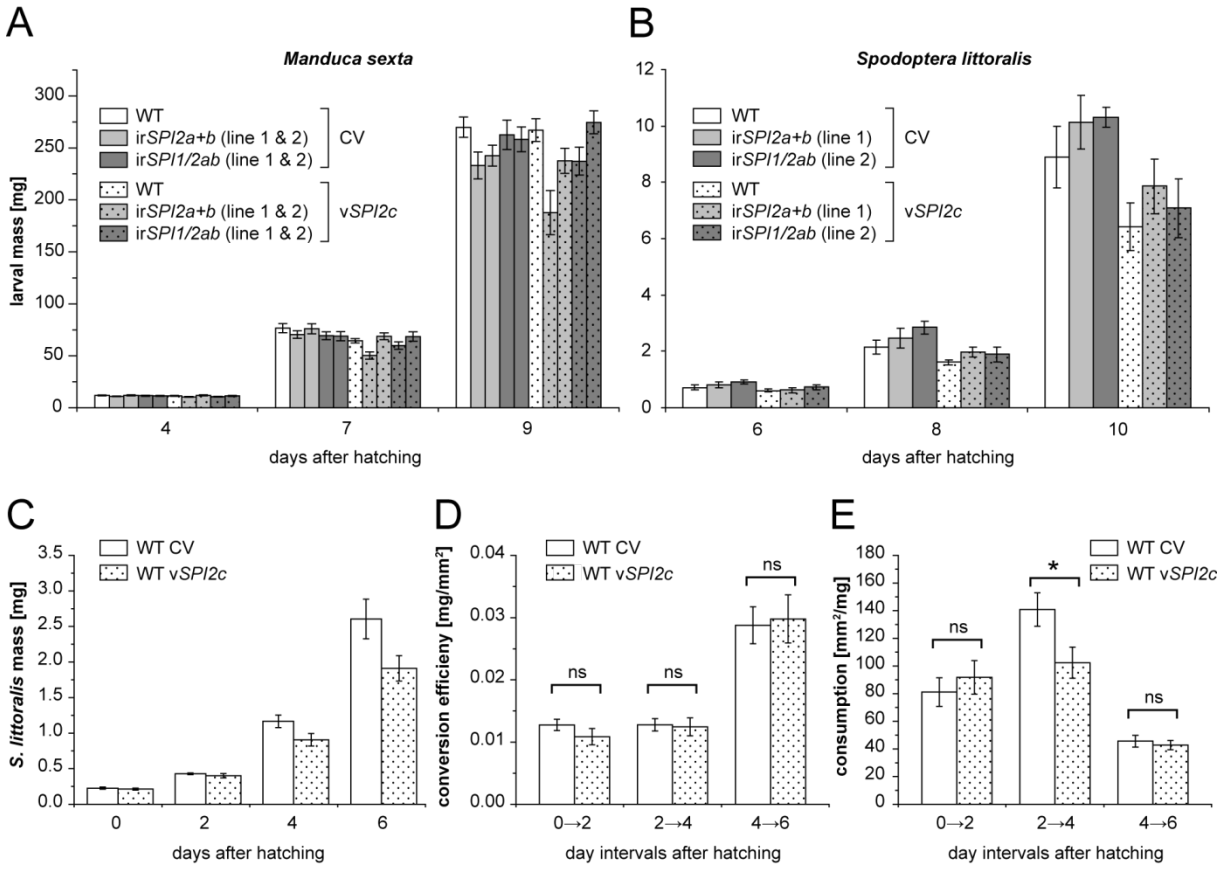

Figure 9. Performance of *M. sexta* and *S. littoralis* larvae feeding on excised leaf discs from WT and SPI-silenced plants, which were additionally silenced for *SPI2c* by virus-induced gene silencing. WT and independently stably transformed lines (ir*SPI2a+b* line 1 and ir*SPI1/2a+b* line 2), were infected with TRV containing a control vector (CV) or a vector for silencing of *SPI2c* (v*SPI2c*). A, mean ± SE larval mass of *M. sexta* (n = 30). We fitted linear mixed-effects models to the data from day 9 and compared them with a maximum-likelihood ratio test. No significant effect could be found among all treatment groups (for details see Supplementary Table S5). B, mean ± SE larval mass of *S. littoralis* over time (n = 12). Silencing of *SnSPI2c* by VIGS has a significant effect on larval mass, the stable silencing of the other SPI genes has no effect (repeated measures ANOVA, $p < 0.01$, see Supplementary table S6). C, Repetition of the experiment in B (n = 15). Larvae were weighed after hatching, transferred to un-induced plant material, and weighed on alternate days. A repeated measures ANOVA showed a significant 'days*v*SPI2c*' interaction ($p<0.05$, see Supplementary Table S7). D, E, mean ± SE food conversion efficiency (D) and consumption (E) corresponding to panel C. Missing values, due to molting of larvae, strongly reduced the power of a repeated measures ANOVA, we calculated individual t-tests for each time-point (D, 0→2: $t_{28} = 1.106$, $p = 0.28$; 2→4: $t_{21} = 0.197$, $p = 0.85$; 4→6: $t_{26} = -0.155$, $p = 0.88$; E, 0→2: $t_{28} = -0.664$, $p = 0.53$; 2→4: $t_{21} = 2.094$, $p = 0.049$; 4→6: $t_{26} = 0.505$, $p = 0.62$; ns: no significant difference; asterisk: $p < 0.05$).

In summary, the insect performance assays revealed just how diverse the responses of leaf-chewing insects are to plant PIs. While *M. sexta* growth is not influenced by the SPIs of *S. nigrum*, *S. exigua* performs significantly better on plants silenced for PI-II genes. *S. littoralis*, however, displays a compensatory feeding response when *SnSPI2c* is present which leads to increased larval growth. The field experiments with generalist herbivores confirmed the defensive properties of *SnSPI2a* and

SnSPI2b but the specialist *E. pubescens* showed only a mild, non-significant response. *SnSPI1* had no effect on any of the tested herbivore species.

DISCUSSION

The combination of activity-based PI profiling and specific gene-silencing enabled us to identify all active SPIs of *S. nigrum* and led to the discovery and purification of the third and most abundant PI-II *SnSPI2c*. In total we isolated five new expressed sequences from *S. nigrum*, two of them belonging to the PI-I family and the remaining three to the PI-II family. Together with the already known *SnSPI2b*, *S. nigrum* expresses three different forms of PI-II and one of PI-I. Each of these SPIs is represented by at least two copies in the genome, resulting in a total repertoire of likely more than ten different SPI genes. The single or combined silencing of each SPI-type in this study represents the first combinatorial and functional characterization of all SPIs in a single plant species. This approach allowed us to dissect the function of native SPIs in plant defense against insect herbivores and in growth and development without having to extrapolate results from experiments with artificial diets or the ectopic overexpression of transgenes.

The large number and close phylogenetic relationship of PI-II inhibitors and their functional similarity to the PI-I inhibitors raised the question to what extent these genes have functionally diversified and which selective forces might have influenced the evolution of this gene family. The analysis of Kong and Ranganathan (2008) revealed that the active sites and the linker regions of PI-II inhibitors bare the signatures of having been under positive selection pressure (K_a/K_s ratios at the active site of PI-II genes with two or three repeats: $K_a/K_s = 2.3$ for the first and $K_a/K_s = 4$ for the second repeat unit; at linker regions of multi-repeat genes: $K_a/K_s = 2.6$). Thus, if neo- or sub-functionalization had occurred in these genes, we would expect differences in substrate specificity, tissue localization, their response to elicitors, or their effect on herbivores. Moreover, if some of these PIs play a physiological role *in planta,* we assumed that plants silenced for single inhibitors or their combinations should display different growth-related or developmental phenotypes. Given that gene diversification represents an ongoing evolutionary process, it is also possible that the genes are in the process of diversification and exhibit functional overlap or multiple roles. Indeed we found evidence for such transition states in functional diversification and we discuss the evidence with respect to the two major functional domains of SPIs: plant development and defense.

The dramatic increases in SPI transcripts and trypsin PI activity after herbivore attack and JA elicitation points to a role of SPI genes in defense against herbivores. It is remarkable that the PI-I and the PI-II families were the only PI genes consistently up-regulated after herbivory by two different insect species and MeJA treatment. Due to the cross-species hybridization and the incomplete representation of genes on both arrays, it is possible that we missed responses of other PI classes [e.g. Bolter & Jongsma (1995) found cysteine PIs induced in potato leaves after Colorado potato beetle attack]. However, we regard this study to be just the first step in the challenging process of

disentangling the intricate functional relationships of PIs in *S. nigrum*. With additional genomic data it will be fascinating to expand the profiling of herbivory related PI expression to all PI families in a single plant species – be it *S. nigrum* or other plants with similarly high PI diversity.

The tissue-specific and induction-dependent expression patterns were consistent with a possible role in plant defense but also indicated differences between the SPIs. In the leaf lamina, the most commonly herbivore-attacked tissue, *SnSPI2b* and especially *SnSPI2c* are most abundant and are highly amplified by W+R or MeJA application. Although *SnSPI2a* and *SnSPI1* also respond to simulated herbivory with a strong fold-increase, their transcript levels remain low compared to the other two SPIs. Why only *SnSPI2c* responds strongly in systemic tissues remains unclear, but it suggests a primary role of these genes for preparing the un-attacked tissue for impending attack. The increased accumulation of *SnSPI2a* and *SnSPI1* in stem and midribs suggests a function in vascular tissues. It has been shown for *SaPIN2a*, a homolog of *SnSPI2a* from *S. americanum*, that it is localized in the phloem (Xu et al., 2001) and that ectopic overexpression of this gene results in chloroplast formation in enucleate sieve elements (Xie et al., 2007), a tissue in which chloroplasts are normally absent. However, it cannot be ruled out that PIs accumulate in these tissues also as a defense response against phloem-sap feeding insects. PI-II inhibitors were shown to be elicited by aphid feeding in *Capsicum annuum* (Tamhane et al., 2009). We did not observe differences in the numbers of *A. fabae* on SPI-silenced lines in the field but small effects on fitness or on other aphid species cannot be excluded. It is clear that mapping the expression of each gene on a finer spatial scale will yield valuable information on their possible functions. *SaPIN2b*, a homolog of *SnSPI2b*, was found to accumulate in trichomes, especially after MeJA treatment (Liu et al., 2006). This might be interpreted as a defense response against herbivores but ectopic overexpression of *SaPIN2a* and *SaPIN2b* in tobacco also led to higher trichome densities and trichome branching (Luo et al., 2009). It is worth remembering that evolution creates SPIs with new functions by tinkering with the genes at hand and that distinctions of "defensive" and "developmental" functions are likely more apparent than real.

Our investigation of the defensive effects against herbivores illustrates that the effects of these SPIs are as diverse as the genes. We found that the performance of specialist herbivores like *E. pubescens* or *M. sexta*, species that naturally occur on solanaceous host plants, were not influenced by SPIs, but we cannot exclude more subtle fitness effects that are realized after pupation (De Leo and Gallerani, 2002). However, when trypsin PIs were silenced in *N. attenuata, M. sexta* mass increased dramatically, demonstrating that the insect is not in general SPI-insensitive (Zavala et al., 2004b). *N. attenuata* contains higher levels of trypsin PIs than *S. nigrum* and it is possible that *M. sexta* counter-defense mechanisms are sufficient to cope with levels of SPIs in *S. nigrum*. The decreased performance and consumption on plant material pre-induced with MeJA demonstrates that *S. nigrum* is able to activate other yet unknown defenses and our results suggest a lack of synergistic mechanism between these unknown defenses and the SPIs. In contrast, generalist herbivores in the field clearly preferred plants silenced in SPIs over WT plants but only after MeJA elicitation. This suggests that

either SPIs alone or in combination with other defenses are effective resistance mechanisms that protect *S. nigrum* against generalist herbivores. As the kinetics of this defense mechanism is relatively slow it will only work when the herbivore pressure is sustained to allow the full response to develop. Rapidly moving and feeding herbivores, such as flea-beetles, could avoid these defenses by switching to other host plants before the defenses accumulate – representing a behavioral adaptation to induced defenses. Lepidopteran larvae, although sometimes mobile at later developmental stages, remain after hatching for a longer period on their host plant and are more exposed to induced systemic defenses.

Our laboratory experiments with generalist herbivores underscore the defensive value of these PIs: *S. exigua* was particularly susceptible to the induced defenses of *S. nigrum*. Even constitutive levels of *SnSPI2a* and *SnSPI2b* were sufficient to affect larval growth. In combination with other induced defenses, including *SnSPI2c*, they barely managed to grow. This corresponds well to a field observation: we repeatedly found *Spodoptera spp.* to oviposit, hatch and feed on WT *S. nigrum* in Utah. However, after an initial feeding period, the number of larvae quickly decreased, an effect that we attribute to up-regulated direct defenses, rather than top-down effects involving the attraction of predators. The problems of *S. exigua* to cope with SPIs of *S. nigrum* are remarkable because Jongsma et al. (1995) reported that the same species did not show a difference in performance when feeding on tobacco ectopically overexpressing potato inhibitor II – an effect that the authors attributed to *S. exigua* larvae responding to the PI-rich diet with the production of insensitive proteases. We speculate that these diet-induced proteases are still sensitive to the SPIs of *S. nigrum*, causing a growth difference already at constitutive levels and giving the plant more time to fully activate its direct and indirect defenses.

The completely different results in the experiments with the other generalist, *S. littoralis,* caution against drawing conclusions about the effects of PIs against generalists from the findings with *S. exigua. SnSPI2c* induced a transient compensatory feeding response in *S. littoralis* which led to more rapid larval growth on WT plants. The increase in consumption could be observed only at the particular growth stage approximately 2 to 4 days after hatching. This coincides with the time when plants were induced with MeJA suggesting that constitutive levels of *SnSPI2c* stimulate feeding, when other inducible defenses are still low. After this, the food conversion efficiency increased strongly but consumption was halved indicating a major shift in the larval digestive physiology. We hypothesize that other induced toxic compounds limited the amount of food which can be ingested making more efficient food conversion necessary. *S. exigua* responds similarly when feeding on *N. attenuata*: the high toxicity of nicotine limits food intake and PIs induce a compensatory feeding only when nicotine synthesis is silenced in the plant (Steppuhn and Baldwin, 2007).

An alternative hypothesis for the transient nature of the effect can be derived from the observation that the response of *S. littoralis* to PIs is concentration-dependent. Low levels of PIs in transgenic Arabidopsis and tobacco induced a compensatory feeding response with increased larval performance but high levels were detrimental for the larvae (De Leo et al., 1998). The stable silencing

of *SnSPI2a* and *SnSPI2b* seems to slightly improve larval performance, although the effects are not statistically significant (Fig. 9B). We saw a similar trend when repeating the experiment which suggests that these two SPIs have a minor negative effect when induced to higher concentrations.

The complexity of defensive functions underscores why the transition between physiological function to defensive function may be a slow and drawn out procedure. Although we see a tendency of *SnSPI2c*, and to some extent *SnSPI2b*, to behave like a typical herbivory-related gene, a single PI might not be sufficient to keep the proteolytic machinery of all naturally occurring herbivores in check. Each herbivore species may require a specific PI cocktail to be kept at bay, with evolution likely selecting for the lowest common denominator, depending on the nature and density of the herbivore populations. This need for combinatorial flexibility might have favored further domain duplication events and driven the evolution of different enzyme specificities in the single domains and of alternative post-translational processing (Christeller, 2005). It could also explain why PIs, although they are required for developmental processes, remain responsive to herbivore attack. Keeping the PI portfolio as diverse as possible and regulating its expression may be evolutionarily advantageous. Additionally our data demonstrates that synergistic effects of SPIs with other induced metabolites are crucial for effective defense and it will be clearly necessary to characterize these unknown defenses if we are to fully understand the defensive function of PIs.

We have shown that the SPIs in *S. nigrum* differ not only in their expression patterns and their effects on herbivores but also in their substrate specificity. We consider this further evidence for functional diversification. *SnSPI2c* is a strong inhibitor of trypsin and chymotrypsin but hardly interacts with subtilisin, whereas *SnSPI2a* and *SnSPI2b* show the opposite patterns of inhibition. While a large number of trypsin- and chymotrypsin-like proteases (serine protease family S1), among others, were found in insects only a handful of subtilase-like proteases (family S8) are known to date (Rawlings et al., 2008). In contrast, subtilases are one of the major serine-protease families in plants whereas trypsin- and chymotrypsin-like proteases are rather rare (van der Hoorn, 2008). This suggests that SPIs with a defensive role against insects are more likely to have high a affinity towards trypsin- or chymotrypsin, SPIs that interact with plant proteases are more likely to bind to subtilases or other predominant serine-protease classes, such as S9, S10, and S33. However, subtilisin PIs could play a role in plant defense against pathogens by inhibiting bacterial or fungal proteases. For example PI-II inhibitors in tomato were shown to be up-regulated after infection with *Pseudomonas syringae* (Pautot et al., 1991) and in the ornamental tobacco *Nicotiana alata* PI-II inhibitors are specifically secreted into the extracellular mucilage of the stigma (Johnson et al., 2007), a tissue of high fitness value which is likely to require special protection against pathogens during pollen tube growth. However, in our field experiments we did not observe any evidence of higher susceptibility to pathogen attack in SPI-silenced plants, although effects on flowers could not be monitored due to legal restrictions.

As a consequence of the differences in substrate specificity, we expected to see developmental phenotypes when silencing the subtilisin-specific *SnSPI2a* and *SnSPI2b*. Sin et al. (2006)

demonstrated that *S. americanum* silenced in the expression of *SaPIN2a* and *SaPIN2b* aborted around 80% of its seeds. Surprisingly, we found this effect to be barely detectable in *S. nigrum*. A reason for this difference might be that the authors used a fragment of *SaPIN2a* for their silencing construct while we used *SnSPI2b* to silence both, *SnSPI2a* and *SnSPI2b*. However when we compared the Northern blots presented in their publication to the transcript levels in our plants, it was obvious that we achieved similar levels of silencing for both genes. Alternatively *SnSPI2c* may have compensated for the silencing effect, assuming that it is not present in *S. americanum*. To investigate the presence of SnSPI2c-like PIs in *S. americanum,* we compared its trypsin PI profile to those of *S. nigrum* and four other species of black nightshades (Supplementary Fig. S5). SPI2c-like bands were found in all investigated species, which rules out the possibility of deficiencies in the potential for compensation in *S. americanum*. As a consequence, we infer that the developmental importance of SPI2a and SPI2b differs between species and is not a conserved feature.

We could not observe any other PI-dependent developmental phenotype which had been reported for other species. There was no significant difference in flower diameter (Sin et al., 2006), number of reproductive units (Zavala et al., 2004a), plant growth (Zavala et al., 2004a; Xie et al., 2007), or trichome morphology and density (Liu et al., 2006; Luo et al., 2009). Again it is possible that *SnSPI2c* compensated for the silencing of *SnSPI2a* and *SnSPI2b* in the stably transformed lines and perhaps the transient silencing of *SnSPI2c* using VIGS did not reveal these phenotypes in the triple knock-downs. Nevertheless, this would not explain why some of these phenotypes occur in *S. americanum* where a homolog of *SnSPI2c* is also highly expressed.

The observed variability of PIs in even closely related species and the completely different SPI pattern of the more distant tomato indicate that even such an important trait as seed viability can be influenced by rapidly evolving genes. Without knowing the exact mechanism by which PIs affect seed development, it will be difficult to explain the variability. Sin et al. (2006) found that *SaPIN2a* is expressed especially in ovules and young seeds. The specificity of *SnSPI2a* and *SnSPI2b* for subtilisin, which was also observed for *SaPIN2a* by Wang et al. (2007), suggests that these SPIs interact with a plant subtilase in the ovary. The constitutively high expression of SPIs in flowers can certainly serve a dual function. In addition to their involvement in seed development, SPIs might accumulate in flowers as a constitutive defense against herbivores to protect these highly fitness relevant tissues. When we dissected flowers and measured the PI activity in the different parts, we discovered that the expression of the different SPIs is tissue specific. By comparing the ratios of subtilisin and trypsin PI activity we can estimate that anthers express high amounts of *SnSPI2a* and *SnSPI2b* but very little *SnSPI2c* (Supplementary Figure S6). Gynoecia however seem to express all three SPIs in high amounts. It may be that these specific expression patterns serve yet unknown functions in the interaction with other organisms. The high PI load could limit the amount of pollen collected by pollinators or pollen robbers. Buzz pollinating bees are able to assess the quantity of pollen in a flower quickly (Buchmann and Cane, 1989) and it would be fascinating to learn if they

could also determine pollen quality as mediated by PI content. On the other hand the high PI activity in ovaries and anthers raises the question if there is a common reason for their expression in sporogenic tissues: perhaps SPIs are not only involved in the formation of embryos and seeds but also in microsporogenesis and pollen formation. In this context, it is interesting to note that a serine-protease was found to be involved in microsporogenesis in anthers of *Lilium longiflorum* (Taylor et al., 1997). Although we could not find any obvious effects on pollen viability (data not shown) it is possible that similarly subtle effects as in seed development occur.

The silencing of *SnSPI1* did not result in any phenotype and its function in *S. nigrum* remains unclear. However, although often described to be wound-induced, the evidence for PI-I to be an effective defense against herbivores is rather limited (Jongsma and Bolter, 1997; Mosolov and Valueva, 2005). Perhaps its higher specificity for chymotrypsin and subtilisin makes it effective against particular insect taxa or pathogens which were not yet investigated and which did also not occur in our field studies. We found that *SnSPI1* is preferentially expressed in flowers but also in vascular tissues which suggests a defensive function against phloem-feeding insects or another unknown function in these tissues similar to that of *SnSPI2a*. Beuning et al. (1994) mentioned that PI-I is an ancient gene family, present in monocots and bacteria, fungi and leech, which displays a lack of hypervariability at the active site [Functional divergence ratios (FDR) at the active site did not differ significantly from 1 or were < 1)]. This unexpected lack of positive Darwinian selection questions the interpretation of PI-I inhibitors as being purely defense-related, which would leave their putative primary function still to be discovered.

To summarize, we have shown that the SPIs of *S. nigrum* exhibit a certain degree of differentiation but also considerable functional overlap. The highly abundant *SnSPI2c* displays typical characteristics of a defense-related gene. The other two, *SnSPI2a* and *SnSPI2b*, show a clear overlap of defensive and developmental properties. Both are very similar to each other and perhaps they represent an early stage in the differentiation of a developmental function for *SnSPI2a* and a defensive function for *SnSPI2b*. Their specificity for subtilisin and their involvement in seed development suggests an interaction with plant subtilases in these tissues and the identification of these target proteases will be an interesting task for future research. The different responses of the herbivores exemplified how dynamic the interaction of PIs with insect proteases can be. Given that a co-evolutionary process evolving several organisms creates a variety of different outcomes, this is to be expected. Such complexity underscores the challenges that the engineering of transgenic crops expressing PIs will face. Single SPIs might be effective against particular herbivore species but might be quickly overcome. Expressing more than one PI increases the chances of inhibiting insect target proteases but also increases the risk of eliciting compensatory feeding responses or of other unintended effects on plant growth and development. The likely synergisms with other defense chemicals are important to counter adaptive responses sustainably but they also greatly complicate the engineering and application in agricultural crops. Although plant PIs have been studied for such a long

time this study shows that we have just scratched the surface of our understanding of their function and regulation. We still know little about how and to what purposes plants fine-tune PI expression and activity. Differences in promoter binding sites, alternative splicing, or variable post-translational processing depending on external stimuli or developmental programs are just a few examples of regulatory mechanisms. But we have also learned that not every SPI plays an essential role in a particular process. Being not absolutely indispensable might explain how SPIs have been able to evolve in many directions, leaving us to witness and wonder about the current situation on this evolutionary playground.

MATERIALS AND METHODS

Plant material and growth

All experiments were conducted with the *S. nigrum* L. inbred line Sn30 (Schmidt et al., 2004). Additionally we used *Solanum spp.* from the seedbank at IPK Gatersleben, Germany, for the comparison of PI profiles presented in Supplementary Figure S5. Seeds were sterilized and incubated overnight in 1 M KNO_3 at 4°C before they were germinated on Gamborg B5 plant agar in Petri dishes, as described by Schmidt et al. (2004). After 10 d seedlings were transferred to Teku trays containing a peat-based substrate ("Tonsubstrat", Klasmann, Germany) and kept in growth chambers for another 10 days (16 h light, 8 h dark, 26°C, 155 µmol m^{-2} s^{-1} PAR at shelf height). The plants were then transferred to 9 × 9 × 9.5 cm or 1 L pots containing the same substrate and put in the glasshouse of the institute (16 h light, supplemental lighting by Philips Master Sun-T PIA Agro 400 W and 600 W sodium lights, 23 to 25°C and 45 to 55% rel. humidity; 8 h dark, 19 to 23°C and 45 to 55% rel. humidity). The plants were automatically watered daily with 0.5 g/L of a combination fertilizer containing phosphate, potassium, and magnesium (Euflor, Germany) and 0.5 g/L $Ca(NO_3)_2$. The seedlings and plants used for VIGS were grown in climate chambers according to Hartl et al. (2008). For field experiments in Germany, plants were germinated and kept in Teku trays as described above, acclimatized for 3 to 5 d outdoors at the institute grounds and then planted at the field site 21 to 24 d after sowing. For the experiments at the field station in Utah, seeds were incubated in 1 M KNO_3 as described above, germinated in Jiffy pots (Alwaysgrows Greenhouse Supplies, Sandusky, OH, USA) and kept in a shade-house. After 3 weeks the plants were transferred to 2 L pots and hardened by reducing the shade until they were exposed to full sunlight.

The release of transformed plants at the field site near Dornburg, Germany, was conducted in compliance with EU and German regulations as administered by the Thüringer Landesverwaltungsamt and the Thüringer Landesamt für Lebensmittelsicherheit und Verbraucherschutz (release application nos. 6786–01–0156 and 6786-01-0187 [ir*SPI2a+b*] and 6786–01–0189 [ir*SPI1/2ab*]). The release of transgenic plants at the Lytle Ranch Preserve, Santa Clara, UT, USA, was conducted under APHIS notification number 06-003-07n and the import of the seeds under 06-003-03n.

In both locations all plants were monitored daily during the whole experiment and all flower buds of each genotype were removed before opening. After the experiments, the plants where harvested, including roots, and autoclaved or incinerated.

Plant treatments

To mimic herbivore feeding we punctured the youngest fully developed leaf with a fabric pattern wheel, creating four rows on each side of the midvein. Immediately after wounding we applied 30 µL of *M. sexta* regurgitate (diluted 1:5 with dH_2O) with a pipette. The regurgitate was collected from third- to fourth-instar larvae feeding on *S. nigrum* WT plants and centrifuged after collection to remove cell debris.

MeJA in lanolin paste was applied with a spatula either to the stem at the first internode (150 µg in 20 µL; Figs. 2B, 7B, 8, and 9) or adaxially across the base of the leaf lamina (75 µg in 10 µL; Figs. 1, 2A, 2D, 3, and 5). Pure lanolin served as a control. To assess a possible response of SPIs to ethylene and salicylic acid, we applied 50 µL of the ethylene releasing compound ethephone (50 µM in 0.05% Tween 20) or 1 mM salicylic acid to leaves after mechanical wounding, as described above. All the chemicals used for elicitations were purchased from Sigma-Aldrich, Germany.

Treatments were performed between 10 and 11 am. The leaves were harvested without the midrib and, as all other tissues, frozen immediately in liquid N_2. For all experiments 4- to 5-week old plants were used, except for the comparison of tissue specificity (Figs. 2B, 6B). In this experiment we harvested young leaves (top leaves of the primary shoot axis and of the two oldest side branches) and mature leaves (leaves at node 5 to 7 counted from the cotyledonary node) 5 weeks after sowing and 2d after treatment with MeJA in lanolin paste or pure lanolin at the stem. After eleven weeks another set of plants was treated with MeJA or lanolin and two days later mature buds, open flowers, and fruits were harvested. For stems we selected a medium-aged portion spanning two internodes at the main branch. Tissues from 9 plants per treatment and time-point were collected and the material from three plants was pooled to obtain three biological replicates. All other replicate numbers, as mentioned throughout the text, represent individual plants, separate for each time-point or treatment, except for the microarray experiment described below.

Microarrays

The experimental design, hybridization, and data analysis of the TIGR microarrays were described in detail by (Schmidt et al., 2005). To summarize shortly, we used twelve plants for each of the four treatment groups: *M. sexta* feeding, MeJA, and their respective controls. Ten freshly hatched *M. sexta* larvae were placed on two leaves and allowed to feed for 24 h. MeJA treatments were performed on a single leaf as described above. Untreated or lanolin treated leaves, respectively, served as controls. We extracted the total RNA individually for each plant and pooled equal amounts of RNA, from four plants each, to obtain three independent replicates. mRNA extraction, cDNA synthesis, differential

labeling, hybridization, scanning, raw data acquisition and LOWESS normalization were performed by TIGR Expression Profiling Service as described on their website (http://jcvi.org/potato/sol_ma_protocols.shtml). We considered a transcript as being differentially regulated when the following criteria were fullfilled: (1) The expression ratio (ER) was significantly different from 1 as determined by a Student's t test ($P < 0.05$); and (2) the ER exceeded the thresholds of either < 0.67-fold or > 1.5-fold for down- and up-regulation, respectively. For this manuscript we selected all clones which were annotated as PIs. The experiment followed MIAME guidelines and the raw and processed data are freely available online.

The custom microarray contains 1421 50-mer oligonucleotides of herbivory-related genes from different solanaceous species, selected according to their regulation patterns in previous studies (Halitschke et al., 2003; Voelckel and Baldwin, 2003; Schmidt et al., 2005). Each oligomer was spotted twice in pairs onto an epoxy-coated glass slide (Quantifoil Microtools, Jena, Germany), resulting in four spots per clone and a total of 5684 spots per microarray. For the MeJA-treatment we used the same six pooled samples as for the TIGR microarray. Samples induced by the natural herbivore *E. pubescens* were collected during a field experiment planted on July 15, 2005, in Dornburg, Germany. 40 3-week-old WT plants were planted in the field plot in four rows of 10 plants each and alternate rows were sprayed with a pyrethroid insecticide (0.05 % FASTAC SC, BASF, Germany) immediately after planting. We observed high numbers of *E. pubescens* on July 16. One day later we harvested infested leaves from 12 plants and corresponding leaves from the neighboring un-infested, insecticide-treated plants. We extracted RNA individually for each plant following the TRIzol® protocol (Invitrogen, CA, USA), and again pooled equal amounts of RNA from four plants each to obtain three independent replicates. mRNA isolation, differential labeling (Cy3 for treatments, Cy5 for controls), hybridization, and scanning were performed as described by Halitschke et al. (2003). Spot intensities (SIs) were extracted from image files using the software AIDA (Raytest, Germany). SIs were cleared of noise by subtracting the local-background (lBg) and lBg-subtracted SIs below 2 × lBg were considered below the detection level, discarded, and set to 0 (signal-to-noise cut off). The raw data was subsequently normalized via LOWESS using the software MIDAS (Saeed et al., 2003) to correct for dye bias. Prior to statistical analyses, 0.01 was added to each normalized SI after which they were $\log_2$-transformed. We performed a nested ANOVA to identify significantly regulated clones across the three replicates, as determined by an average $\log_2$-ratio > 0.585 or < -0.585 (equivalent to 1.5-fold up- or 0.65-fold down-regulation). We adjusted the P-value < 0.05 using FDR, according to the Benjamini and Hochberg step-up procedure for controlling the false discovery rate (Benjamini and Hochberg, 1995). ERs reported throughout this manuscript are the $\log_2$-transformed ratios (except ERs in the Supplementary Table S2 which show anti-$\log_2$ back-transformed ratios). Gene annotations, raw and processed data are presented in Supplementary Table S2.

Cloning of SPI genes and generation of transgenic lines

We used primers designed on sequences from other solanaceous species to clone fragments of *SnSPI1a*, *SnSPI1b*, *SnSPI2a* and *SnSPI2c* by standard PCR and 3'RACE (Scotto-Lavino et al., 2007) from cDNA and gDNA (all primer sequences are listed in Supplementary Table S8). A fragment of *SnSPI2b* was cloned into the vector pSOL3, described by Bubner et al. (2006), to generate the inverted repeat construct pSOL3PIS for silencing *SnSPI2a* and *SnSPI2b* (ir*SPI2a+b*). Similarly we combined fragments of *SnSPI1a* and *SnSPI2b* to form the plasmid pSOL3PIN12, silencing *SnSPI1*, *SnSPI2a*, and *SnSPI2b* simultaneously (ir*SPI1/2ab*). *Agrobacterium*-mediated plant transformation was carried out as described before (Krügel et al., 2002; Schmidt et al., 2004). T_1 plants were screened on hygromycin-containing media and homozygous lines were selected in T_2, based on segregation analysis. For each construct we characterized two independently transformed, homozygous lines, which harbored a single transgenic insert (as determined by Southern blot), and which displayed reduced transcript levels (ir*SPI2a+b*: line 1, S03-111-3; line 2, S03-114-1. ir*SPI1/2ab*: line 1, S06-336-8; line 2, S06-356-10).

Southern blots

We isolated genomic DNA from *S. nigrum* seedlings and leaves using a modified CTAB procedure (Bubner et al., 2004). The gDNA was digested with BamHI, EcoRI, and EcoRV to assess the native SPI copy numbers and with EcoRV to determine the number of transgenic inserts. The plasmid pSOL3PIS was digested with XhoI and used as a positive control on the blot containing the transgenic lines. We separated 30 µg DNA by 0.8% agarose gel electrophoresis and blotted it onto a nylon-membrane (Genescreen Plus, Perkin Elmer, USA) according to Brown et al. (1999). The blots were hybridized with ^{32}P-labeled probes (Rediprime II kit, GE Healthcare) coding for the hygromycin-resistance gene *hptII* or specifically for *SnSPI1*, *SnSPI2a*, and *SnSPI2b* (Brown, 1993; for primers used to generate probes see Supplementary Table S8). The specificity of the three SPI probes was determined by hybridization to slot blots of three plasmids containing corresponding regions of the three genes (Brown, 1999; Halitschke and Baldwin, 2003; Supplementary Fig. S1).

Virus-induced gene silencing (VIGS)

We amplified a fragment of *SnSPI2c* (for primers see Supplementary Table S8) and ligated it into the vector pYL156 (Liu et al., 2002) to obtain the vector pTRVPIN2c (= v*SPI2c*). *Agrobacterium* cultivation, vacuum-infiltration, and plant cultivation were done as described previously (Hartl et al., 2008).

Quantitative real-time PCR (QPCR)

Total RNA was extracted from flash-frozen and ground plant material following the TRIzol® protocol. Reverse transcription of 500 ng of total RNA was performed using SuperScript II Reverse

Transcriptase (Invitrogen, Germany) and a poly-T primer to obtain cDNA. Twenty nanograms of cDNA were applied to 20 µl SYBR Green reactions (qPCR Core Kit for SYBR Green I; Eurogentec, Belgium), which were run on an ABI PRISM 7700 sequence detection system (Applied Biosystems, CA, USA; cycler conditions: 10 min at 95°C, 40 cycles of 30 s at 95°C and 30 s at 60°C). Each plate was run with a standard curve, no template control, and pools of RNA samples to check for DNA contaminations. Primers were designed using Primer 3 v.0.4.0 (Rozen and Skaletsky, 2000), checked for specificity using a melting curve analysis (ABI PRISM 7700 Dissociation Curve Software) and are summarized in the Supplementary Material, Table S8. For data analysis we calculated relative transcript abundance by efficiency-correcting for each primer pair and normalizing to the *S. nigrum* elongation factor EF1α (*SnEF1α*) according to Pfaffl et al. (2002).

PI activity assays

The samples were ground in liquid N_2 and extracted with 2 mL cold extraction buffer (Jongsma et al., 1993) per g plant tissue by vortexing for 5 min. After repeated centrifugation and transfer of the supernatant we determined the total protein concentration with the Bradford method (Protein Assay, Bio-Rad, Germany) using bovine serum albumin (Sigma-Aldrich) as a standard. SPI activity was determined by a radial immunodiffusion assay (Jongsma et al., 1993; van Dam et al., 2001) using bovine trypsin, bovine α-chymotrypsin type II, and Subtilisin Carlsberg type VIII (all from Sigma-Aldrich). If samples from the same type of tissue were compared the concentration of active PIs was expressed relative to mg total protein, if different tissues were compared relative to g fresh mass.

Native PAGE and gel-x-ray-film contact prints (GXCP)

The same extraction procedure as for the PI activity assays was used for native PAGE. We loaded equal amounts (150 µg) of total protein to 12% native vertical slab gels (Hoefer, USA) in a discontinous buffer system, run at 30 mA constant current. After electrophoresis
the gels were processed for activity visualization using the gel-xray-film contact print method (GXCP; Pichare and Kachole, 1994). We washed the gels 2 x 15 min in 0.1 M Tris-HCl buffer, pH 7.8, followed by incubation in 0.1 mg/mL bovine trypsin in the same buffer for 15 min at RT on a rotary shaker. The gels were subsequently washed 3 x 10 min in the same buffer and then placed on x-ray film, coated with gelatin. We repeated the exposures with new film and increasing exposure times from 2 - 20 min. After exposure the films were immediately rinsed in warm tap water until the hydrolyzed gelatin was washed off and bands appeared as un-hydrolyzed gelatin against the background. We photographed the dried films under a strong uniform light source, with bands reflecting light differently than the remaining area. The pictures were converted to grayscale, inverted, and contrast and brightness were adjusted equally for the whole picture, using Adobe Photoshop CS.

SPI2c purification and MS-MS de-novo sequencing

For the isolation of SnSPI2c we collected 50 g of flowers from WT plants, froze and ground them in liquid N_2 and extracted them with 250 mL PI extraction buffer by stirring for 1 h on ice. After repeated centrifugation (10000 x g, 30 min) to clear the extract from cell debris we incubated the supernatant for 30 min at 60°C in a water bath, and centrifuged again to precipitate the denatured proteins. The supernatant was dialyzed against dH_2O at 4°C overnight (Spectra/Por 7, 1 kD MWCO; Spectrum Europe, Netherlands) and then freeze-dried. We took up the remaining protein in 10 mL of dH_2O and ultra-filtrated the solution through centrifugation columns with a 30 kD MWCO (Vivaspin 15, Sartorius, Germany). The filtrate and the concentrate were tested for TPI activity and analyzed using native PAGE followed by GXCP. Since the filtrate contained the unknown bands and showed high acitivity and less contamination by other proteins, we continued with this fraction. After freeze-drying we dissolved the sample in 150 mM KCl, 10 mM Tris-HCl buffer, pH 8, incubated it with trypsin affinity agarose (Sigma-Aldrich) on a rotary shaker for 30 min at RT, and then loaded the slurry on a gravity column. After washing with 10 vol. of incubation buffer we eluted with 5 vol. of 7 M urea, pH 3, and neutralized the eluate immediately with 1 M Tris-HCl, pH 8. The eluate was again dialyzed, freeze dried, dissolved in dH_2O, and concentrated in a vacuum concentrator (Eppendorf, Germany) before electrophoresis. The sample was loaded together with aliquots of the purification steps on a 12% discontinuous native PAGE. The gel was stained with Coomassie Blue (BioSafe Coomassie, Bio-Rad, Germany) except for one lane containing raw extract which was subjected to GXCP (Fig. 4). The stained gel was dried on filter paper and sent for MS-MS *de novo* sequencing.

The sample preparation for ESI-MS and the measurements were conducted by OMX Gmbh, Munich, Germany, using the following protocol. All organic solvents and chemicals used for sample preparation and ESI-MS were of p.a. grade. Formic acid was purchased from Baker (NJ, USA). DTT and iodoacetamide were obtained from Sigma-Aldrich, trypsin (NB sequencing grade modified trypsin, Cat.-No. 37283) from Serva (Germany).

An aliquot of each gel spot was crushed into small particles and destained using 50 mM ammonium bicarbonate in 50% ACN. The samples were reduced with 10 mM DTT for 45 min at 56°C and SH-groups were subsequently alkylated with 55 mM iodoacetamide. Gel particles were dehydrated in acetonitrile, swelled in digestion-buffer containing 12.5 ng/µL trypsin in 50 mM ammonium bicarbonate, and incubated for two hours at 50°C. Peptides were extracted into the digestion buffer during the incubation time. The supernatant was collected, acidified with a final concentration of 1% formic acid and stored at 8°C for 24 hours maximum.

Static nanoESI-MS was performed on a Q-TOF Premier tandem mass spectrometer (Waters, Manchester, UK) as described before with minor modifications (Granvogl et al., 2007). Peptide MS-spectra were scanned in a mass range of 400-1500 m/z. Corresponding data were collected at a scan rate of 2 s. For external calibration, the fragmentation pattern from 500 fmol/µl [Glu1]-Fibrinopeptide B (Sigma-Aldrich) in 50% ACN and 0.1% FA was used. The signal at 421.759, which resulted from

autolysis of trypsin was used for internal calibration of MS spectra and to monitor enzyme activity. Fragment ion spectra were recorded at a scan time of 3 s for at least 1 min or until a fragmentation pattern appeared which was adequate for *de-novo* sequencing of peptides. Manual interpretation of MS-MS spectra was assisted by signal deconvolution using the MaxEnt3 algorithm of the MassLynx 4.1 software.

Herbivore performance in the field

The field site in Germany was situated near Dornburg on a former agricultural field. We planted 20 pairs of WT plants and ir*SPI2a+b* line 1 in each of the two experiments (June 6 and July 15 2005). The plants were watered when necessary and the flower buds were removed on all genotypes before opening. We quantified leaf damage as described by Schmidt and Baldwin (2006a). To summarize shortly, the herbivore damage of each individual leaf was categorized according to damage classes and then a mean damage level was calculated for each plant. After arcsine-transformation the data were tested with a paired t-test.

The experiments in the USA were carried out at the field station in the Lytle Ranch Preserve, Utah. The 5-week-old, potted wild-type plants and ir*SPI2a+b* line 1 were transferred to three mesh tents. The plants were induced with MeJA 3d before the experiment (13 plants per genotype) or were left untreated (6 plants per genotype); both treatments and genotypes were evenly distributed to the three tents. We collected 175 beetles (*Epitrix spp*., Chrysomelidae), 75 leafhoppers (*Empoasca spp*., Cicadellidae), and 57 grasshoppers (*Trimerotropis sp*., Acrididae), all species that we previously observed to feed on *S. nigrum*. We distributed them evenly over the three tents, allowing them to feed for five days. Since the overall percent damage levels were low, we determined the percentage of damaged leaves per plant. The two genotypes were compared separately for control and MeJA-treated plants with t-tests after arcsine-transformation.

Herbivory performance in the glasshouse

M. sexta on stably silenced plants (Fig. 8A, B, C): *M. sexta* eggs were obtained from our in-house culture. Freshly hatched larvae were reared on un-induced leaf tissue, separated by genotypes in boxes (three boxes per genotype). The leaf material was exchanged daily and after 3 d 14 to 20 larvae per genotype and treatment were weighed and separated in individual containers containing two leaf discs (25 mm), from either untreated or MeJA-induced plants (treated on day of hatching), on moist filter paper. Four leaf discs were cut from a single fully expanded leaf, supplying two caterpillars with material from one plant (10 plants per genotype and treatment). The boxes were randomized and kept at 23 to 25°C in a shaded area in the glasshouse. Leaf discs were exchanged daily and the larval mass was recorded on days 4, 5, and 7. The leaf discs from day 4 were scanned on day 5 and the consumed leaf area determined using ImageJ 1.38 (http://rsb.info.nih.gov/ij/index.html). As a measure of consumption we related the consumed leaf area to larval mass at day 4. To estimate food conversion

efficiency the mass gain between day 4 and 5 was related to the leaf area consumed in this period. ir*SPI1/2ab* line 2 was not included in the setup with un-induced material to make the experiment more manageable. The experiment was repeated three times with the ir*SPI2a+b* lines, also allowing larvae to feed directly on the plants, with similar results. The statistical analysis (ANOVA and repeated measures ANOVA) is explained in detail in Supplementary Tables S3 and S4.

M. sexta on VIGS-silenced plants (Fig. 9A): the experiment was carried out as above but with 30 caterpillars per genotype and VIGS vector. All larvae were transferred to MeJA-induced material in individual boxes on day 4. The experiment was repeated two times with similar results. For statistical analysis see Supplementary Table S5.

S. exigua performance on stably silenced lines (Fig. 8D): neonates, freshly hatched from eggs supplied by the Plant Protection Center of Bayer AG (Monheim, Germany), were reared communally in boxes on un-induced leaf tissue at RT, as described above. After 3d we transferred 21 larvae per genotype and treatment to individual containers (3 larvae per plant replicate). From day 6 on half of the caterpillars were fed on MeJA-induced leaf material and after 9 d they reached a size which allowed weighing. ir*SPI2a+b* line 2 and ir*SPI1/2ab* line 2 were not included in the setup with un-induced material to make the experiment more manageable. For statistical analysis see Supplementary Table S3.

S. littoralis performance on VIGS-silenced plants (Fig. 9B-E): Eggs were obtained from the Plant Protection Center of Bayer AG, Germany. The experiments were carried out as with *M. sexta*, transferring 12 larvae per genotype and vector to individual boxes on day 6, but only supplying them with un-induced tissue throughout the experiment (Fig. 9B). The experiment was repeated two times with similar results. To measure the consumption early in the larval development we repeated the experiment with larvae feeding only on WT plants inoculated with CV or v*SPI2c* (Fig.9C-E). The larvae were weighed after hatching and then transferred to individual containers containing leaf discs. Larval mass was recorded on alternate days using an analytical balance with a readability of 0.01 mg and the consumed leaf area was determined as described above. For statistical analysis see Supplementary Tables S6 and S7.

Statistics

The software package SPSS 17.0 for Windows (SPSS Inc., IL, USA) was used for all statistical analyses if appropriate. For the linear mixed-effects model fitted to the *M. sexta* performance data (Fig. 9A) we used R 2.9.0 in combination with the package lme4 (R Development Core Team; http://www.R-project.org). If necessary, data were transformed to meet the assumption of homogeneity of variances, as specified for each test. Further details of the tests and applied models are described in the figure legends and in the supplementary material.

ACCESSION NUMBERS

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers GU133368 (*SnSPI1a*), GU133369 (*SnSPI1b*), GU133370 (*SnSPI2a,* gDNA), GU133371 (*SnSPI2a* mRNA), GU133372 (*SnSPI2c-R2*), and GU133373 (*SnSPI2c-R3*).

Microarray data from the TIGR potato cDNA array is available online under the accession ID 53 at the TIGR Solanaceae Genomics Resource hosted by JCVI (http://jcvi.org/potato/sol_expression.shtml).

ACKNOWLEDGEMENTS

We thank H. Merker, A. Prehl, S. Lindermann, and A. Hackett for assistance in the lab and in the field, K. Gase, A. Wissgott, W. Kröber, and S. Kutschbach for the design and generation of silencing constructs and for plant transformation, D. Kessler, A. Berg, and D. Rosenberger for insect culture, J. Kellmann and K. Groten for managing the legal and practical obstacles to conducting field experiments with GMOs in Germany, Brigham Young University for the use of the Lytle Ranch Preserve field station, German and US authorities for constructive regulatory oversight, and the Max Planck Society for funding.

SUPPLEMENTAL DATA

The following materials are available in the online version of this article:

Supplementary Table S1. Annotated list of all PI clones, spotted on the TIGR microarray, and their expression ratio.

Supplementary Table S2. Expression ratios, gene annotations, and raw and processed data of the custom microarrays.

Supplementary Table S3. Statistical analysis for data in Figures 8A and 8D

Supplementary Table S4. Statistical analysis for data in Figures 8B and 8C

Supplementary Table S5. Statistical analysis for data in Figure 9A

Supplementary Table S6. Statistical analysis for data in Figure 9B

Supplementary Table S7. Statistical analysis for data in Figure 9C

Supplementary Table S8. Primer sequences.

Supplementary Figure S1.Copy number of SPI genes in *S. nigrum*.

Supplementary Figure S2. Number of T-DNA inserts in the genomes of the transgenic lines.

Supplementary Figure S3. Plant height, flower diameter, and number of viable and non-viable seeds in WT and SPI-silenced *S. nigrum* plants.

Supplementary Figure S4. Multiple sequence alignment (Clustal W) of translated cDNA sequences from all three SnSPI2 genes containing two sequence repeats.

Supplementary Figure S5. TPI profiles of different *Solanum spp.* using GXCP.

Supplementary Figure S6. Trypsin and subtilisin PI activity in different flower tissues.

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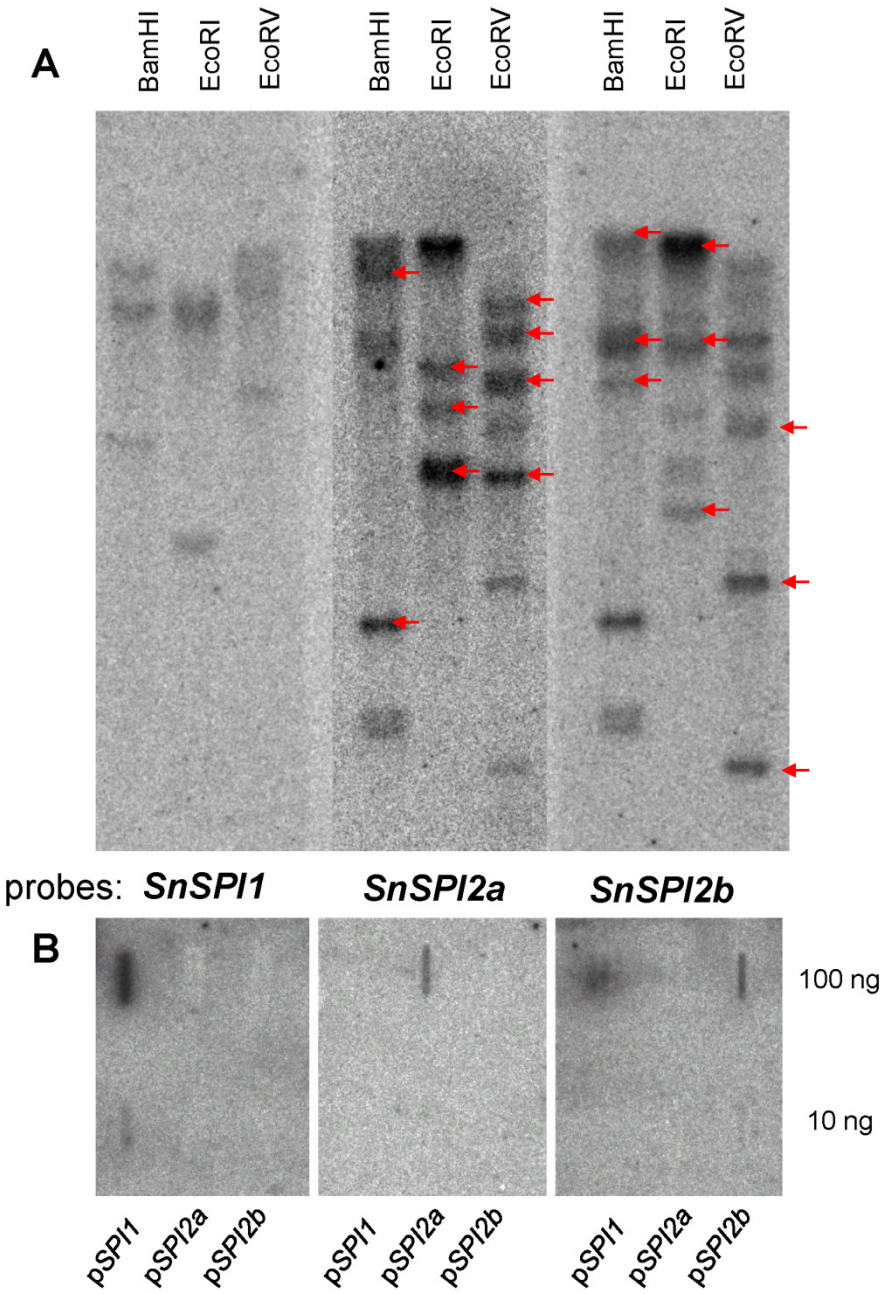
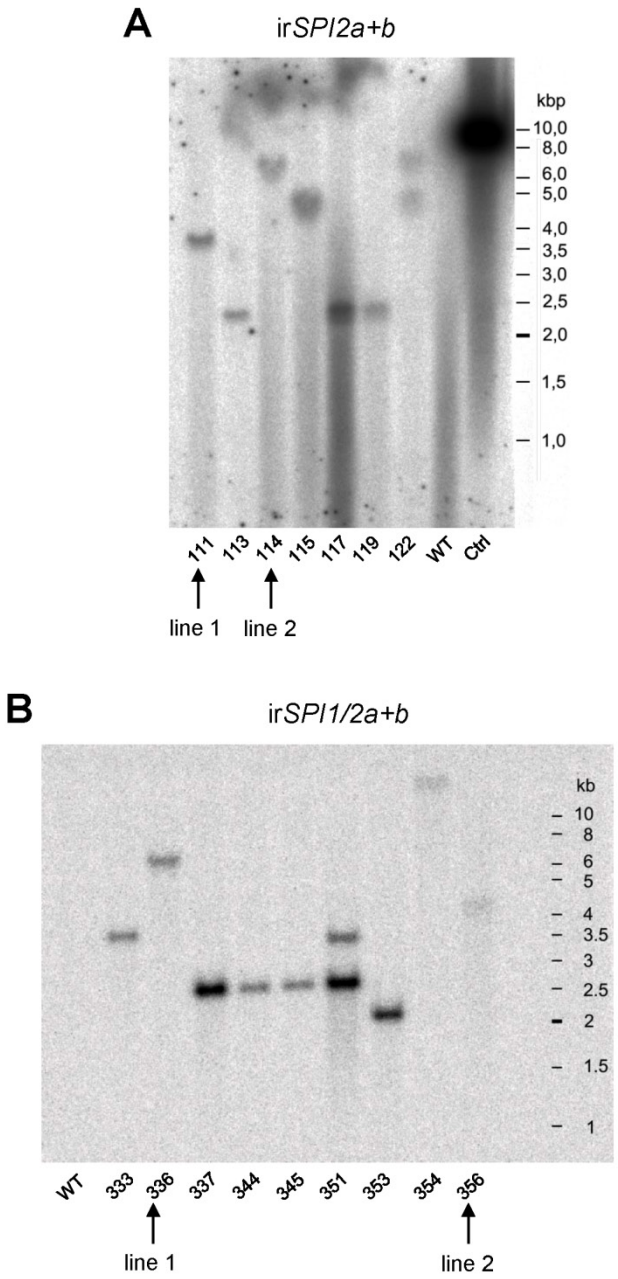
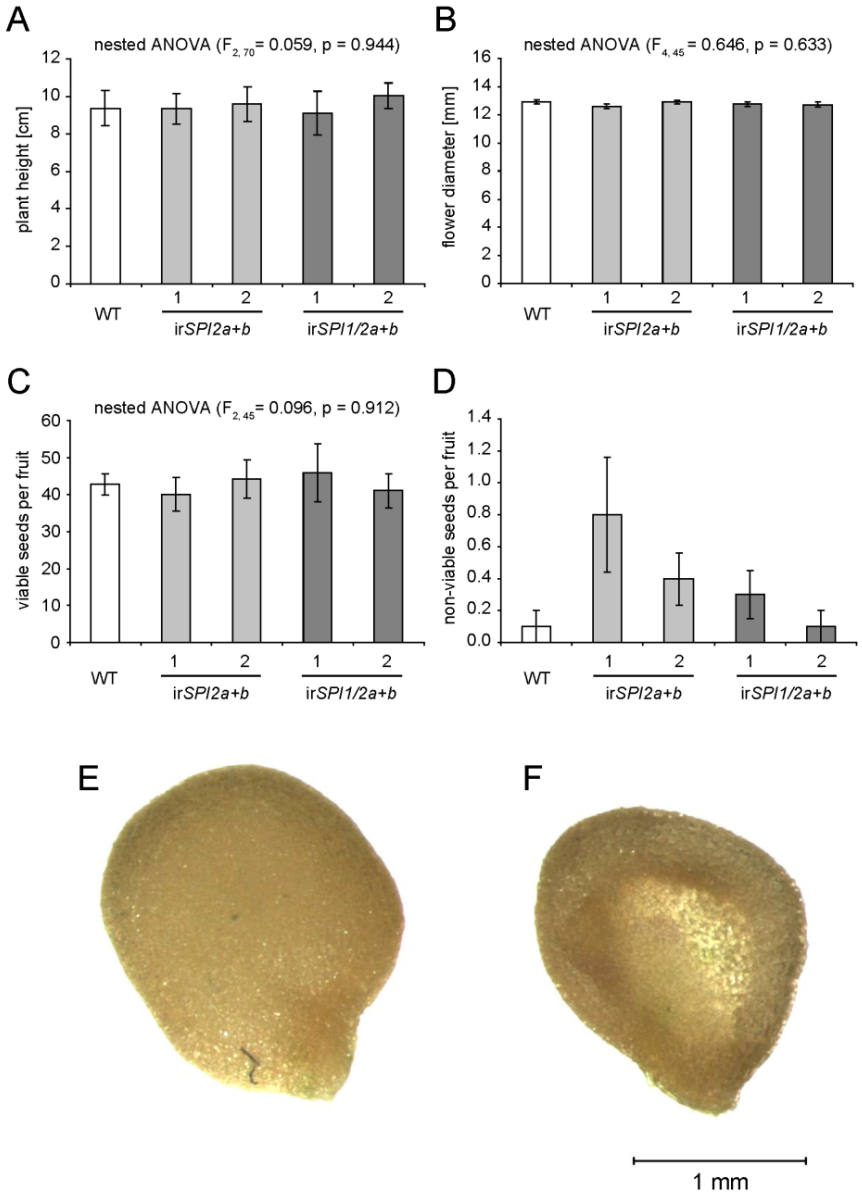


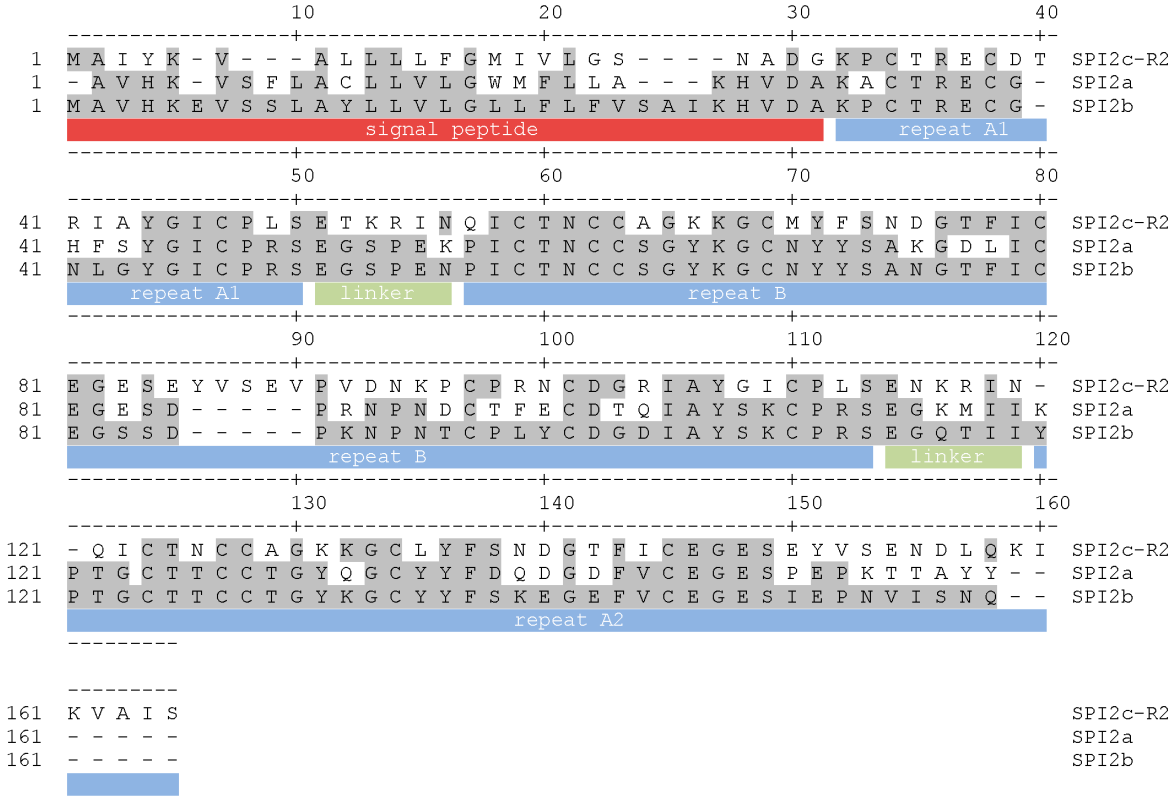
Supplementary Figure S1. Copy number of SPI genes in *S. nigrum*. A, Southern blot of genomic DNA digested with the enzymes BamHI, EcoRI, and EcoRV and hybridized with probes specific for *SnSPI1*(a+b), *SnSPI2a*, and *SnSPI2b*. Red arrows indicate signals which are stronger either on the blot hybridized with *SnSPI2a* or with *SnSPI2b.* B, specificity of probes. A slot blot of plasmids containing fragments of *SnSPI1a*, *SnSPI2a*, and *SnSPI2b* (p*SPI1*, p*SPI2a*, p*SPI2b*) was hybridized with the same probes as in A. Two concentrations per plasmid were blotted (10 and100 ng).



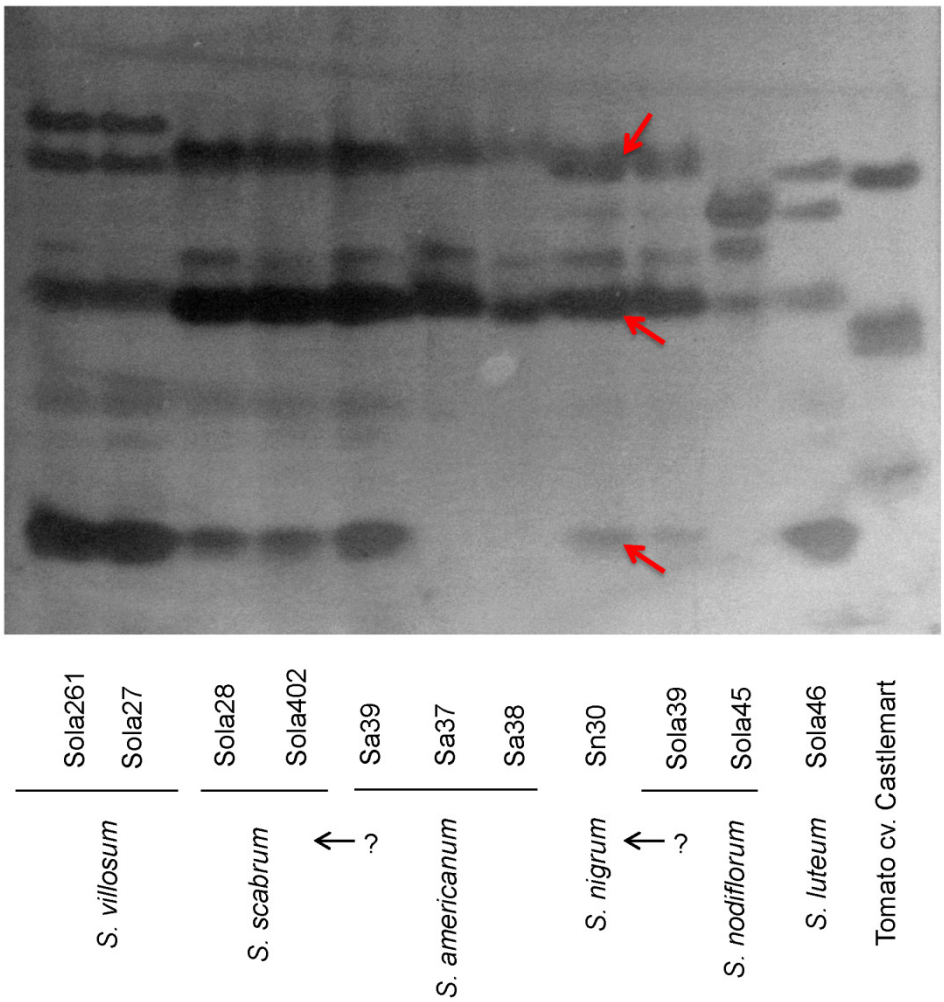
Supplementary Figure S2. Number of RNAi constructs inserted in the genomes of the transgenic lines. A, Southern blot of genomic DNA from ir*SPI2a+b* line 1 and 2, hybridized with a probe coding for the hygromycin resistance gene. B, Southern blot of genomic DNA from ir*SPI1/2a+b* line 1 and 2, hybridized with the same probe as in A. Arrows indicate the lines selected for further experiments.



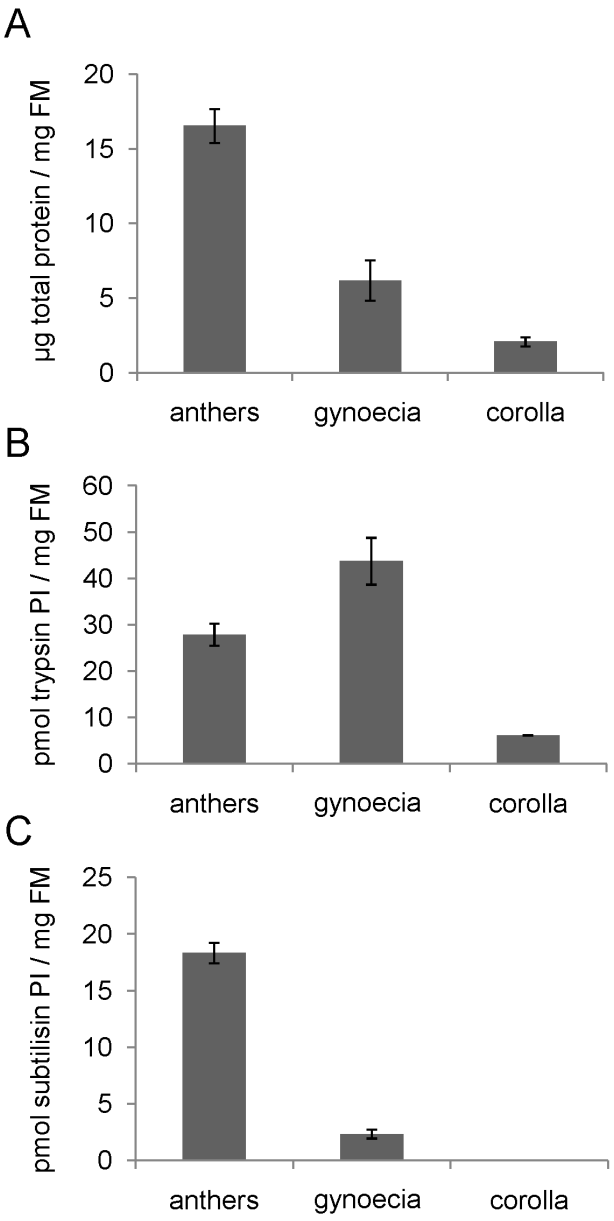
Supplementary Figure S3. Plant height (A), flower diameter (B), and number of viable and non-viable seeds (C, D) in wild-type and SPI-silenced *S. nigrum* plants. A, mean ± SE plant height of 4-wk-old plants measured from the cotyledonar node to the apex (n = 15). B, mean ± SE flower diameter measured between the tips of the two most distant petals (10 plants per genotype, 3 flower replicates per plant). Nested univariate ANOVA-model: diameter ~ genotype flower-replicate (genotype). C, D mean ± SE number of viable (C) and non-viable (D) seeds per berry (n = 10). E, viable wild-type seed. F, non-viable ir*SPI2a+b* seed (line 1).



Supplemental Figure S4. Multiple sequence alignment (Clustal W) of translated cDNA sequences from all three *SnSPI2* genes containing two sequence repeats. *SnSPI2c-R3*, which contains three repeats with 97% similarity to *SnSPI2-R2*, was not included to facilitate the illustration. Residues that match the sequence of *SnSPI2b* are shaded in gray. Colors show the putative functional units of the proteins as derived from a comparison with other PI-II inhibitors in public databases and literature (Barta et al., 2002; Horn et al., 2005; Kong & Ranganathan, 2008).



Supplementary Figure S5. Native PAGE (12%) of extracts from different *Solanum spp.* visualized with GXCP for TPI activity. The red arrow indicates the location of the SPI2c-type bands in *S. nigrum*. All seeds except *S. nigrum* and tomato were obtained from the seedbank of IPK Gatersleben, Germany, and the corresponding accession numbers are given in the figure. The taxonomic situation of the black nightshades is difficult and for this reason the species identities are not completely certain. For example Sa39 is listed in the database as *S. americanum* but when compared morphologically and in the PI profile it resembles rather *S. scabrum*. Similarly Sola39 is classified as *S. nodiflorum* but it is more likely to be *S. nigrum*. This putatively correct taxonomic classification is indicated by black arrows.



Supplementary Figure S6. Mean ±SE (n = 3) total protein concentration (A), trypsin PI activity (B), and subtilisin PI activity(C) in flower tissues of *S. nigrum* wild-type plants. FM: fresh mass.

Supplementary Table S1. Annotated list of all PI clones, spotted on the TIGR microarray, and their expression ratio (ER) in *S. nigrum* in response to *M. sexta* herbivory and treatment with methyl jasmonate. The mean 2log ER was calculated from three microarrays (each representing three independent biological replicates) where every clone was present twice on each microarray. 'UP' or 'DOWN' indicate significantly up- or down-regulated genes (> 1.5 or < 0.67, t-test: $p < 0.05$). PI-I: potato inhibitor I; PI-II: potato inhibitor II; CPI: cysteine PI; MCPI: metallocarboxypeptidase inhibitor; API: aspartate PI; KPI: Kunitz PI; Thio: thionin-like PI.

Clone ID	PI family	Annotation	*M. sexta* mean $\log_2$ ER	significantly regulated	MeJA mean $\log_2$ ER	significantly regulated
STMCK27	PI-I	(Q43648) Proteinase inhibitor I	1.27	**UP**	0.68	ns
STMCO50	PI-I	(Q43651) Proteinase inhibitor I (Fragment)	1.29	ns	1.12	ns
STMGY25	PI-I	Proteinase inhibitor I precursor	2.10	**UP**	0.84	**UP**
STMIU61	PI-I	(Q43651) Proteinase inhibitor I (Fragment)	1.92	**UP**	0.91	**UP**
STMCR35	PI-II	serine proteinase inhibitor - potato (fragment)	0.01	ns	-0.11	ns
STMCC57	PI-II	Proteinase inhibitor type II CEVI57 precursor, Lycopersicon esculentum	0.08	ns	0.74	ns
STMJA26	PI-II	Proteinase inhibitor type II CEVI57 precursor, Lycopersicon esculentum	0.21	ns	-0.04	ns
STMCP16	PI-II	Proteinase inhibitor type II TR8 precursor, Lycopersicon esculentum	-0.17	ns	-0.17	ns
STMIY57	PI-II	Proteinase inhibitor type II TR8 precursor, Lycopersicon esculentum	0.26	ns	-0.15	ns
STMCX74	PI-II	(Q8W2K6) Proteinase inhibitor IIa	0.26	ns	0.84	ns
STMCL26	CPI	Cysteine protease inhibitor 1 precursor (PCPI 8.3)	-0.19	ns	0.06	ns
STMCV60	CPI	Cysteine protease inhibitor 1 precursor (PCPI 8.3)	0.38	ns	-0.14	ns
STMCX33	CPI	Cysteine protease inhibitor 1 precursor (PCPI 8.3)	-0.36	ns	0.03	ns
STMHQ67	CPI	Cysteine protease inhibitor 1 precursor (PCPI 8.3)	-0.14	ns	-0.03	ns
STMCG57	MCPI	metallocarboxypeptidase inhibitor IIa precursor	-0.29	ns	0.53	ns
STMJP33	MCPI	metallocarboxypeptidase inhibitor IIa precursor	-0.75	**DOWN**	0.19	ns
STMGH73	MCPI	probable metallocarboxypeptidase inhibitor PFT3	-0.34	ns	0.26	ns
STMHJ22	MCPI	probable metallocarboxypeptidase inhibitor PFT3	-0.17	ns	0.12	ns
STMGL75	MCPI	(O24372) Metallocarboxypeptidase inhibitor	-0.81	ns	0.14	ns
STMCQ55	API	Aspartic protease inhibitor 10; wound-induced cathepsin D inhibitor	NA	na	0.27	ns
STMHV19	KPI	(Q8L5X6) Putative kunitz-type proteinase inhibitor	0.46	ns	0.03	ns
STMCM05	Thio	Probable protease inhibitor P322 precursor.	NA	na	0.15	ns

Supplementary Table S3. Repeated measures ANOVA to detect effects of stably silencing SPIs, of methyl jasmonate elicitation, and of their interaction on larval mass of *Manduca sexta* (a) and *Spodoptera exigua* (b, c). We defined 'genotype' (WT, ir*SPI2a+b*, ir*SPI1/2a+b*), 'line' nested within 'genotype', and 'treatment' (control or MeJA) as factors in the model: genotype line(genotype) treatment genotype*treatment (a, b). For *S. exigua* feeding on un-induced tissue only one line per plant genotype was used. This reduced the power to detect a genotype-specific effect in the complete model. Thus we computed a second ANOVA for larvae feeding on un-induced material only (c). Data were log-transformed to improve homogeneity of variances. Mauchly's test indicated a violation of the assumption of sphericity ($\chi^2_{(5)} = 20.43$ (a), $\chi^2_{(5)} = 72.99$ (b), $\chi^2_{(5)} = 30.16$ (c), $p < 0.05$). Therefore Greenhouse-Geisser- ($\varepsilon = 0.911$, (a)) or Huyn-Feld-estimates ($\varepsilon = 0.728$ (b), $\varepsilon = 0.724$ (c)) were used to correct the degrees of freedom. (SS, sum of squares type IV; df, degrees of freedom; MS, mean squares; F- and p-value; s indicates significant differences $p < 0.05$)

	SS	df	MS	F	P	
(a) *Manduca sexta* larval mass						
Within-Subjects Effects						
day	29.74	2.73	10.88	1339.74	0.000	s
day * genotype	0.08	5.47	0.01	1.70	0.127	
day * line (genotype)	0.09	5.47	0.02	1.97	0.076	
day * treatment	0.56	2.73	0.21	25.37	0.000	s
day * genotype * treatment	0.02	5.47	0.00	0.34	0.901	
Error(day)	2.71	333.35	0.01			
Between-Subjects Effects						
Intercept	1161.21	1	1161.21	20484.15	0.000	
genotype	0.02	2	0.01	0.20	0.822	
line (genotype)	0.20	2	0.10	1.79	0.172	
treatment	1.31	1	1.31	23.12	0.000	s
genotype * treatment	0.04	2	0.02	0.31	0.736	
Error	6.92	122	0.06			
(b) *Spodoptera exigua* larval mass						
Within-Subjects Effects						
day	16.72	2.06	8.14	279.54	0.000	s
day * genotype	0.20	4.11	0.05	1.69	0.152	
day * line (genotype)	0.05	4.11	0.01	.45	0.774	
day * treatment	0.39	2.06	0.19	6.53	0.002	s
day * genotype * treatment	0.21	4.11	0.05	1.76	0.137	
Error(day)	4.85	166.47	0.03			
Between-Subjects Effects						
Intercept	40.70	1	40.70	223.58	0.000	
genotype	1.04	2	0.52	2.84	0.064	
line (genotype)	0.70	2	0.35	1.92	0.153	
treatment	2.35	1	2.35	12.93	0.001	s
genotype * treatment	0.38	2	0.19	1.04	0.360	
Error	14.75	81	0.18			
(c) *Spodoptera exigua* larval mass, un-induced plants only						
Within-Subjects Effects						
day	16.54	2.17	7.61	267.58	0.000	s
day * genotype	0.21	4.35	0.05	1.70	0.152	
Error(day)	2.53	89.10	0.03			
Between-Subjects Effects						
Intercept	49.24	1	49.24	257.16	0.000	
genotype	1.60	2	0.80	4.17	0.022	s
Error	7.85	41	0.19			

Supplementary Table S4. General linear mixed-effects model to detect effects of stably silencing SPIs, MeJA elicitation, and their interaction on leaf consumption (a) and food conversion efficiency (b) of *Manduca sexta*. We defined 'genotype' (WT, ir*SPI2a+b*, ir*SPI1/2a+b*), 'line' (for the independent lines 1 and 2 of each genotype, nested within 'genotype'), and 'treatment' (control or MeJA) as factors in the model: genotype line(genotype) treatment genotype*treatment (using sum of squares type IV). (SS, sum of squares; df, degrees of freedom; MS, mean squares; F- and p-value; s indicates significant differences $p < 0.05$)

a. ANOVA for leaf consumption by *M. sexta* (days 4-5)

		SS	df	MS	F	P	
Between-subjects effects							
Intercept	Hypothesis	31490.94	1	31490.94	163.97	0.004	
	Error	416.04	2.17	192.06[a]			
genotype	Hypothesis	358.94	2	179.47	0.79	0.566	
	Error	418.88	1.84	227.67[b]			
line(genotype)	Hypothesis	415.22	2	207.61	3.07	0.050	s
	Error	7780.81	115	67.66[c]			
treatment	Hypothesis	1829.02	1	1829.02	27.03	0.000	s
	Error	7780.81	115	67.66[c]			
genotype * treatment	Hypothesis	294.90	2	147.45	2.18	0.118	
	Error	7780.81	115	67.66[c]			

b. ANOVA for efficiency of food conversion by *M. sexta* (days 4-5)

		SS	df	MS	F	P	
Between-subjects effects							
Intercept	Hypothesis	3.62E-07	1	3.62E-07	262.93	0.002	
	Error	3.12E-09	2.27	1.38E-9[d]			
genotype	Hypothesis	1.79E-09	2	8.94E-10	0.55	0.648	
	Error	2.98E-09	1.85	1.61E-9[e]			
line(genotype)	Hypothesis	3.02E-09	2	1.51E-09	2.58	0.080	
	Error	6.85E-08	117	5.85E-10[f]			
treatment	Hypothesis	3.07E-10	1	3.07E-10	0.53	0.470	
	Error	6.85E-08	117	5.85E-10[f]			
genotype * treatment	Hypothesis	9.48E-09	2	4.74E-09	8.10	0.001	s
	Error	6.85E-08	117	5.85E-10[f]			

a. 0.89 MS(line(genotype)) + 0.11 MS(Error)
b. 1.14 MS(line(genotype)) - 0.14 MS(Error)
c. MS(Error)
d. 0.86 MS(line(genotype)) + 0.14 MS(Error)
e. 1.11 MS(line(genotype)) - 0.11 MS(Error)
f. MS(Error)

Supplementary Table S5. Likelihood ratio test on linear mixed-effects models (a) and a corresponding ANOVA (b) to test effects on *Manduca sexta* larval mass after silencing *SPI2c* using VIGS in wild-type plants and SPI-silenced genotypes (ir*SPI1/2a+b*, ir*SPI2a+b*). a, the models included 'genotype' and 'v*SPI2c*' as fixed effects and 'box' and 'line' as random effects. The factor 'box' indicated the container in which caterpillars were pre-reared in batches for three days before being transferred in individual containers. Three such boxes containing 10 to 15 caterpillars per genotype and VIGS construct were used (30 boxes in total). The factor 'line' accounted for possible differences between the two lines per genotype. Another possible random effect could have emerged from differences in VIGS efficiency between plant individuals. However, when this 'plant'-effect was included in the model comparison it did not show any effect and was thus removed for further analysis, so that the final model was: (larval mass ~ genotype + v*SPI2c* + genotype*v*SPI2c* + (1|box) + (1|line)). b, as an alternative we computed a univariate ANOVA, neglecting the 'line' factor and just using 'box' as an error term (larval mass ~ genotype + v*SPI2c* + genotype:v*SPI2c* + Error(box) as multiple error terms are not allowed in ANOVA). For both types of analyses the data was transformed to the power of 1.5 to meet requirements of homoscedasticity.

a. Linear mixed-effects model for larval mass on day 9

	logLik	Chisq	Chi df	P
genotype	-2421.6	5.5343	2	0.06284
v*SPI2c*	-2421.2	0.8272	1	0.36309
genotype*v*SPI2c*	-2421.1	0.3158	2	0.85393

b. ANOVA for larval mass on day 9

	df	SS	MS	F	P
genotype	2	41533183	20766592	2.4995	0.1033
v*SPI2c*	1	3976912	3976912	0.4787	0.4957
genotype*v*SPI2c*	2	1478833	739417	0.0890	0.9151
Residuals	24	199396260	8308178		
Error: within	248	464566587	1873252		

Supplementary Table S6. Repeated measures ANOVA to detect effects on larval mass of *Spodoptera littoralis* after feeding on plant material silenced in *SPI2c* using VIGS in different genetic backgrounds (wild-type or SPI-silenced genotypes ir*SPI1/2a+b*, ir*SPI2a+b*). We defined 'genotype' (WT, ir*SPI2a+b*, ir*SPI1/2a+b*) and 'v*SPI2c*' as factors in the model: genotype + v*SPI2c* + genotype*v*SPI2c* (using sum of squares type III). Mauchly's test indicated a violation of the assumption of sphericity ($\chi^2_{(5)} = 148.9$; $p < 0.001$). Therefore Huynh-Feldt estimates ($\varepsilon = 0.566$) were used to correct the degrees of freedom. (SS, sum of squares; df, degrees of freedom; MS, mean squares; F- and p-value; s indicates significant differences $p < 0.05$)

	SS	df	MS	F	P	
Within-Subjects Effects						
day	2116.64	1.13	1871.1	424.99	0.000	s
day * genotype	9.23	2.26	4.08	0.93	0.411	
day * v*SPI2c*	53.42	1.13	47.23	10.73	0.001	s
day * genotype * v*SPI2c*	1.39	2.26	0.61	0.14	0.893	
Error(day)	288.87	65.61	4.40			
Between-Subjects Effects						
Intercept	2734.47	1	2734.47	532.05	0.000	s
genotype	15.51	2	7.75	1.51	0.230	
v*SPI2c*	66.53	1	66.53	12.94	0.001	s
genotype * v*SPI2c*	1.68	2	0.84	0.16	0.849	
Error	298.09	58	5.14			

Supplementary Table S7. Repeated measures ANOVA to detect effects on larval mass of *Spodoptera littoralis* after feeding on wild-type plant material silenced in *SPI2c* using VIGS. Although log-transformation improved homogeneity of variances Mauchly's test indicated a violation of the assumption of sphericity ($\chi^2_{(5)} = 101.54$; $p < 0.001$). Therefore Huynh-Feldt estimates ($\varepsilon = 0.728$) were used to correct the degrees of freedom. (SS, sum of squares type III; df, degrees of freedom; MS, mean squares; F- and p-value; s indicates significant differences $p < 0.05$)

	SS	df	MS	F	P	
Within-Subjects Effects						
day	15.916	1.986	8.014	696.138	0.000	s
day * v*SPI2c*	0.084	1.986	0.042	3.691	0.032	s
Error(day)	0.594	51.637	0.012			
Between-Subjects Effects						
Intercept	3.963	1	3.963	86.818	0.000	s
v*SPI2c*	.166	1	0.166	3.642	0.067	
Error	1.187	26	0.046			

Supplementary Table S8. Primer sequences used for cloning, construct generation, and QRT-PCR.

Primer name	Sequence (5' -> 3')	Target	
PIS5-21 (fwd)	GAAAACAAAGGTGGCCAGAAC	SnSPI1	cloning
PIS6-21 (rev)	CATTATATATAGTAAGAGTAC	SnSPI1	
Sa_pin2a_F1	TGGCTGTTCACAAAGTTAGCTTC	SnSPI2a	
Sa_pin2a_R1	AGTCCACATTACAGTAACCAGCA	SnSPI2a	
Sl_PI2_F4	AATGTGACACTCGAATTGACTATG	SnSPI2c	
Sl_PI2_R5	CATAGTCAATTCGAGTGTCACATT	SnSPI2c	
Sl_PI2_R6	CTGCACAACAGTTGGTGCA	SnSPI2c	
Sl_PI2_R7	TTCAGATTCTCCYTCACAAATAAAAG	SnSPI2c	
SnPIN2c_F1_3'R	CTATGGGATTTGCCCACTT	SnSPI2c (3' RACE)	
SnPIN2c_F2_3'R	GTGAGGTACCGGTGGACA	SnSPI2c (3' RACE)	
SnPIN2c_R2	CGTACACACTTAAGTAGCACAAACC	SnSPI2c	
PIS1-33 (fw)	GCGGCGCCATGGATTAGAAATAACGTTGGGTTC	SnSPI2b (for generating pSOL3PIS)	inverted-repeat construct generation
PIS2-32 (rev)	GCGGCGCTGCAGGCTGTTCACAAAGAAGTTAG	SnSPI2b (for generating pSOL3PIS)	
PIS3-33 (fw)	GCGGCGCTCGAGATTAGAAATAACGTTGGGTTC	SnSPI2b (for generating pSOL3PIS)	
PIS4-33 (rev)	GCGGCGGAGCTCGGCTGTTCACAAAGAAGTTAG	SnSPI2b (for generating pSOL3PIS)	
PIS9-32 (fw)	GCGGCGCTGCAGAAAACAAAGGTGGCCAGAAC	SnSPI1 (for generating pSOL3PIN12)	
PIS10-40 (rev)	GCGGCGCCATGGATGCATGACATTATAATCAACATGAACC	SnSPI1 (for generating pSOL3PIN12)	
PIS11-33 (fw)	GCGGCGGTATACGAAAACAAAGGTGGCCAGAAC	SnSPI1 (for generating pSOL3PIN12)	
PIS12-38 (rev)	GCGGCGCTCGAGCTCGACATTATAATCAACATGAACCC	SnSPI1 (for generating pSOL3PIN12)	
PIN2c1-33	GCGGCGCTCGAGTATGTAAGTGAGGTACCGGTG	SnSPI2c with restriction site	VIGS construct generation
PIN2c2-33	GCGGCGGAATTCAAGTGGGCAAATTCCATAGGC	SnSPI2c with restriction site	
Sn_pin1_F1	TACCAGCAAAGCTTGCTAAGG	SnSPI1 (only for wild-type)	QRT-PCR
Sn_pin1_R1	TTGTGACATTATAATCAACATGAACC	SnSPI1 (only for wild-type)	
Sn_pin1_F2	TTGATGTAATTAGCAGCCACACA	SnSPI1	
Sn_pin1_R2	CATTATATATAGTAAGAGTACATTGTGAC	SnSPI1	
Sn_pin2a_F3	ATTGTACCTTCGAATGTGATAC	SnSPI2a	
Sn_pin2a_R4	GATAGATAACACAACAGATGATTG	SnSPI2a	
Sn_pin2b_F2	TGCCCTCTATATTGTGATGG	SnSPI2b	
Sn_pin2b_R2	ACAGTGATCATTAGCATATATTGC	SnSPI2b	
Sn_pin2c_F1	GATCTCCAGAAAATCAAGGTTGC	SnSPI2c	
Sn_pin2c_R1	GCCATGGCAGAAATATATCATCA	SnSPI2c	
Sol_EF1a_fp	GTTTCACTGCCCAGGTCATCATC	SnEF1α	
Sol_EF1a_rp	TGGGCTTGGTGGGAATCATC	SnEF1α	
Sn_pin1_prF1	GCCAGAACTTGTTGGTGTAC	SnSPI1	Southern probes
Sn_pin1_prR1	CATGTGTGGCTGCTAATTACA	SnSPI1	
Sn_pin2a_prF1	TGAACCCAAGACCACTGCTTAT	SnSPI2a	
Sn_pin2a_prR1	GTCCACATTACAGTAACCAGCAT	SnSPI2a	
Sn_pin2b_prF2	AATGATATGCGTTGTAGTTTTTA	SnSPI2b	
Sn_pin2b_prR2	CATATTACAGTGATCATTAGCAT	SnSPI2b	

7. DISCUSSION

Coevolutionary theory, which we outlined briefly in the introduction, predicts that the species diversity of plants and herbivorous insects, and the metabolic diversity which these species produce have evolved as the consequence of a continuous cycle of adaptation and coadaption between interacting organisms. This means that species diversity, with all its observable variations in form, size, color, etc., is accompanied by a similarly varied diversity of biotic interactions. Taxonomists, ecologists, and chemists have investigated and mapped many aspects of diversity from different angles, but it remains to be understood how it evolved and which selection pressures have shaped it.

Higher plants seem to have evolved common defense signaling pathways, which depend mainly on the hormones jasmonic acid (JA, and its derivatives), ethylene, and salicylic acid, among others (Howe and Jander, 2008; Bari and Jones, 2009). These hormones are integrated in a signaling network of upstream and downstream processes, including mitogen-activated protein kinases (MAPKs), calcium-calmodulin systems, other phosphatases and kinases, ubiquitylation and proteasome-dependent protein degradation, and the activation and repression of transcription factors (Howe and Jander, 2008; Browse, 2009). The ubiquity of this signaling network suggests that it is the backbone of induced plant defenses; however we know little about the variability and specificity of this network in different species. The JA-dependent pathways of *A. thaliana* and Solanaceae seem to differ substantially with respect to temporal and spatial hormone accumulation (Schmidt and Baldwin, 2006; Glauser et al., 2008; Stork et al., 2009), which suggests that the system has undergone adaptive variation in response to different selection pressures. It is not surprising that the defense responses downstream of these signaling cascades are even more diverse than the pathways themselves. The multivariate nature of plant-herbivore interactions could have promoted species radiation and led to the convergent evolution of analogous "defense syndromes" (Agrawal, 2007), which are similar in their mode of action but differ in the characteristics of their constituents. We can hypothesize that a novel trait which confers resistance to herbivores thus promotes adaptive radiation and could be the starting point of a whole clade, such as the Solanaceae ("escape and radiate", Ehrlich and Raven, 1964).

The evolution of new traits builds on changes in genes which are already present in an organism's genome. As outlined in the introduction, this could create adaptive conflicts in the case of genes which are important for plant development, growth, or reproduction. We will need to study the defense mechanisms of different taxa in full detail to understand what facilitated the evolution of new traits, how these traits influenced plant survival and diversification, and whether qualitatively different defenses have analogous functions in distantly related clades. Although there are many studies on chemical defenses, both direct and indirect, against herbivores (Bennett and Wallsgrove, 1994), e.g. on glucosinolates in Brassicaceae (Hopkins et al., 2009), nicotine in *Nicotiana spp*. (Steppuhn et al., 2004), or terpenoids in several families and species (Cheng et al., 2007), we do not yet understand

how these interact with other defenses to counter the different herbivores feeding on a plant[4]. It will be necessary to identify all the components of plant defense in several species and study how they act in response to the plant's natural enemies (Futuyma and Agrawal, 2009). Such an in-depth analysis could allow for comparisons among defense mechanisms, with respect to the natural history of the particular species, to understand if there are general macro-evolutionary trends behind the evolution of these traits.

The present thesis attempts to understand the plant defenses of *S. nigrum* in more detail. The microarray study in Manuscript I estimates the degree of conservation between the defense responses of two species from the same family to be fairly low. Moreover, it identifies a number of interesting candidate genes in both species that might have important functions in plant defense, which paves the way for the subsequent work. The methods developed in Manuscripts II to IV allow the role of SPIs in plant defense and development to be precisely dissected, as is done in Manuscript IV. Finally, we demonstrate that even a group of well-studied proteins such as SPIs can be surprisingly diverse in its effects on herbivores, as its members depend heavily on their interaction with other defenses and display a number of functions that extend beyond plant defense, with interesting implications for their evolution. Clearly, much effort will be necessary to fully understand plant defenses in an ecological and evolutionary context, especially when we turn our attention to less well-studied defense components. In the following pages, we will discuss the main findings of this thesis from an evolutionary and ecological standpoint, relate them to the recent literature, and outline the future perspectives which our work has opened up.

7.1 TRANSCRIPTIONAL RESPONSES TO HERBIVORE ATTACK

The transcriptional comparison of defense responses in two solanaceous species, presented in Manuscript I, estimates that the overlap of commonly regulated genes in both species is just around 10% of all regulated genes. This finding revealed that even quite closely related species may differ substantially in their defense responses. Although the JA pathway is a key regulator of induced defenses in Solanaceae, the JA accumulation patterns of *S. nigrum* and *N. attenuata* were reported to differ (Schmidt and Baldwin, 2006; Stork et al., 2009), which suggests that evolution is constantly fine-tuning even the central mechanisms of the signaling system. The transcript data in Manuscript I also highlight other signaling components which differed between the two species but which seem to be important constituents of the defense response. For example, calcium-dependent and inositol-phospholipid signaling, which were only regulated in *N. attenuata*, are known to be important

[4] At this point, it should be mentioned that the focus on plant-herbivore interactions, even if it includes multitrophic levels of predators, parasitoids and hyperparasitoids, still neglects interactions of plants with microbial or viral pathogens or symbionts which are introduced at wound sites or already present in the plants. There is evidence that strong cross-signaling occurs among herbivore- and pathogen-elicitied responses. However, as we are currently struggling to fully understand the interaction of one herbivore species with its plant host, it will take some time to integrate all the levels of complexity into a single model.

regulators of defenses against microbial and fungal pathogens (Lecourieux et al., 2006) but recently emerged as influential in plant-herbivore interactions (Ryan, 2000; Maffei et al., 2004; Arimura et al., 2008). The role of heterotrimeric G proteins in plant defense was largely unexplored when Manuscript I was published in 2005, but in 2006 Trusov et al. (2006) found them to be involved in responses to necrotrophic fungi and in JA signaling in *A. thaliana*. Furthermore, Okamoto et al. (2009) identified the Gα subunit to be important for regulating responses downstream of JA. The importance of cytokinins for plant defense is poorly understood (Bari and Jones, 2009), but there is some evidence that they play a role in plant-pathogen interactions. The specific expression signature that we detected in *S. nigrum* indicates that cytokinins might also contribute to signaling processes related to herbivore attack. In summary, our study pinpoints a number of interesting but poorly understood signaling components in both species and thus provides promising starting points for future research.

The observed up-regulation of genes related to ubiquitin-dependent proteolysis might indicate the activation of the SCF^{COI1}-complex, a multi-protein E3-ubiquitin ligase. After binding to JA-Ile, a derivative of JA which accumulates in herbivore-elicited tissues, this complex leads to the ubiquitylation and subsequent degradation of jasmonate ZIM domain (JAZ) proteins by the 26S-proteasome (reviewed by Browse, 2009). The discovery of the JAZ proteins and their targeted degradation was a milestone in the jasmonate field, as it revealed how the JA signal is perceived and transduced downstream. At least some of the JAZ proteins act to repress the transcription factors of JA-responsive genes, and their degradation thus leads to the activation of numerous defense genes. Although we could not detect JAZ transcripts with our microarray approach – owing perhaps to several technical issues discussed below – we could still identify proteasome-related transcripts. However, we cannot rule out the possibility that this transcriptomic signature is at least partially independent of SCF^{COI} or that the proteasome and other protein-degrading processes influence the defense response in other ways.

The high up-regulation of leucine-aminopeptidase (LAP) in *S. nigrum* is an example of another proteolytic enzyme involved in activating defense responses. Whereas the inducible LAP-A seems to be restricted to the genus Solanum, many other species outside Solanaceae express the homologue LAP-N, which is not regulated after herbivory (Chao et al., 2000). This specificity with regard to genus was reflected in the microarray data, and the very high up-regulation of LAP-A in *S. nigrum* made it an ideal candidate gene for assessing the efficiency of our VIGS system, as described in Manuscript II. The strong effect of LAP on herbivore performance, which we found after silencing LAP in *S. nigrum*, was the first direct evidence that LAP is involved in plant defense against herbivores. Recently Fowler et al. (2009) found that LAP-A acts as a JA-dependent regulator of late defense responses in tomato, confirming the herbivore phenotype that we had observed for *S. nigrum*. Although the mechanism is still unclear, LAP-A appears to positively regulate the gene expression of SPIs and polyphenol oxidases (PPOs), among others. It is interesting that a genus-specific gene has such a profound effect on defense gene expression and herbivore fitness. We know little about the

function of ubiquitous LAP-N in plants, but that it is conserved argues for its essential role in plant physiology or development. Perhaps LAP-A represents the trace of another gene duplication event which has gained influence on plant defense through adaptation.

The surprisingly large differences between the herbivore- and the MeJA-induced transcriptomes of *S. nigrum* underscore the specificity of the response to insect feeding. Obviously, it is not sufficient just to turn on the JA pathway to attain responses similar to those that follow herbivore attack. The characteristic way in which insects mechanically damage plant tissue (Mithöfer et al., 2005) and the recognition of herbivore-specific elicitors by the plant (Howe and Jander, 2008) lead to the simultaneous activation of several signaling pathways of which JA-signaling is just one important component. This finding should also safeguard us from over-interpreting results that rely on only a few marker genes but make predictions for entire pathways. Similarly, we have to be careful when drawing conclusions from our microarray data. First, we have to consider that we used only one particular herbivore species for the comparison. Other herbivores will probably yield different results (Voelckel and Baldwin, 2004; Voelckel et al., 2004). Second, because we compared the transcriptome at one timepoint, only a particular set of transcripts was shown to be active. Third, the technology itself is not free of flaws. Although microarrays are generally quite reliable, they might yield false positive or negative signals for some transcripts, depending on the sequence that is spotted on the microarray, the chip architecture, the number of clones per gene, the applied hybridization and labeling technique, the abundance of the gene of interest relative to the whole transcriptome, and ultimately even data acquisition and normalization procedures (Clarke and Zhu, 2006). Fourth, our approach of cross-species hybridization and the incomplete representation of the genome on the microarray might have led to false or missing signals.

Indeed, the comparison of microarray data in Manuscript IV showed that PI-II transcripts did not give signals on the cDNA array, while the custom oligo-array showed a strong up-regulation of these genes; interestingly, in the case of PI-I transcripts, it was the opposite. As a consequence, it might be that the TIGR cDNA-array was biased towards conserved genes and missed signals from more diverged genes. However, even if this is the case, the large differences that we observed are even more striking, as conserved genes are expected to display conserved functions. With the cost of sequencing plummeting and the availability of high-density microarrays, custom-printed on demand, increasing, more and more detailed and precise genomic and transcriptomic information on non-model species such as *S. nigrum* is within reach. Our study has already provided us with a huge amount of interesting data, many promising candidate genes, and novel results, but the insights and possibilities the new tools offer us seem limitless. A single microarray study usually reveals more new questions than it provides answers, and with the depth and precision that the new technologies deliver, a well-planned experiment might yield enough material for a whole scientific career.

7.2 Optimizing virus-induced gene silencing

Microarrays and other descriptive approaches yield informative insights into regulatory networks and transcriptomic changes. However, it is difficult to validate and test hypotheses derived from such information without the suitable techniques of genetic manipulation. The discovery and application of methods for silencing and overexpressing genes were essential for the recent huge advances in molecular biology. Similarly, the stable transformation method we used for silencing genes in this thesis was key for understanding the function of SPIs. However, as it takes about nine to twelve months to obtain stably silenced lines of *S. nigrum* for experiments, a faster method to manipulate gene expression was necessary. Without the application of VIGS, the data presented in Manuscript IV could not have been obtained in a reasonable timeframe. Both the confirmation of the identity of SPI2c and the investigation of its role in plant defense depended heavily on this method and demonstrated its usefulness in functional and ecological screens. Although the viral infection in a VIGS experiment might not resemble typical conditions in nature and thus might also affect plant-herbivore interactions, we are confident that valid and valuable information concerning herbivore performance can be obtained with this method when the proper controls are used – as was shown by the confirmation of the LAP-phenotype by Fowler et al. and also in other studies (Wu et al., 2007; Steppuhn et al., 2008). There is a growing interest in *S. nigrum* because of its ability to hyper-accumulate cadmium and other toxic elements from contaminated soils (Wei et al., 2005), its partial resistance to *Phytophthora infestans* (Colon et al., 1992; Zimnoch-Guzowska et al., 2003; Lebecka, 2009), and its medicinally interesting metabolites (Lee and Lim, 2006; Jeong et al., 2007), and thus we believe that our optimized VIGS protocol will be of general use for the scientific community.

In addition to demonstrating LAP's direct involvement in the defense against herbivores, our VIGS procedure also offered methodological advantages with general consequences for other VIGS approaches. The use of vacuum infiltration at the seedling stage allows plants to be silenced much earlier in their development than when other infiltration methods are used and thus might be useful when early life stages of a plant are studied. The only comparable approach ("Agrodrench", Ryu et al., 2004) was found to require more preparatory work and is much less effective, which was also confirmed by Ho et al. (2009) for tomato.

The advantages of VIGS, the rapidity of the procedure, and the possibility it offers to investigate genes that are lethal when silenced by stable transformation come at the cost of the side-effects caused by infecting the plants with *A. tumefaciens* and a virus, such as TRV. The VIGS vector designed by Liu et al. (2002) is characterized by the strong expression of TRV, which might enhance viral side-effects and which makes proper controls essential. To our knowledge, our study was the first to investigate the effect of transgene insert size on virus and plant performance. The finding that the empty vector (EV) shows faster and more efficient virus replication when compared to a vector with a non-coding insert of about 300 bp ("control vector", CV) has important consequences for the design of VIGS experiments. The higher virus load, as we have shown, correlated with a reduction in plant

growth, and we can assume that increasing the load affects other physiological processes as well. We have evidence that plants inoculated either with EV or CV differ in the amount of JA they accumulate after simulated herbivory (H. Merker, unpublished data). As a consequence, we strongly advise the use of a CV containing a non-coding insert. This insert should have a similar length as the gene fragment which is used for the silencing vector.

7.3 Detecting new SPIs and measuring their activity

Without genomic information, characterizing a whole gene family is difficult. Moreover, even if all genes are known, it is impossible to predict the number of active proteins they code for, in particular, if the genes code for multi-domain proteins that can be alternatively processed as in the case of SPIs. Hence, it is necessary to characterize not only the transcripts but also the active proteins. In the case of SPIs, we found activity gels with subsequent gel-x-ray-film contact prints, adapted from Pichare and Kachole (1994), to be a reliable and sensitive method.

However, without stable and transient gene silencing, it would have been difficult to unambiguously identify the different active bands, because the amount of SPI protein in the activity gels was small and the level of purification on the one-dimensional gels was low. Thus a large-scale purification was necessary to identify the new SPI SnSPI2c. Because such purifications are cumbersome, a method that combines activity data with qualitative information would be extremely useful. The dual fluorescence/MALDI-TOF platform described in Manuscript III is a first step in this direction. It combines high sensitivity with low sample consumption, allows the detection of proteolytic activity and simultaneously yields qualitative information in terms of molecular masses of substrate peptides and SPIs remaining after digestion. This fast, sensitive technique allows high-throughput screens to be developed to monitor activity of (novel) PIs and can be adapted for any kind of sample, regardless of its origin. The value of the approach was also demonstrated by its ability to detect residual peptides in extracts of MeJA-induced plants silenced in the expression of *SnSPI2a* and *SnSPI2b*. These peptides most likely represent SnSPI2c, which accounted for the remaining inhibitory activity and which was later identified in Manuscript IV.

7.4 SPIs between defense and development

The accumulation of PIs after herbivore attack is supposedly one of the best-described induced defense responses. Nevertheless, studies that have tried to investigate the importance of PIs for plant survival in a natural setting are rare (Zavala et al., 2004; Steppuhn et al., 2008). Moreover, so far no study has tried to address the importance of the simultaneous expression of multiple PIs for plant defenses. Dissecting this problem requires a detailed analysis of all PIs involved in plant defense in a

natural model system. To perform such an analysis on the SPIs of *S. nigrum* represented the main objective of this thesis.

We have discussed the advantages of *S. nigrum* as an ecological model system and described the successful development of the tools that we required to investigate the functions of SPIs in this plant. In retrospect, the choice of this species was fortunate, as it expresses a large variety of PIs and, in particular, SPIs. The transcriptomic analysis revealed that two main SPI families are typically and strongly expressed after herbivore attack: PI-I and PI-II. As mentioned above, the microarray approach might have missed the response of PIs from other families, such as cysteine PIs or aspartate PIs, and future studies are needed to close this gap. Given the complexity of the functions of SPIs alone in *S. nigrum*, investigating all PI families at once would have exceeded our scope; however, the complexity suggests interesting directions for future research.

Combining genetic tools with activity-based profiling techniques enabled us to identify all active SPIs expressed in *S. nigrum* and helped identify the novel inhibitor SnSPI2c, which had been missed by previous screening approaches based on DNA sequence similarity only (Xu et al., 2001; Schmidt et al., 2004). Functional analyses revealed that the functions of SPIs are often different and sometimes overlapping with regard to defense against herbivores and plant development; this is seen in the accumulation of SnSPI2c and SnSPI2b following herbivore attack, as well as in the different tissue-specific expression patterns and the strongly separated enzyme specificities of all four SPIs. Although we tried to cover as many aspects as possible, clearly we were able to analyze just the tip of the iceberg. It is likely that SPIs influence not only other interacting organisms, be they herbivores, pathogens, or pollinators, but also other physiological or developmental processes *in planta.* Further studies will certainly reveal an even more complex picture. As mentioned above, it will be necessary to assess the synergistic effects of SPIs with other PI families and other direct or indirect defenses to develop an integrated view of the defense system “protease inhibition”. The different types of alkaloids in Solanaceae (steroidal, tropane or pyridine alkaloids) might indicate that the coexpression of PIs with toxic compounds is a selective advantage (as demonstrated by Steppuhn and Baldwin, 2007).

A holistic approach takes the evolutionary perspective on plant defenses into account. Although it is presently difficult to prove, we speculate that PIs originated from ancestral genes whose primary functions were somehow related to plant physiology or development (Barta et al., 2002; Christeller, 2005). Their main property, the ability to inhibit proteases, might then have turned into a selective advantage under growing pressure from herbivores. It is noteworthy that PIs with similar properties convergently evolved in different plant families (e.g. PI-I and PI-II in Solanaceae, and Kunitz-PI in Fabaceae), and it is tempting to suggest that, for example, a gene duplication or a mutation of an ancestral PI gene is one of the evolutionary causes for the success and the species radiation of these families (Christeller, 2005; Kong and Ranganathan, 2008).

The diversity of the PI-II family in Solanaceae (be it structural as in *Nicotiana spp.* or genetic as in other genera) is obviously the result of several gene or domain duplications and subsequent adaptive changes, probably because of selective pressures exerted by different herbivores. These duplications can be interpreted as a means of escaping adaptive conflict (Conant and Wolfe, 2008; Flagel and Wendel, 2009), if the PI in question has multiple functions that are under different selection pressures. However, emphasizing the function of every gene and every gene product neglects the possibility that (duplicated) selectively neutral genes might be retained in a genome, increasing the potential for adaptation in the future. This means it is difficult to find out if an ancestral gene escaped an adaptive conflict or if it first became duplicated and then one copy functionally diverged. While some SPIs in our study clearly influence herbivore performance or seed development, others displayed surprisingly mild or undetectable phenotypes. This suggests either that the function of these genes is visible only under specific circumstances, which were not encountered during our experiments in the lab or in the field, or that these genes have no or only very subtle functions. Perhaps, these genes are exemplifying the silent potential of *S. nigrum*, potential that might benefit the species through future mutation and selection. The current focus on mechanistic explanations for the function of each gene might sometimes blind us to the imperfections that every evolving organism carries and which are the evolutionary basis for new traits.

7.5 SYNOPSIS

We have demonstrated that even closely related plants differ substantially in their response to herbivory. Although they share common mechanisms – for example, JA's role as a central signaling hormone – the exact nature of the underlying processes has varied at every level as a result of constant adaptation. Thus, general conclusions that are based on examining one or a few model species should be treated with care. Similarly, we should be careful when assigning functions to new genes based exclusively on sequence similarity. As SPIs have taught us, similar proteins can have very different properties and functions. The *in planta* physiological functions of PI-II inhibitors have remained undiscovered for decades, because they were primarily viewed as plant defenses. Our study clearly shows that the simple label “plant defense” that was attached for so long to SPIs represents a dangerous reductionism and does not reflect the multiple roles these proteins can play. As a consequence, I hope this thesis contributed to a more nuanced understanding of SPIs and plant defenses in general. The research presented here is a step in the investigation of plant defenses as a multi-dimensional phenomenon constantly shaped by multiple ecological interactions over evolutionary time.

8.1 SUMMARY

The amazing diversity of plants and the herbivores that consume them is thought to have emerged from a continual process of adaptation and counter-adaptation between these two groups. As a result of this coevolution plants have evolved sophisticated defenses which protect them against herbivory in several ways. These include direct resistance mechanisms, such as mechanical or chemical defenses, indirect defenses, which recruit the herbivores' natural enemies, or tolerance mechanisms, which enhance the plant's ability to recover from herbivore attack. But the deployment of these defenses is costly: plants face a trade-off between allocating resources and energy to growth and reproduction, or to defense.

The evolution of induced defenses, which are only produced after a plant is attacked, may present a solution to this dilemma. Different clades of plant species are known to produce specific and specialized arsenals of biochemical weapons that can be activated upon herbivore attack. While it has long been clear that these downstream defense responses differ among plant taxa, their activation mechanisms were thought to be fairly well conserved. The signaling cascades which control the release of induced defenses after herbivore attack have been extensively investigated in a few model plant species. However, many of them represent domesticated crop plants which are likely to have lost parts of their coevolutionary defense signature as a result of artificial selection for yield-enhancing traits. It remained an open question whether these model systems could yield general predictions which could explain how ecological responses are elicited in plants in general.

In the first part of the thesis (Manuscript I) we investigated this question in more detail by analyzing transcriptional responses of two native solanaceous species to the attack of the same herbivore species. With a 10k-cDNA potato (*Solanum tuberosum*) microarray, we compared the transcriptional responses of *Nicotiana attenuata* with those of *S. nigrum* when both were attacked by the solanaceous generalist herbivore *Manduca sexta*. 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. Given that attack from the same herbivore elicits profoundly different responses in two solanaceous species, we conclude that blueprints for commonly regulated responses to plant-herbivore interactions appear unlikely. It rather seems that evolution fine-tunes even central mechanisms of the defense signaling system leading to relatively poor conservation. Furthermore this study pinpointed genes such as heterotrimeric G proteins and cytokinins whose role in defense against herbivores is currently poorly understood and which represent interesting candidates for future research in defense signaling.

The second defense-related part of the thesis (Manuscript IV) concentrated on a well-known downstream response to herbivory which was also highlighted by the microarray study, namely the accumulation of serine protease inhibitors (SPIs). Solanaceae produce diverse SPIs, which have long been known to act as anti-digestives in the herbivore gut, and are a classic example of induced

defense. However, despite the large number of studies that have been conducted to understand the defensive function of SPIs, many questions remained unexplored. Why do the Solanaceae in particular produce so many different SPIs, and which forces influenced the evolution of this diversity? How much do SPIs actually contribute to plant defense in a natural system? Are the different SPIs specific for particular herbivores or do they act nonspecifically or synergistically with other PIs or defenses?

Moreover, a recent study on *S. americanum* and our own results suggested that some SPIs also interact with endogenous proteases in planta. Thus, the diversity of SPIs in Solanaceae could partly be due to their different functions in plant defense or development. How plants balance the conflicting requirements for functional stability in the regulation of growth and development, and for adequate defenses against the coadaptations of herbivorous attackers is unknown. Gene duplication and subsequent neofunctionalization, or sub-localization are possible scenarios, but were not well studied with respect to defense and development.

To address these questions, we examined *S. nigrum's* complete SPI profile, comprising four different active inhibitors, of which the most abundant proved to be novel, to understand their functional specialization in an ecological context. Transcript and activity characterization revealed tissue-specific insect-elicited accumulation patterns, which indicated that the different SPIs are likely to have functionally diversified. Through stable and transient gene silencing of all SPIs we discovered the four SPIs to have different specificities for target proteinases: defense-related inhibitors had high specificity for trypsin- and chymotrypsin, while development-related inhibitors were highly active against subtilisin. In field and lab experiments we discovered that SPIs have strongly herbivore-specific defensive properties, with opposite effects on closely related species. In contrast to observations in *S. americanum*, developmental phenotypes resulting from the silencing of SPIs were weak or not apparent *in S. nigrum*, suggesting that SPIs' developmental functions are not fully conserved in the genus *Solanum*.

In summary this study revealed that a simple interpretation of SPIs as plant defense falls short of describing the multi-faceted functions they actually serve. SPIs are likely to influence a number of physiological processes in the plant or ecological interactions with non-herbivorous organisms, which in turn affect SPI evolution. SPIs are thus an example of the challenges that lie ahead us, if we want to understand the origin, evolution, and mechanisms of plant defenses.

The investigation of these questions required the development of tools which allowed fast genetic functional screens and quantitative and qualitative activity profiling of PIs. The development of these methods constitutes the remaining part of the thesis (Manuscripts II and III) without which the findings in Manuscript IV would not have been possible.

In Manuscript II we describe the first application and optimization of VIGS in *S. nigrum*, which allowed efficient gene silencing. The development of an appropriate control vector and the optimization of the infiltration technique constitute substantial improvements to the method which are of general importance for all TRV-based VIGS approaches. The silencing of LAP as a proof of

principle experimentally revealed for the first time the long-postulated involvement of this enzyme in direct defense against herbivores.

Manuscript III describes a dual fluorescence/MALDI-TOF platform which permits rapid screening for proteolytic enzyme activity and the corresponding inhibitors. It combines high sensitivity with low sample consumption, facilitates the quantitation of proteolytic activity and simultaneously yields qualitative information in terms of molecular masses of substrate peptides and SPIs remaining after digestion. The fast and sensitive technique allows high-throughput screens for the activity of (novel) PIs and can be adapted for any kind of sample, regardless of its origin.

8.2 ZUSAMMENFASSUNG

Die Entwicklung der erstaunlichen Vielfalt der Pflanzen und ihrer Fraßfeinde wird gemeinhin als das Produkt eines kontinuierlichen Prozesses von Anpassung und Gegen-Anpassung zwischen diesen beiden Gruppen interpretiert. Als Folge dieser Koevolution haben Pflanzen höchst spezialisierte Verteidigungssysteme entwickelt, mit deren Hilfe sie sich auf vielfältige Weise gegen Herbivoren schützen. Die Abwehrmechanismen können dabei auf verschiedene Art ihre Wirkung entfalten: als direkte Resistenz, z.B.in Form mechanischer Barrieren oder chemischer Abwehrstoffe, als indirekte Abwehr, durch die Anlockung der natürlichen Feinde der Herbivoren, oder in Form von Toleranzmechanismen, die die Fähigkeit einer Pflanze optimieren sich nach Beschädigung durch einen Herbivoren wieder zu erholen und den Gewebe- und Energieverlust auszugleichen. Der Einsatz solcher Abwehrstrategien bedeutet allerdings in vielen Fällen einen erheblichen Mehraufwand von Energie und Ressourcen für die Pflanze und hat somit Einschränkungen für Wachstum und Reproduktion zur Folge.

Induzierbare Abwehrsysteme, die ausschließlich bei Bedarf, also bei Herbivorenbefall, aktiviert werden, stellen eine Möglichkeit dar diesen Ressourcen-Konflikt zu lösen. Uns ist mittlerweile eine fast unüberschaubare Vielzahl taxon-spezifischer und spezialisierter Abwehrsysteme bekannt, die erst nach Herbivorenbefall aktiviert werden. Es ist daher offensichtlich, dass sich die Qualität und Ausprägung der Abwehrsysteme, in Abhängigkeit des koevolutionären Hintergrundes, zwischen verschieden pflanzlichen Taxa stark unterscheidet. Die Signalwege, die zur Aktivierung der Abwehrantworten führen, wurden jedoch lange Zeit als evolutionär konserviert betrachtet. Zahlreiche Studien haben sich in einigen pflanzlichen Modellsystemen intensiv mit den Signaltransduktionswegen, die die induzierte Abwehr steuern, beschäftigt. Bei vielen dieser Systeme handelt es sich jedoch um domestizierte Nutzpflanzen, deren Abwehrverhalten unter Umständen durch die gezielte Selektion auf ertragssteigernde Merkmale nicht mehr dem natürlichen Zustand entspricht. Es blieb daher unbekannt, ob die Erkenntnisse, die mit diesen Pflanzenarten über Signalwege gewonnen wurden, allgemeine Rückschlüsse darauf zulassen, wie Abwehrreaktionen in (Wild-) Pflanzen gesteuert werden.

Im ersten Teil dieser Dissertation (Manuskript I) widmen wir uns dieser Frage näher, indem wir die transkriptionellen Reaktionen zweier wild vorkommender, ungezüchteter Arten der Solanaceae nach Befall durch dieselbe Herbivorenart vergleichen. Dazu verwendeten wir einen cDNA-microarray mit über 10.000 verschieden Gensequenzen der Kartoffel (*Solanum tuberosum*) und hybridisierten diesen mit cDNA von *Solanum nigrum* und *Nicotiana attenuata*, 24 Stunden nach Befall durch Larven des Tabkschwärmers *Manduca sexta*. Abgesehen von den transkriptionellen Unterschieden, die aufgrund der bereits aus der Literatur bekannten verschiedenen Sekundärmetaboliten erwartet werden konnten, zeigten beide höchst unterschiedliche Reaktionsprofile, die sich nur zu etwa 10% überschnitten. Diese geringe Übereinstimmung weist darauf hin, dass sich offensichtlich nicht nur die nachgeordneten Abwehrsysteme sondern auch die übergeordneten Signalwege beträchtlich

unterscheiden. Mit Hinblick auf diese großen Unterschiede zwischen nahe verwandten Arten erscheint die Existenz einheitlich konservierter Signalmechanismen, die eine generelle Aussage über Pflanzen verschiedener Abstammungslinien erlaubten, sehr unwahrscheinlich. Offenbar führt der konstante Selektionsdruck sogar in den zentralen Signalsystemen zu evolutiven Anpassungen und Feinjustierungen, die die beobachtete Diversifikation bedingen. Diese Unterschiede beinhalteten einige interessante Gene, deren Funktion in bezug auf pflanzliche Abwehr noch nahezu unbekannt ist, und die daher vielversprechende Kandidaten für zukünftige Forschung auf diesem Gebiet darstellen.

Im zweiten auf pflanzliche Abwehr bezogenen Teil der Dissertation (Manuskript IV) untersuchten wir Serin-Protease-Inhibitoren (SPI). Diese gelten als eine bereits gut erforschte induzierte Abwehr gegen Herbivorie, die sich auch in der transkriptionellen Analyse von *S. nigrum* als eindeutig aktiviert erwies. Solanaceae produzieren bekanntermaßen eine Vielzahl von SPI, die die Eigenschaft besitzen Verdauungsenzyme von Herbivoren zu hemmen, und die als eines der klassischen Beispiele für induzierte Abwehr gelten. Obwohl zahlreiche Studien zur Wirkungsweise einzelner Inhibitoren durchgeführt wurden, blieben einige zentrale Fragen bisher nahezu unbeantwortet. Warum finden wir gerade in den Solanaceae eine derartige Vielfalt von SPI und welche Faktoren haben dies beeinflusst? Wie relevant sind SPI für die Verteidigung gegen Herbivoren in einem natürlichen System? Ist die Wirkung der SPI spezifisch abgestimmt auf einzelne Herbivorenarten oder ist sie eher unspezifisch oder synergistisch mit anderen PI oder Abwehrmechanismen?

Unsere eigenen Ergebnisse und kürzlich veröffentlichte Studien an *Solanum americanum* weisen außerdem darauf hin, dass SPI auch Funktionen in der Pflanze selbst erfüllen, indem sie mit endogenen Proteasen interagieren. Diese Beobachtung eröffnet die Möglichkeit, dass die Diversität der SPI in den Solanaceae zum Teil auf die mehrfachen Rollen, die sie in der Abwehr von Herbivoren und in Entwicklung und Physiologie der Pflanze zu erfüllen haben, zurückzuführen sind. Die notwendige Erhaltung der Funktion für physiologische Prozesse und die stetige Neuanpassung an veränderte Koadaptionen der Herbivoren führen zu einem evolutionären Konflikt, dessen Lösung in Pflanzen nicht bekannt ist. Duplikation von Genen und anschließende funktionelle Diversifizierung sind bekannte mögliche Strategien, aber wurden noch nicht in Bezug auf Gene getestet, die gleichzeitig Funktionen in Abwehr und Entwicklung erfüllen.

Zur Bearbeitung dieser Fragen untersuchten wir alle aktiven SPI in *S. nigrum,* wobei wir vier verschiedene Inhibitor-Typen identifizierten, wovon einer bisher unbekannt war. Eine Charakterisierung der Transkriptmengen und der Inhibitor-Aktivität ergab, dass die vier SPI gewebespezifische und von Induktion durch Insektenfraß abhängige Akkumulationsmuster aufweisen, die bereits auf eine funktionelle Diversifizierung der einzelnen Inhibitoren hinweist. Mittels stabiler und transienter RNAi-Methoden konnten wir außerdem nachweisen, dass die SPI stark unterschiedliche Enzymspezifitäten aufweisen. Abwehrrelevante SPI haben eine hohe Aktivität gegen Trypsin und Chymotrypsin, während Entwicklungsrelevante SPI eine starke Spezifität für Subtilisin

zeigen. In Feld- und Labor-Experimenten konnten wir nachweisen, dass die Wirkung der SPI auf Herbivoren stark artspezifisch ist und teilweise zu sogar entgegengesetzten Effekten in nah verwandten Insektenarten führt. Im Gegensatz zu den Ergebnissen in *S. americanum* hatten die SPI allerdings nur einen schwachen oder gar nicht erkennbaren Einfluss auf die Physiologie oder Entwicklung von *S. nigrum*. Dies lässt vermuten, dass derartige Funktionen in der Gattung *Solanum* nur zum Teil konserviert sind.

Zusammenfassend zeigte dieser zweite Teil der Dissertation, dass wir, wenn wir SPI ausschließlich als Abwehr interpretieren, Gefahr laufen durch diese Vereinfachung wichtige Aspekte ihrer Funktionen zu übersehen. Der Einfluss von SPI auf pflanzliche physiologische und entwicklungsbezogene Vorgänge, beziehungsweise auf Interaktionen mit nicht-herbivoren Organismen, hat weitreichende Konsequenzen für das Verständnis der Funktionen und Evolution dieser Gene. In dieser Gesamtheit betrachtet, bieten SPI ein Beispiel für die Herausforderungen, die uns erwarten, wenn wir den Ursprung, die Entwicklung und die Mechanismen von Pflanzenabwehr verstehen wollen.

Die Untersuchung all dieser Fragen machte die Entwicklung einiger Methoden notwendig, die im dritten Teil dieser Dissertation behandelt wird (Manuskript II und III). In Manuskript II beschreiben wird die erste Anwendung und Optimimierung einer Methode des „virus-induced gene silencing“ (VIGS) in *S. nigrum*, das eine effiziente und gezielte Reduktion von Transkripten durch RNAi erlaubt. Die zusätzliche Entwicklung eines geeigneten Kontroll-Vektors und die Optimierung der Infiltrationstechnik stellen wichtige Verbesserungen der Methode dar und sind somit von allgemeinem Interesse für die Anwendung von auf TRV basierendem VIGS. Um die Funktion unserer Methode zu demonstrieren haben wir die Transkriptmenge der Leucin-Aminopeptidase (LAP) reduziert und anschließend in einem Fraßversuch mit *M. sexta* den ersten experimentellen Nachweis für den Einfluss dieses Enzyms auf die direkte Abwehr von Pflanzen gegen herbivore Insekten erbracht.

Manuskript III beschreibt die Entwicklung einer dualen Fluoreszenz/MALDI-TOF-Plattform, die eine schnelle Analyse proteolytischer Aktivität erlaubt. Sie vereinigt hohe Sensitivität mit sehr geringem Probenaufwand und ermöglicht nicht nur die Quantifizierung proteolytischer Aktivität sondern auch gleichzeitig die Gewinnung qualitativer Informationen über die Molekülmassen der Substratpeptide und etwaiger Inhibitoren, die in der Probe nach dem Verdau verbleiben. Diese schnelle und sensitive Technik ermöglicht Analysen mit hohem Probendurchsatz und könnte daher für breit angelegte Suchen nach (neuen) Inhibitoren verwendet werden. Da die Technologie einfach und unabhängig vom Ursprungsgewebe für jede Art von Probe adaptiert werden kann, ist sie für einen weiten Bereich an Fragestellungen von der Pflanzenbiologie bis hin zur Medizin anwendbar.

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10. DANKSAGUNG

Eine Dissertation ist in erster Linie eine eingehende wissenschaftliche Auseinandersetzung mit einem bestimmten Themenbereich. Da Forschung allerdings in den seltensten Fällen ein geradliniger von stetigen Erfolgen geprägter Prozess ist, lernt man im Laufe seiner Arbeit nicht nur das Thema, sondern auch sich selbst bedeutend besser kennen. Ich glaube daher in den letzten Jahren als Wissenschaftler aber auch als Mensch enorm viel gelernt zu haben. Dieser Reifungsprozess wäre allerdings ohne die Unterstützung vieler Menschen unmöglich gewesen, die ich daher an dieser Stelle erwähnen möchte.

Ich danke **Ian Baldwin** dafür, dass er mir die Möglichkeit gegeben hat in seiner großartigen Gruppe unter seiner Führung arbeiten zu dürfen. Er hat mir die Freiheit und das Vertrauen geschenkt, meine eigenen Wege bestreiten zu können, stand stets für Rat und interessante Ideen zur Verfügung und vermittelte mir, dass es auch in den schwierigsten Situationen der Wissenschaft und des Lebens stets mehr als eine Lösung gibt.

Andrea Loferer danke ich dafür, dass sie mir ihre Liebe und Geduld geschenkt hat, auch wenn das bei einem Naturwissenschaftler nicht gerade immer leicht ist. Sie hat mir geholfen darauf zu achten, dass mir der Tellerrand nie zu hoch wurde und ohne ihre Unterstützung und ihr Lachen hätte so mancher experimentelle Totalschaden eine allzu tragische Dimension erhalten.

Ich danke **meinen Eltern und Brüdern**, dass sie immer für mich da sind, wenn ich sie brauche und dass sie mir stets vertrauen, auch wenn ihnen nicht immer so ganz klar ist, was ich in meiner Wissenschaft eigentlich mache.

Die Arbeit mit *Solanum nigrum* hält immer mehr Überraschungen bereit, als man sich auszudenken vermag. Da ist es günstig, Menschen um sich zu haben, die sich mit derselben Pflanze herumschlagen. **Silvia Schmidt** war während der ersten Jahre nicht nur eine kompetente Ansprechperson, die im Notfall stets zur Stelle war, sondern wurde auch zu einer wertvollen Freundin. **Arjen van Doorn** und **Holger Merker** danke ich für ihre nicht mindere Hilfsbereitschaft, für unzählige gute Ideen, aber auch für den Spaß im Büro, am Feld und im Gewächshaus und für ein Bier oder ein Glas Whisky zur richtigen Zeit. **Dominik Schmidt** hat durch seine Pionierarbeit an *S. nigrum* meine Arbeit erst ermöglicht und mir mit seiner Erfahrung beim Schreiben meines ersten Manuskripts zur Seite gestanden. Auch wenn **Antje Wissgott** zwar nicht direkt zur Solanum Gruppe gehört, so hat sie sich durch ihre Hilfsbereitschaft, ihr Wissen und ihre fröhliche Art schon längst eine Ehrenmitgliedschaft verdient.

Ashok Giri hat mir mit seinem theoretischen und praktischen Erfahrungsschatz wichtige Starthilfe für das PI-Projekt gegeben. Außerdem erachte ich es als einen Glücksfall ihn persönlich näher kennengelernt zu haben, da er für mich mit seiner ruhigen, überlegten und lebensfrohen Art ein Vorbild darstellt. Ich bin ihm unendlich dankbar dafür, dass er mir die indische Küche nähergebracht hat und es ermöglicht hat am Proteomics-Workshop in Pune teilzunehmen.

Adriana Prehl danke ich für die Hilfe mit langwierigen Raupen-Experimenten und für die dabei geführten Gespräche. **Harleen Kaur**, **Austin Hackett** und **Susanne Linderman** boten mir eine

großartige Unterstützung bei zahlreichen Experimenten und haben mir, mit all ihrer Nachsicht, die Möglichkeit gegeben, meine Fähigkeiten als Betreuer besser zu entwickeln.

Merijn Kant danke ich für seine wertvolle Hilfe und Initative bei der Auswertung der Microarrays und für seine Bereitschaft sich über anderer Leute wissenschaftliche Probleme ernsthaft den Kopf zu zerbrechen. **Rayko Halitschke** und **Caroline von Dahl** waren zwei der besten Quellen für hilfreiche wissenschaftliche Informationen und außerdem verantwortlich dafür, dass ich für einige Zeit im Klettern einen idealen Ausgleich zum wissenschaftlichen Alltag gefunden habe.

Jens Schwachtje und **Michael Stitz** danke ich dafür, dass sie sich für nahezu alles interessieren und ihr Wissen auch auf lebhafte und sehr unterhaltsame Art mitzuteilen vermögen. Das hat so manchen Weg zur Mensa und so manchen Abend zu kurz erscheinen lassen.

Ohne die Hilfe von **Jan Kellmann** und **Karin Groten** wären die Feldversuche in Dornburg ungleich schwieriger geworden. Durch sie konnten wir uns auf die Wissenschaft konzentrieren.

Hendrik Wünsche danke ich für die Hilfe im Proteomicslabor und zahlreiche interessante Diskussionen. **Alexander Muck** und **Ales Svatos** danke ich für ihre große Hilfsbereitschaft und die vielen Versuche PIs per LC-MS/MS zu identifizieren.

Meine Arbeit wäre ohne **Tamara Krügel** und ihr **Gärtnerteam** unmöglich gewesen. Ich danke Ihnen für die Hilfsbereitschaft und die Freundlichkeit, mit der sie versuchen auch noch im unmöglichsten Moment irgendwo einen Platz für ein paar Pflanzen zu finden, und für die professionelle Arbeit, durch die es den Pflanzen fast nie an etwas mangelt.

Die äußerst gründliche Arbeit von **Klaus Gase**, **Thomas Hahn**, **Susan Kutschbach** und **Wibke Kröber** in Klonierung, Transformation, Microarray-Hybridisierung, uvm. ist ein wichtiges Fundament der ganzen Abteilung Molekulare Ökologie und für diesen Dienst kann man ihnen nicht genug danken.

Christian Kost danke ich für viele interessante Diskussionen, gute Ratschläge in statistisch kniffligen Fragen und für seine positive Einstellung.

Emily Wheeler und **Meredith Schuman** bin ich für ihr außerordentliches sprachliches Gefühl und für ihre Geduld, die ewig gleichen dummen sprachlichen Fehler in meinen Manuskripten gewissenhaft auszubessern zu größtem Dank verpflichtet.

Ich danke **Heinz und Irmgard Loferer** für ihre herzliche Art, die vielen schönen Stunden und die ausgezeichnete Verpflegung, durch die ich immer wieder etwas Kraft schöpfen konnte.

Meinen Freunden in Wien bin ich dankbar dafür, dass sie den Kontakt niemals abreißen lassen haben, auch wenn ich mich monatelang nicht gemeldet habe.

Abschließend danke ich der **Max-Planck-Gesellschaft** für die Finanzierung dieses großartigen Institutes, **allen Mitgliedern der Abteilung Molekulare Ökologie** für die Hilfsbereitschaft und die gute Stimmung und allen Personen, die mir in den letzten Jahren zur Seite standen, aber die in dieser Auflistung keinen Platz mehr gefunden haben.

11. SELBSTSTÄNDIGKEITSERKLÄRUNG

Ich erkläre, entsprechend § 5 Abs. 3 der Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich Schiller Universität Jena, dass mir die geltende Promotionsordnung der Fakultät bekannt ist. Ich habe die Dissertation selbstständig und nur unter Zuhilfenahme der im Text angegebenen Quellen und Hilfsmittel angefertigt, wobei alle von Dritten übernommenen Textabschnitte entsprechend gekennzeichnet wurden. Personen, die zu den Experimenten, der Datenauswertung oder der Verfassung der einzelnen Manuskripte beigetragen haben, sind im Kapitel 2 (Manuscript overview) unter Angabe ihrer jeweiligen Beiträge zur Arbeit aufgeführt, oder werden, im Falle von Beiträgen geringeren Ausmaßes, in den Danksagungen am Ende der entsprechenden Manuskripte genannt. Gemäß Anlage 5 zum § 8 Abs. 3 wurde die Beschreibung des von mir geleisteten Eigenanteils von Prof. Ian T. Baldwin, dem Betreuer der Dissertation, mit Unterschrift bestätigt und der Fakultät bei Einreichung dieser Dissertation vorgelegt. Ich habe weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte unmittelbar oder mittelbar geldwerte Leistungen von mir für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Diese Dissertation wurde von mir niemals zuvor als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht. Desweiteren habe ich keine in wesentlichen Teilen ähnliche oder eine andere Abhandlung bei einer anderen Hochschule als Dissertation eingereicht.

_____

Markus Hartl

Jena, am 7. Januar 2010

12. CURRICULUM VITAE

PERSONAL DETAILS

Name:	Markus Herbert Hartl
Date of birth:	February 16, 1978, Vienna
Nationality:	Austria

EDUCATION

Since 2004	PhD thesis: "From plant defense to development: Serine protease inhibitors and their multiple functions in *Solanum nigrum*." Max Planck Institute for Chemical Ecology, Jena Supervisor: Prof. Ian T. Baldwin, Dept. Molecular Ecology Member of the International Max Planck Research School (IMPRS) "The Exploration of Ecological Interactions with Molecular and Chemical Techniques"
1996 – 2003	Diploma studies of ecology at the University Vienna, with emphasis on botany, plant ecology and ecological biochemistry. Diploma thesis: *"Antifungale Stilbenoide der Gattung Stemona unter besonderer Berücksichtigung der stressinduzierten Derivate"*, Supervisor: Prof. Harald Greger, Institut of Botany
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PUBLICATIONS

Hartl M, Giri A, Kaur H, Baldwin IT: Between development and defense: functionally diversified serine protease inhibitors play multiple roles in *Solanum nigrum*. Submitted to The Plant Cell.

Halim V, Muck A, **Hartl M,** Ibáñez AJ, Giri A, Erfurth F, Baldwin IT, Svatoš A (2009): A dual fluorescent/MALDI chip platform for analyzing enzymatic activity and for protein profiling. Proteomics 9, 171-181.

Hartl M, Merker H, Schmidt D, Baldwin IT (2008): Optimized virus-induced gene silencing in *Solanum nigrum* reveals the defensive function of leucine aminopeptidase against herbivores and the shortcomings of empty vector controls. New Phytologist 179, 356-365.

Hartl M, Baldwin IT (2006): The ecological reverberations of toxic trace elements. Current Biology 16, R958-R960 (editorial material).

Schmidt D, Voelckel C, **Hartl M**, Schmidt S, Baldwin IT (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.

Brem B, Seger C, Pacher T, **Hartl M**, Hadacek F, Hofer O, Vajrodaya S, Greger H (2004): Antioxidant dehydrotocopherols as a new chemical character of Stemona species. Phytochemistry 65, 2719–2729.

Hartl M & Kiehn M (2004): Chromosome numbers and other karyological data of four Stemona species (Stemonaceae) from Thailand. Blumea 49, 457-460.

SELECTED ORAL PRESENTATIONS

Hartl M & Baldwin IT (2008): You reap what you sow: how to plant questions about biotic interactions using *Solanum nigrum*. 5th Solanaceae Genome Workshop, Köln.

Hartl M & Baldwin IT (2006): The endogenous and ecological functions of serine-protease inhibitors in *Solanum nigrum*. Workshop under the Max-Planck-India Partnership Program: Proteomic Insights into Plant-insect Interactions, National Chemical Laboratory, Pune, Indien.

Hartl M & Baldwin IT (2006): Studying plant defenses using microarrays. BIORHIZ Workshop: Rhizosphere processes and induced defense, Jena.

SELECTED POSTER PRESENTATIONS

Hartl M & Baldwin IT (2009): The many faces of plant protease inhibitors. FEBS Workshop: Adaptation Potential in Plants, Wien, Österreich.

Hartl M & Baldwin IT (2005): Indigestible diversity in plant defense - unraveling the importance of protease inhibitors in Solanum nigrum. 2nd Solanaceae Genome Workshop, Ischia, Italien.

FIELD EXPERIENCE

2004 – 2007	Ecological field experiments with transgenic *Solanum nigrum* in Dornburg, Thuringia, Germany
2006	Ecological field experiments with transgenic *Solanum nigrum* in the Great Basin Desert, Utah, USA
2001 / 02	Project on ecophysiological life strategies of epiphytes and hemi-epiphytes during a field course on diversity and ecophysiology of tropical plants in Esquinas National Park, Costa Rica. Dept. of Chemical Plant Physiology, University of Vienna.
2000 / 05	International course in tropical ecology hosted by the Tropical Biology Association (University of Cambridge) in Amani Nature Reserve, Tanzania.
1998 / 08	Assistance in experiments on interstitial flow in the hyporheic zone of a mountain stream and its impact on invertebrate distribution. Biological Station Lunz, Austria

Markus Hartl

Jena, am 7. Januar 2010