"… All we have to decide is what to do with the time that is given to us. …"

Bag End, April 3018

Herbivore-Induced Changes in the Transcriptome of *Nicotiana attenuata*

Dissertation

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1. Introduction

1.1. Why Study the Transcriptome?

By means of induced responses plants can defend themselves directly (bottom-up) or indirectly (top-down) against herbivores or compensate for the consequences of herbivory (Karban and Baldwin 1997). The mechanisms of these induced responses can be examined at any stage in the transition from genotype to phenotype, starting from genome organization (genomics) and gene expression (transcriptomics) over protein levels and enzyme activities (proteomics) to metabolite contents (metabolomics).

This thesis focuses on the transcriptional events in plants following attack from different herbivore species. Plants discriminate between mechanical wounding and herbivory (manuscript I) – but can they also recognize by whom they are attacked and tailor their response accordingly? The reasons for addressing this question by studying the transcriptome rather than the proteome or metabolome are diverse. First of all, specificity in gene expression is mediated by the binding of *trans*-activating factors (proteins) to *cis*-acting elements (distinct DNA sequence motifs) in gene promoters, which leads to enhanced or suppressed transcription of the respective gene (manuscript I). Thus specific interactions involving transcription factors can be followed directly by measuring mRNA levels. Second, in most cases of induced responses increases in gene expression precede increases in metabolite levels. Exceptions are preformed defenses such as (1) glucosinolates, which upon caterpillar feeding come into contact with separately stored myrosinase enzymes and are metabolized to repellent and toxic thiocyanates, isothiocyanates and nitriles (‘the mustard oil bomb’, Ratzka et al. 1999) or (2) constitutively produced prosystemin peptides, which release mobile systemin after wounding, which, in turn, mediates systemic wound-inducible proteinase inhibitor production (McGurl et al. 1992). Third, not all transcriptional responses may translate to higher level phenotypic responses, but indicate the perception of environmental signals that is not measurable downstream of transcriptional events. In such cases, antagonistic regulation may play a role. Lastly, a technique developed in Stanford in 1995 - DNA microarray technology - became a standard tool for genome-wide monitoring of gene expression and allows to compare in detail how plants respond to different aggressors as well as to identify new defense-related genes (Reymond 2001).

1.2. Needle(s) in a Haystack – How to Find the Relevant Genes?

The annotations of the nuclear *Arabidopsis* genome (haploid chromosome number 1n = 5) predict between 25,470 and 29,804 genes (Crowe et al. 2003, Schiex et al. 2001, AGI 2000); those for draft sequences of two different rice cultivars expect 33,000-50,000 (Torrey Mesa Research Institute) and 55,000-65,000 (Beijing Genomics Institute) genes and thus place rice (1n=12) on top of all sequenced organisms so far (Bennetzen 2002). Genome sequencing projects for solanaceous crops (tomato, potato, tobacco, 1n=12) are underway and hence no prediction for gene numbers are available yet for close relatives of the ecological model plant *Nicotiana attenuata* (1n=12). Estimates on the proportion of the genome involved in defense ('defensome': pathogen perception, signaling pathways, meatobolite biosynthetic pathways) are summarized by Reymond (2001). For example, an analysis of 1.9Mb contiguous sequence of *A. thaliana* chromosome 4 classified 14% of the genes as being involved in resistance. A microarray analysis revealed 4.3% of 7,000 *Arabidopsis* genes to be involved in systemic acquired resistance (SAR) to pathogen attack.

If we are interested in comparing plant transcriptional responses to different herbivore species, how can we identify the genes that are most likely showing such responses? To find the relevant genes without a complete transcriptome microarray at hand (compare CATMA project, Crowe et al. 2003) two approaches are feasible, a biased and an unbiased one. *The biased approach uses prior knowledge*: for example, herbivores have been found to activate pathogen defense pathways as well as wound response pathways and for many of the genes up and downstream of defense signals (e.g. jasmonic acid - JA, salicylic acid - SA, ethylene, and reactive oxygen species) a role in resistance mechanisms is already established (Walling 2000). Moreover, a wide range of herbivore-induced changes in chemical constituents, including phenolics, terpenes, alkaloids, glucosinolates, cyanogenic glycosides, defensive proteins, and others (Karban and Baldwin 1997), has been measured in many plant species. Based on these findings, numerous hypotheses about putative transcriptional changes may be proposed and many candidate genes identified. In contrast, *the unbiased approach ignores prior knowledge*: plants are attacked by herbivores and changes in the transcriptome of herbivore-treated plants as compared to untreated plants are analyzed by differential screening procedures, such as Differential Display (DDRT-PCR) or subtractive libraries (SHMB). By separating radioactively labeled, randomly amplified fractions of mRNA pools originating from differentially treated plants on polyacrylamid gels (DDRT-PCR) or eliminating commonly expressed transcripts between two pools of mRNA by several hybridizations of driver cDNA (control) with tester mRNA (treatment) (SHMB),

putative differential genes can be cloned. Using these techniques and cDNA-AFLP, a related procedure, 234 expressed sequence tags (ESTs) had been isolated from *Manduca sexta*-infested *N. attenuata* plants (Hermsmeier et al. 2001, Hui et al. 2003, Halitschke et al. 2003). Many of these ESTs represented genes with no previously described role in plant-insect interactions, such as genes involved in photosynthesis, primary metabolism, or transcriptional regulation.

To initiate this project's comparative analysis, the unbiased approach was taken: (1) a subset of *N. attenuata*'s transcriptome, which had been isolated from plants subjected to herbivory by *M. sexta* larvae and *Tupiocoris notatus* bugs, was examined by multiple DDRT-PCR in order to clone new genes and reveal different elicitations between both herbivores and (2) the transcriptome of control plants had been subtracted from that of *T. notatus*-treated plants by SHMB to identify more *T. notatus*-induced genes in addition to the 234 *M. sexta*-responsive genes. A list of putative 'differentials' obtained with both procedures, results from a Northern blot analysis to confirm differential expression of a randomly selected set of 'differentials', and an evaluation of the strengths of both methods are reported in manuscript II.

Further comparative transcriptional analyses were conducted with two customized microarrays, which differed in concept and design. The first array was entirely unbiased in its gene collection, i.e. it consisted of cDNAs of the 234 putative *M. sexta*-responsive clones and six positive control genes. Each cDNA was represented by two independent PCR fragments, which were spotted four times. In addition to these 240 genes, the second array contained sequences from *N. attenuata* and related species comprising genes with known roles in plant defense (e.g. phenylpropanoid synthesis, ethylene synthesis and perception, systemin perception, and pathogen resistance), carbohydrate metabolism (e.g. aldolase, fructokinase, triose-phosphate-isomerase), and nitrogen metabolism (e.g. nitrate reductase, glutamate synthase, asparagine synthetase); genes from cDNA libraries of *N. attenuata* trichomes and flowers; and lastly, the putative *M. sexta*- and/or *T. notatus*-responsive genes cloned by DDRT-PCR and SHMB. Hence, the gene collection for this array was compiled uniting both the unbiased and the biased approach towards gene selection. Moreover, the second array served to verify or falsify differential expression of the putative 'differentials' stemming from DDRT-PCR and SHMB analyses. Instead of cDNAs, gene tags were 50mer oligonucleotides, which were spotted in quadruplicate.

Both arrays were used to analyze shifts in *N. attenuata*'s transcriptome when plants were (a) exclusively, (b) sequentially, and (c) simultaneously attacked by *M. sexta* larvae

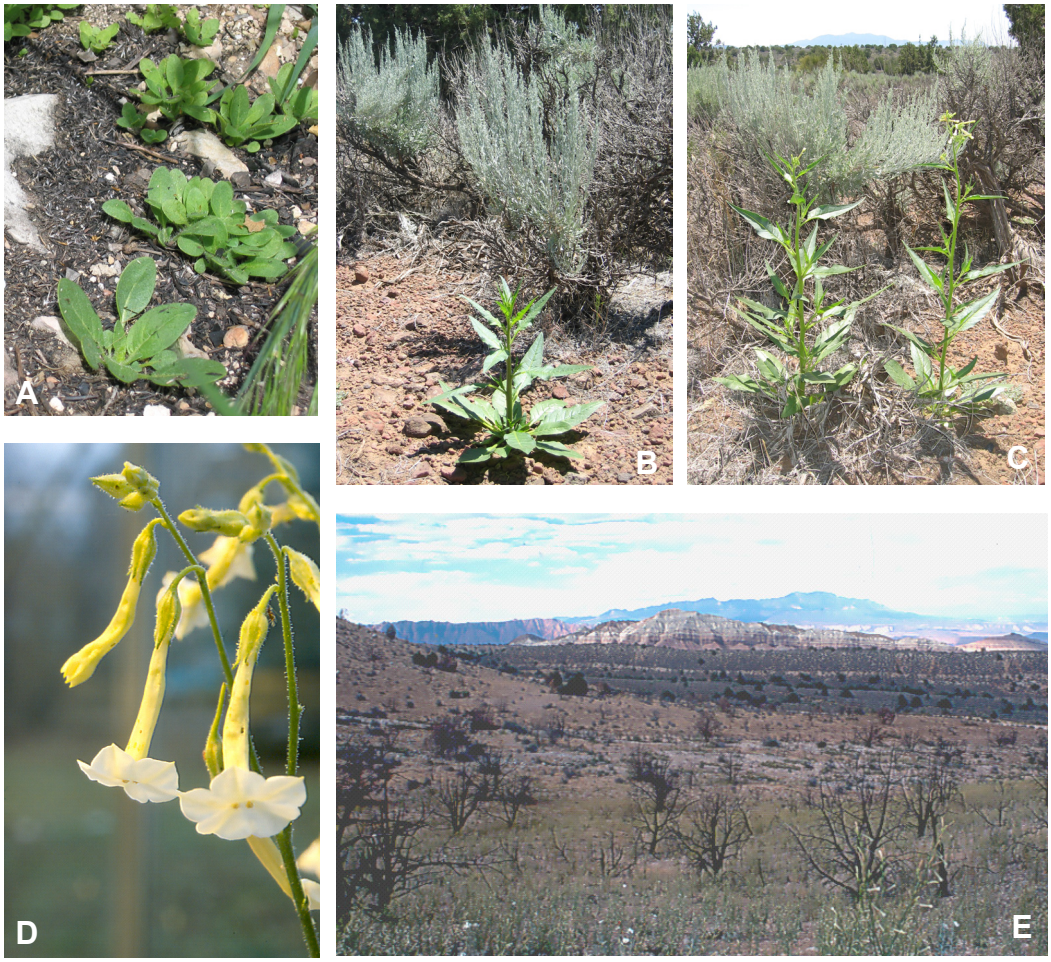
and *T. notatus* bugs (manuscript III). The cDNA array was used to examine a potential transcriptional basis for source-sink manipulation of tobacco metabolism by *Myzus nicotianae* aphids (manuscript IV) and to test whether tobacco plants exhibit a generalized transcriptional response to Lepidoptera attack (manuscript V). Detailed cDNA and oligonuclotide spotting schemes, gene descriptions including accession numbers of *N. attenuata* or foreign sequences, and various data compilation files – all of which are referred to as Supplementary Material in manuscripts III, IV, and V – can be found on the companion CD ROM.

1.3. Introducing the Plant – *Nicotiana attenuata* as a Model System in Chemical Ecology

Nicotiana attenuata Torr. Ex Wats. (synonymous with *N. torreyana* Nelson and Macbr., Solanaceae, 'coyote tobacco') plants native to the Great Basin Desert of North America evolved in the primordial agricultural niche: the immediate post-fire environment. Dormant seeds respond to a combination of germination stimulants in wood smoke and inhibitors from the unburned litter of dominant vegetation and as a consequence synchronously germinate into the nitrogen-rich soils of a post-fire environment. The initially high population densities of this ephemeral pioneer plant decline with the immigration of stronger competitors. Potential herbivores have to recolonize burned areas and establish new populations with every new generation of plants. Hence, this native tobacco encounters highly variable herbivore and pathogen challenges. Nutrient rich soils, high intra-specific competition, variable pathogen loads - these are the habitat parameters *N. attenuata* shares with many crops. The latter, having been extensively bred for yield-enhancing traits, frequently lack the large amount of morphological and chemical phenotypic plasticity found in *N. attenuata.* Elucidating the genetic basis of this plasticity may provide the tools to engineer herbivore resistance back into crops (Baldwin 2001). Molecular research with *N. attenuata* is facilitated by the increasing amount of sequencing information that is available for solanaceous crops (see design of the oligonucleotide array, manuscript III).

N. attenuata (2n=24) is largely self-compatible, but has maintained features for outcrossing. Occasionally it is pollinated by hawkmoths (Sime and Baldwin 2003). Selfing and generation times of 2-3 months predestine this plant for laboratory studies in general and genetic engineering in particular. From *N. tabacum* more than 2,500 secondary metabolites have been identified (Nugroho and Verpoorte 2002), among them isoprenoids, alkaloids, cinnamoylputrescines, and flavonoids, all of which have also been found in *N.*

attenuata. Moreover, *N. attenuata* exhibits natural genotypic variation in defense traits – a prerequisite for studying the evolution of these traits. The Arizona genotype shows no constitutive and jasmonate-induced production of proteinase inhibitors and no herbivore-induced elicitation of the volatile cis-α-bergamotene (Glawe et al. 2003).



Nicotiana attenuata **A** Rosettes on burned soil **B, C** Elongating and flowering plants in front of sagebrush (*Artemisia tridentata*) **D** Flowers **E** Population between burned juniper trees at the Apex mine field site (Saint George, Utah) - Sources: A-C by Rayko Halitschke; D, E by Claudia Voelckel

Lastly, previous investigations of germination cues (Preston and Baldwin 1999), reproductive biology (Sime and Baldwin 2003), induced resistance to insect herbivores (Baldwin 2001), interactions with the third trophic level (Kessler and Baldwin 2001), and competitive interactions and costs of defense (van Dam et al. 2001, Glawe et al. 2003, Zavala et al. 2004) have provided a large amount of information for further studies to build upon. Taken together, studying plant-herbivore interactions in undomesticated *N. attenuata* will copiously complement research of plant-pest interactions in domesticated tomato, potato, and tobacco performed elsewhere.

1.4. Introducing the Herbivores – Guilds, Clades, Host Range, Interactions

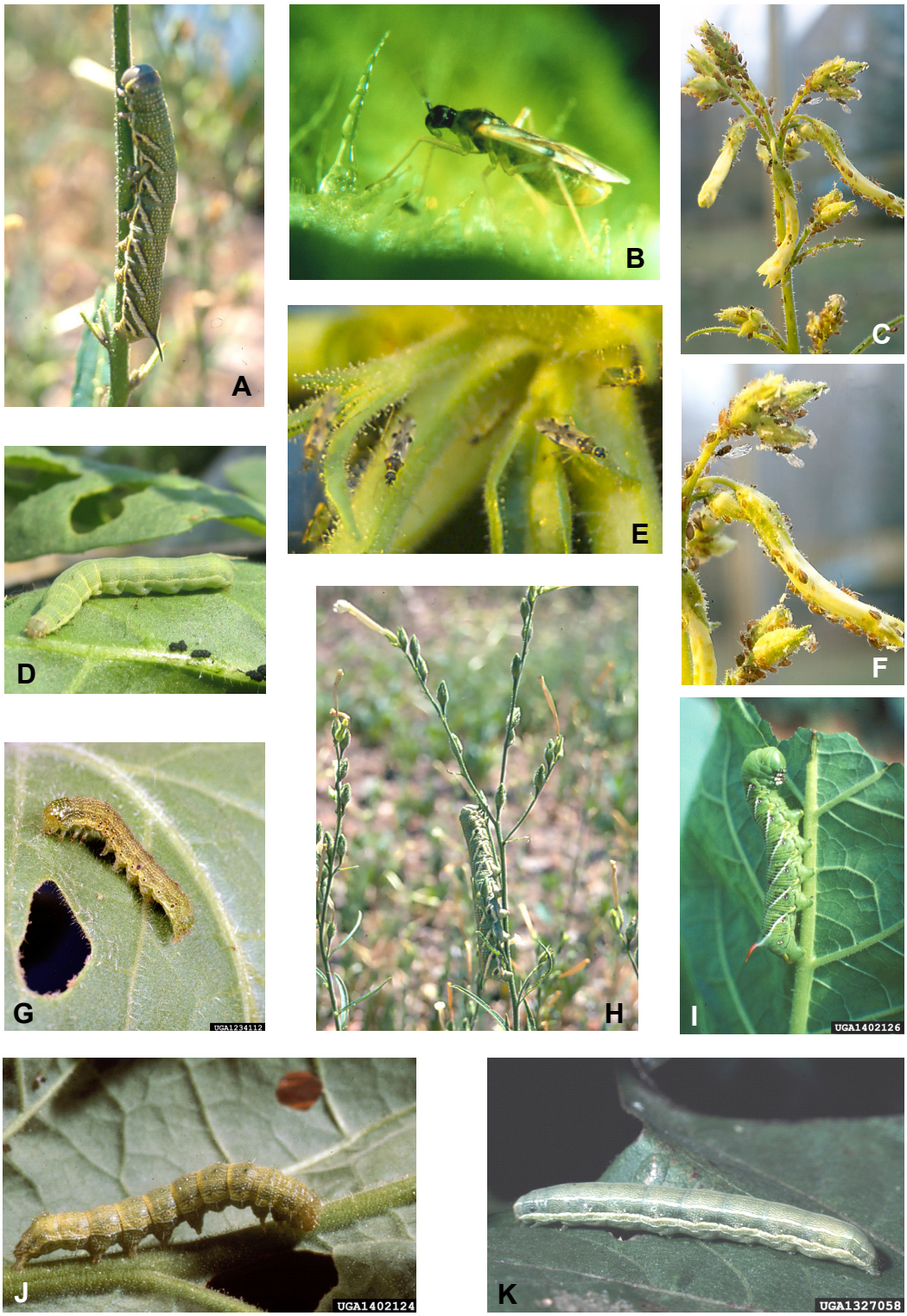
N. attenuata is attacked by herbivores from more than 20 different taxa, including mammalian browsers consuming entire plants as well as intracellular feeding insects, and functions as a host likewise to polyphagous and oligophagous organisms. In other words, it has to cope with herbivores from different feeding guilds, phylogenic clades, and with a different host breadth, all of which may confer specificity to a given herbivore-tobacco interaction. Therefore, what follows is a summary on the feeding behavior, life cycle, phylogeny, indirect interactions, and other characteristics of those herbivores that were chosen for this thesis' transcriptional analyses of host plant responses.

Leaf chewing *Manduca sexta* (Linnaeus) and *Manduca quinquemaculata* (Haworth) larvae are major defoliators of wild *N. attenuata* and a variety of nightshade crops, such as tomato, tobacco, potato, pepper, and eggplant. Two *Proboscidea* species (Martyniaceae) were recently reported as exceptional hosts for *M. sexta* (Mechaber and Hildebrand 2000). An average egg stage of five days, 5-6 larval instars, an average larval development time of twenty days, pupation and diapause in soil, nocturnal moths imbibing nectar from hawkmoth-pollinated flowers and depositing single eggs on the lower leaf surface, a life cycle of 30-50 days, 2-4 generations per year, and geographic ranges from Canada to the southern US (tomato hornworm) and Argentina (tobacco hornworm) characterize these two holometabolous sister species of the sphingid family (Lepidoptera, Sphingidae). Despite being adapted to tobacco foliage, the performance of *M. sexta* larvae was impaired on jasmonate-elicited plants (van Dam et al. 2000), on trypsin proteinase inhibitor (TPI) producing plant genotypes as opposed to genotypes lacking TPIs (Glawe et al. 2003, Zavala et al. 2004), and on wild type plants as compared to plants with reduced nicotine production (Voelckel et al. 2001, Steppuhn et al. in press) and reduced jasmonic acid levels (Halitschke et al. 2003). Its responsiveness to jasmonate-inducible and genetically engineered phenotypic variation renders *M. sexta* an important herbivore in bioassays. Moreover, *M. sexta* has been found to modulate the plant's wound response by (1) an ethylene mediated suppression of nicotine accumulation (Kahl et al. 2000, Winz and Baldwin 2001), (2) TPI induction above wound-induced levels (unpublished results), and (3) larval elicitor-mediated emission of terpenoid volatiles (Halitschke et al. 2001). These manipulations anticipate a large transcriptional reorganization *in planta* in response to *M. sexta* attack.

Tupiocoris notatus (Distant; Heteroptera, Miridae, Dicyphina) is a 'lacerate and flush' feeder on the mesophyll of *N. attenuata* leaves and an abundant herbivore on native plant populations, solanaceaous crops (*N. tabacum, L. esculentum*), and wild relatives (*Datura*

wrightii, Solanum carolinense, S. viarum, and *N. rustica*). Eggs of this hemimetabolous species are laid singly in plant tissue, hatch in about four days, and mold five times before becoming adults. *T. notatus* and relatives have adapted to glandular hosts using specialized claws enabling them to move freely on surfaces with glandular trichomes. They actively ingest trichome exudates containing repellent acylsugars. Whether they exploit the exudates as an extra food source or sequester some of the compounds therein for their own defense remains to be investigated (Van Dam and Hare 1998). Similarly to *M. sexta*, *T. notatus* responds to phenotypic variation in plant defense traits: it prefers genotypes with low or no TPI activity over genotypes with high TPI activity (Glawe et al. 2003, Zavala et al. 2004). Comparable to the *Manduca* sister species, *T. notatus* elicits elevated levels of TPIs, phenolics, diterpene glycosides, and the emission of methylsalicylate, C6 and terpenoid volatiles while feeding on *N. attenuata* plants (Kessler and Baldwin 2001, 2004). Again, these alterations in the plant's proteome and metabolome anticipate preceding and parallel transcriptional signatures.

To sum up, both the hornworms and *T. notatus* are specialist feeders on solanaceous plants and elicit similar changes in metabolite profiles, but differ in feeding modes and herbivorous elicitors. A hornworm's regurgitate (R) contains fatty acid-amino acid conjugates (FACs), which collectively mediate *Manduca*'s modifications of the plant's wound response (Halitschke et al. 2001), but these FACs have not been found in *T. notatus* (A. Roda, A. Steppuhn and I. T. Baldwin, unpublished results) and potential elicitors from mirid saliva remain to be discovered. From a plant's perspective, the fitness consequences of *T. notatus* feeding are negligible while a *Manduca* larva can leave a plant with nothing to reproduce. Interestingly, *Manduca* larvae perform worse and *Manduca* moths oviposit less on plants previously infested with mirids. Direct and indirect mirid-induced defenses slow larval growth and increase predation of the larvae by predatory *Geocoris pallens* bugs. Due to minor reductions of plant fitness caused by *T. notatus* herbivory, mirid feeding 'vaccinates' *N. attenuata* plants against *Manduca* attack in environments where both herbivores are present (Kessler and Baldwin 2004). This plant-mediated competitive interaction between hornworms and mirids may explain the lack of co-occurrence of both herbivores in a given plant population. Because of (1) their high abundance in the field, (2) their specialization on solanceous hosts, including important crops, (3) representing different feeding guilds, (4) eliciting a variety of induced responses, and (5) their (plant-mediated) interspecific competition, *M. sexta* and *T. notatus* were of great interest in this comparative analysis.



Herbivores of *Nicotiana attenuata*. **A, H** *Manduca quinquemaculata* (tomato hornworm) **I** *Manduca sexta* (tobacco hornworm) **B, E** *Tupiocoris notatus* (suckfly) **D, K** *Spodoptera exigua* (beet armyworm) **G, J** *Heliothis virescens* (tobacco budworm) **C, F** *Myzus nicotianae* (tobacco aphids) - Sources: D by Rayko Halitschke; G, I, J, K taken from http://www.ipmimages.org; A-C, E, F, H by Claudia Voelckel

Heliothis virescens (Fabricius) and *Spodoptera exigua* (Huebner) larvae (Lepidoptera, Noctuidae) feed similar to *Manduca* larvae, but are both polyphagous and serious pests to many crops and only occasionally observed on native *N. attenuata* plants. Beet armyworms (*S. exigua*) that originated in Southeast Asia and now occur worldwide are famous in chemical ecology, since the first FAC, volicitin, was isolated from their R (Alborn et al. 1997). The eggs are laid in clusters and young larvae feed gregariously and skeletonize foliage but become solitary as they mature and eat large holes into leaves. Tobacco budworms (*H. virescens*) can be found from Canada to South America. Larvae primarily feed on buds, which leads to big regular holes as leaves expand, but may also tunnel into stalks and midribs of leaves or feed on seed pods. *H. virescens* closely resembles the corn earworm (*Helicoverpa zea*) in appearance and feeding habits.

Phloem-feeding *Myzus nicotianae* (Blackmann) and *Myzus persicae* (Sulzer) aphids (Hemiptera, Aphididae) are important pests, which are barely distinguishable morphologically. Before being described as *M. nicotianae*, tobacco aphids were considered a tobacco-adapted form of the highly polyphagous *M. persicae* (host plants in over forty plant families). Both species exist in several color morphs, are globally distributed, and capable of interbreeding. Meanwhile genetic, biochemical, and behavioral data suggest the synonymy of *M. nicotianae* and *M. persicae* (Clements et al. 2000a, b). *M. persicae* (the green peach aphid) can have over twenty generations in mild climates and complete a generation within 10-12 days; it overwinters in the egg stage on *Prunus* spp. and after several generations winged dispersants deposit nymphs on summer hosts. In cold climates, adults return to *Prunus* spp. in autumn where mating and oviposition occurs. All except for the autumn generation are parthenogenetic. In spite of being a serious pest on tobacco fields, *M. persicae* has not yet been found on *N. attenuata*. Aphid stylets move intercellularly before tapping phloem veins and secrete gelling saliva along the stylet path. Little injury and suppression of plant responses by components of the stylet sheath have been suggested to contribute to an aphid's 'stealthy' feeding behavior (Miles 1999). Although there are reports for a mirid (*Cyrtopeltis nicotianae*) transmitting velvet tobacco mottle virus to *Nicotiana velutina* (Gibb and Randles 1988), among the insects studied here, the aphids were the most likely ones to vector viral plant diseases: with over hundred different transmitted viruses *M. persicae* is considered the most important vector of plant viruses worldwide.

Unless differently cited, most of the information assembled in this section can be accessed via http://ipm.ncsu.edu/AG271/tobacco/tobacco.html and http://creatures.ifas.ufl.edu.

1.5. A New Alliance – Seeking Answers to Ecological Questions with Molecular Tools

Does secondary plant chemistry play a role in determining insect host choice? What are the costs of resistance? Do indirect defenses work in nature? Is herbivore-mediated elicitation equatable to induced defenses? Does a negative correlation between metabolite levels and herbivore performance identify the metabolite as a defensive one? Questions like these have one thing in common: eventually they address the evolutionary forces shaping chemically mediated ecological interactions and are pursuable using techniques from molecular biology and genetics, particularly via the isolation, characterization, and manipulation of genes (Mitchell-Olds et al. 1998). For example, correlative and elicitation studies suggested compounds such as proteinase inhibitors or phenolics to mediate resistance to herbivores. Gene transformation studies, in which the expression of the putative defense genes was manipulated, however, either failed to demonstrate resistance or proved a defensive function (references in Roda and Baldwin 2003).

This thesis measures and compares transcriptional signatures characteristic of different plant-insect interactions from a 'phyto-centric' view. In doing so it produces the prerequisites for manipulative studies addressing the biological function and evolution of plant defense traits, which, in turn, are a prerequisite for the improvement of herbivore resistance in crops.

Manuscript I illuminates the field of gene expression profiling in plant-insect interactions by reviewing related studies conducted before or in parallel to this work and introducing the molecular methods used in the field. It closes with an outlook on how the findings from such studies may generate innovative tools to examine the role of insects in ecosystem function.

Manuscript II describes the cloning of *N. attenuata* genes, whose expression presumably changes in response to *M. sexta* or *T. notatus* attack, by two unbiased, 'ask the plant' cloning procedures.

Manuscript III centers on the question whether wild tobacco plants tailor their responses to a particular attacker by repetitively analyzing transcriptional imprints after 24 hours of *M. sexta* or *T. notatus* attack with a cDNA array and an oligonuclotide array. Additional single species treatments were designed to reveal the decay of the transcriptional response after the attack had ceased or continued. Treatments in which both species sequentially colonized a plant with symmetrical and asymmetrical colonization times were designed to reveal the persistence and erasability of a particular imprint. Treatments in which both species simultaneously colonized a plant with short and long colonization times were designed to

examine plant responses to multiple challenges. Single and multiple herbivore treatments should reveal whether responses are tailored to a specific sequence of events rather than to a particular attacker. Moreover, a multivariate statistical procedure – a principal components analysis – was proven useful in 'extracting' the transcriptional imprint from multidimensional datasets.

Manuscript IV and V both use arrays to test ecological and evolutionary hypotheses. Manuscript IV investigated whether an aphid's feeding preference is correlated with constitutive plant gene expression and if there is transcriptional evidence for an aphid-mediated manipulation of sink-source metabolism known from metabolome studies. Moreover, the manuscript describes how microarray hybridization strategies can be adapted to the relevant biological question. Manuscript V compares *N. attenuata*'s transcriptional response to three lepidopteran larvae, which belong to the same feeding guild and all have FACs in their oral secretion but differ in host range: two are generalists, one is a specialist feeder. Based on the results, the manuscript discusses the mechanism by which specialist herbivores may exploit plant resources more efficiently or tap resources not accessible to generalists and suggests further experiments to test its predictions.

2. List of Manuscripts – Contents and Author's Contributions

Manuscript I

"Herbivore-Specific Transcriptional Responses and Their Research Potential for Ecosystem Studies"

Claudia Voelckel and Ian T. Baldwin

In: Insects and Ecosystem Function, eds. Wolfgang W. Weisser and Evan Siemann,

Springer Berlin Heidelberg, Ecological Studies Vol. 173: 357-379

Section IV - Methods: Reducing, Enhancing and Simulating Insect Herbivory, Chapter 17

This review summarizes mechanisms of transcriptional regulation in plants, studies profiling plant transcriptional response to herbivorous insects (status January 2003), and sketches the methods used in expression profiling. Thus the review provides the groundwork for this thesis' experiments. Being a chapter of the book on "Insects and Ecosystem function" it envisions how a molecular understanding of plant-insect interactions can be used when it comes to monitoring and manipulating these interactions in the field. I am responsible for the selection and integration of the reviewed studies, the compilation of the graphs and tables and wrote the first draft of the manuscript, which was optimized after intense discussions with the co-author Ian T. Baldwin.

Manuscript II

"Detecting Herbivore-Specific Transcriptional Responses in Plants with Multiple DDRT-PCR and Subtractive Library Procedures"

Claudia Voelckel and Ian T. Baldwin

Physiologia Plantarum 2003, 118: 240-252.

This manuscript describes the identification of 77 *Nicotiana attenuata* genes cloned by differential display-reverse transcriptase PCR (DDRT-PCR, 45 genes) and magnetic bead-assisted subtractive hybridization (SHMB, 32 genes), which are putatively differentially expressed in response to either *Manduca sexta* or *Tupiocoris notatus* attack. For a random selection of seven 'differentials' herbivore-induced gene expression was examined by Northern blot analysis (NA). Out of this gene collection four genes were suggested as candidates for further research. The entire collection was part of this thesis' central

transcriptional analysis described in manuscript III. Experimental designs were developed by me and Ian T. Baldwin. Experimental work, RNA isolation, DDRT-PCR, SHMB, cDNA cloning, sequence assembly, and NA were done by me with support from people listed in the acknowledgements. I wrote a first draft of the manuscript, which was refined in close collaboration with Ian T. Baldwin.

Manuscript III

"Herbivore-Induced Plant Vaccination. Part II. Array-Studies Reveal the Transience of Herbivore-Specific Transcriptional Imprints and a Distinct Imprint from Stress Combinations"

Claudia Voelckel and Ian T. Baldwin

The Plant Journal 2004, 38: 650-663

This manuscript represents the principal comparative transcriptional analysis of my thesis. With the combined application of microarray technology and multivariate statistics, it investigates *N. attenuata*'s response to single, sequential, and simultaneous attack by its two most abundant insect herbivores, *M. sexta* and *T. notatus*. While I am responsible for the experimental work, RNA isolations, and statistical analyses, the planning of the experiments and the compilation of the manuscript was a joint effort by me and Ian T. Baldwin. Both the cDNA array and the oligonucleotide array were fabricated by Quantifoil Micro Tools (Jena, Germany). CDNA labeling of RNA samples, chip hybridizations, raw data acquisition, and data normalization were done by Susan Kutschbach, Klaus Gase, Matthias Held, and Thomas Hahn. I was involved in oligonucleotide array design by providing cDNA clones (manuscript II) and selecting heterologous sequences from primary metabolism as templates for oligonucleotide synthesis.

Manuscript IV

"An Analysis of Plant-Aphid Interactions by Different Microarray Hybridization Strategies"

Claudia Voelckel, Wolfgang W. Weisser, and Ian T. Baldwin

Molecular Ecology, in press

Accepted: 02.07.2004

This manuscript examines *N. attenuata*'s local and systemic transcriptional responses to phloem-feeding *Myzus nicotianae* aphids with a cDNA microarray employing two different

hybridization strategies. In addition to testing for an aphid-mediated manipulation of source-sink relations, results are compared those obtained with insects from other feeding guilds (manuscript III). Wolfgang W. Weisser provided the aphids and helped me conducting the experiments; I was responsible for RNA isolation and data analysis; the experimental set up and hybridization strategies were planned by me and Ian T. Baldwin. I wrote a first draft of the manuscript, which was optimized after discussions with both co-authors. Array fabrication and hybridizations were done as described for manuscript III.

Manuscript V

"Generalist and Specialist Lepidopteran Larvae Elicit Different Transcriptional Responses in *Nicotiana attenuata,* Which Correlate with Larval FAC Profiles"

Claudia Voelckel and Ian T. Baldwin

Ecology Letters, 7: 770-775

This manuscript contrasts transcriptional changes elicited by oligophagous *M. sexta* larvae to those from polyphagous *Heliothis virescens* and *Spodoptera exigua* larvae in *N. attenuata* plants. Results are related to larval FAC profiles reported in the literature. I am responsible for data collection and analysis while planning of the experiment and compilation of the manuscript was a joint effort by me and Ian T. Baldwin. Array fabrication and hybridizations were done as described for manuscript III.

Manuscript I

Herbivore-Specific Transcriptional Responses and Their Research Potential for Ecosystem Studies

Claudia Voelckel[1] and Ian T. Baldwin[1*]

In: Insects and Ecosystem Function, eds. Wolfgang W. Weisser and Evan Siemann,
Springer Berlin Heidelberg, Ecological Studies Vol. 173: 357-379
Section IV - Methods: Reducing, Enhancing and Simulating Insect Herbivory, Chapter 17

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24 pages
6012 words
2 Figures
1 Table
65 References

Contents:

17.1. Summary

While simulations of herbivory with mechanical clipping provide many experimental advantages over true herbivory for ecological research, faithful mimicking can be onerous. Not only do herbivores differ in how and what they remove from a plant, they differ in saliva and regurgitate composition, microbial commensalists, pathogen vectoring, feeding phenology, and tritrophic interactions, all of which can dramatically alter a plant's response. These differences in response emerge from alterations in primary and secondary metabolism that are activated by specific signaling pathways and signal recognition systems. Frequently, these responses are under transcriptional control and affect genes involved in hormone biosynthesis and perception, volatile organic compound and secondary metabolite synthesis, photosynthesis, and transcriptional and translational processes, in other words, in metabolism *sensu lato*. We review studies that have identified herbivore-specific transcriptional responses, introduce the molecular techniques used to measure these changes, and argue that research into molecular mechanisms provides ecologists with tools to monitor and manipulate the subtle effects that insects have on ecosystem function.

17.2. The Subtle Effects of Insects on Ecosystem Function

When insects eat leaves, suck sap, shred litter, pollinate plants, they consume net primary productivity (NPP), recycle nutrients, and influence ecosystem function directly through their activities. In addition to these direct effects, the activities of insects are known to alter plant phenotypes, which can indirectly influence ecosystem function. These phenotypic alterations have primarily been described as changes in the concentrations of secondary metabolites (Karban and Baldwin 1997), which, in turn, can influence the 'afterlife' of plant parts and thereby the cycling of nutrients in an ecosystem (Haettenschwiler and Vitousek 2000; Driebe and Whitham 2000; Hartley and Jones - chapter 2). More subtle are the effects of insect-attack on plant metabolism. In order to meet the metabolic demands of the large investments in secondary metabolism and/or structural defenses (thorns, spines), plants reconfigure their metabolism, which frequently has consequences for plant growth and the outcome of competitive interactions (Baldwin 1998; Heil and Baldwin 2002; Glawe et al. 2003). These indirect effects of insect attack on NPP (the 'costs' of resistance) can be large (for a review on cost estimates see Strauss et al. 2002) and add to the direct effects of NPP consumption by insects (compare 10 % annual removal of NPP by herbivores, references in Coley et al. 1985). Since plants play a fundamental role in most ecosystems by providing the energy, nutrient, and material input for all other trophic levels, processes, which alter plant metabolic function, will, in turn, influence ecosystem function. In this chapter, we explore the mechanisms by which insect attack influences plant gene expression and how an understanding of these mechanisms can be used by ecologists to understand ecosystem function.

17.3. Transcriptional Regulation of Plant Responses

All cellular biological processes, including maintenance of metabolic and physiological balance (homeostasis), and responses to the environment are controlled at the level of metabolite production/concentration, enzyme activity, or gene expression. The latter is realized through a cascade comprising transcriptional and posttranscriptional, translational and posttranslational regulation, as well as regulation through protein degradation (Libbert 1993). Since plants are largely immobile, they have evolved a large degree of physiological plasticity to cope with fluctuating environments. For example, *Arabidopsis* dedicates 5.9% of its genome to the production of more than 1,500 transcription factors, which is considerably more than the amount dedicated by the genomes of *Caenorhabditis elegans* (3.5%) and *Drosophila melanogaster* (4.5%), respectively (Riechmann et al. 2000).

Transcription factors can regulate the expression of many genes and are themselves activated by complex signaling pathways, which, in turn, are triggered by various internal and external stimuli. Through such networks of signal transduction cascades, environmental stimuli can alter a plant's 'transcriptome' (the expressed portion of the genome) by both fine-tuning responses of individual genes to specific challenges as well as activating specific sets of genes. Since changes in the transcriptome may not result in altered protein or metabolite phenotypes, studying these transcriptional changes gives researchers the ability to monitor the stimuli that a plant perceives but chooses to ignore (not respond to). In short, transcriptional regulation in all its complexity confers the means to specifically tailor responses to the environment and reveals new insights in the behavioral repertoire of a plant. In the following, we provide a short primer (in the terminological turgidity that characterizes this literature) on transcriptional regulation and an example of a signal transduction cascade that mediates the transcriptional regulation of wound-induced alkaloid production.

A typical eukaryotic gene is composed of several parts. Its transcribed region serves as a template for RNA and protein synthesis and is interspersed with non-coding regions (introns), which are eliminated before translation of the coding regions (exons) (Fig 1A). The transcribed region is flanked on either side by non-coding sequences that can play a role in the regulation of the gene. The first 1 kilo base pairs (kbp) or so of the 5'flanking region are referred to as the gene promoter and contain sequence motifs (*cis*-acting elements, e.g. TATA) that recruit proteins (*trans*-activating factors) that modulate the rate of initiation of mRNA synthesis by the RNA polymerase II complex. *Cis*-acting elements outside the promoter region can either enhance or suppress transcription (Buchanan et al. 2000). See Figure 1 for additional details.

Jasmonic acid (JA), the plant analogue of prostaglandin in animals, is a member of the oxylipin family of signaling molecules that mediate increases in the synthesis of defensive proteins, such as proteinase inhibitors (Farmer et al. 1992), and protective metabolites, such as furanocoumarins (Miksch and Boland 1996), terpenoids (Martin et al. 2002), and alkaloids (nicotine, Baldwin 1999; terpenoid indole alkaloids, TIAs, Aerts et al. 1994) after herbivore attack or wounding. In *Catharanthus roseus*, JA increases the expression of genes involved in TIA synthesis and TIA-precursor formation via *trans*-activating factors called ORCAs (octadecanoid-responsive *Catharanthus*AP2-domain proteins). ORCAs bind to a *cis*-acting element called JERE (jasmonate and elicitor response element) in the promoter region of JA-inducible genes, such as strictosidine synthase (*str*), which catalyzes the initial

Fig. 1

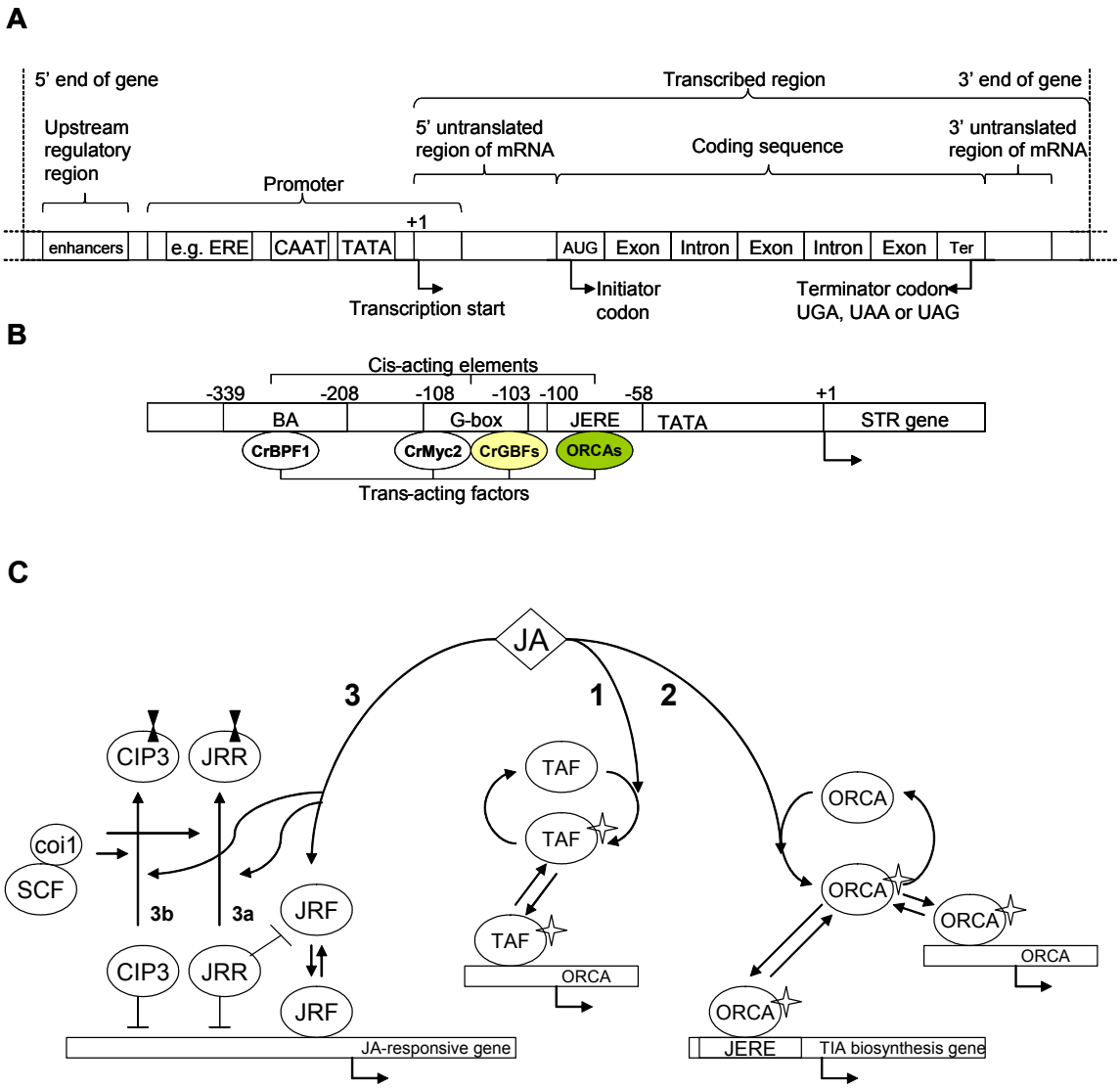

Fig. 1. A Structure and organization of a eukaryotic gene after Buchanan et al. (2000). Basic promoter sequence motifs such as TATA and CAAT, additional promoter elements such as ERE (ethylene response element), and up- or downstream regulatory regions on the same strand as the coding region are called *cis*-elements. Before the RNA transcript (mRNA) serves as a template for protein biosynthesis, non-coding sequences (introns) are eliminated, coding sequences (exons) are fused (referred to as 'splicing'), and the 5' and 3' untranslated regions are posttranscriptionally modified. Open reading frames (ORFs) that are translated into a protein always start with the initiator codon AUG and end with one of the terminator codons UGA, UAA, or UAG. **B** The promoter region of the jasmonate (JA)-inducible strictosidine synthase (*str*) gene after Gantet and Memelink (2002). JA- and elicitor responsive ORCAs (octadecanoid-responsive *Catharanthus*AP2-domain proteins) bind to JERE (jasmonate- and elicitor responsive element) leading to *str* expression; CrGBFs (*C. roseus* G-box-binding factors) bind to the G-Box (5'-CACGTG-3') leading to *str* repression. JA-responsive CrMYC2 (*C. roseus* MYC-type basic helix-loop-helix transcription factor) and elicitor-responsive CrBPF1 (*C. roseus* box P-binding factor 1 homologue) bind to the G-box and the BA-box, respectively, but their

transcriptional activity remains to be discovered. **C** Putative mechanisms regulating transcription factors after Vom Endt et al. (2002) and Devoto et al. (2002). **1.** Regulation of factor abundance by adjusting the production of the encoding mRNA through transcriptional cascades involving transcription activating factors (TAFs). **2.** Regulation of ORCA activity by posttranslational modification (✧) of pre-existing transcription factor protein, which leads to the interaction with cognate elements of responsive genes and/or genes coding for the factors themselves (auto-regulation). **3.** Regulation of factor abundance by adjusting protein turnover rate through COI1-mediated ubiquitination (⧗) and subsequent proteolysis of jasmonate response repressors (JRRs, **3a**) or a histone deacetylase (RPD3b = COI1-interacting protein3, CIP3, **3b**), resulting in the activation of jasmonate response factors (JRFs) and/or increased access of JRFs or other regulators to the regulatory regions, respectively.

step in TIA biosynthesis. However, at least three more types of transcription factors interacting with *cis*-elements other than JERE have been described to regulate the expression of the *str* gene (Gantet and Memelink 2002 and references therein, for details see Fig. 1).

While the expression of a gene is controlled by several transcriptional regulators, the activity of the transcriptional regulators themselves can be controlled by several mechanisms. For example, the peaks of JA-induced ORCA expression precede maximal induction of target genes such as *str,* suggesting the existence of a transcriptional cascade in which a putative transcription activating factor (TAF) promotes ORCA expression (Fig. 1C, 1). However, JA-induced *str* expression is not susceptible to protein synthesis inhibitors, indicating that JA does not induce TIA gene expression simply by increasing ORCA protein abundance, but rather activates pre-existing ORCA protein, which, once activated, binds to the promoters of TIA genes and the ORCA gene itself (Fig. 1C, 2). Protein activity can be regulated through posttranslational modifications (e.g. phosphorylation, acetylation, glycosylation, and others) and/or interactions with other proteins. In the case of ORCA, JA-responsive *str* expression is sensitive to protein kinase inhibitors, suggesting that ORCA phosphorylation is required for ORCA activation and TIA gene expression (Vom Endt et al. 2002).

In addition to regulating transcription factor production, changes in transcription factor abundance can stem from modifications of stability and turnover of these proteins. In *Arabidopis,* JA-induced biosynthesis of indole glucosinolates requires a functional COI1 (coronatine-insensitive1) protein, which is part of the SCF (Skp1/Cul1/F-box protein) complexes that specifically recognize and target proteins for ubiquitin-mediated proteolysis. By analogy to what is known about auxin responses, Vom Endt et al. (2002) propose that JA promotes the binding of JA response repressors (JRR) to the SCF^{COI1} ubiquitin ligase,

which leads to their ubiquitination and subsequent proteasome-mediated degradation. This results in the activation of JA response factors (JRFs) and the de-repression of JA-responsive genes (Fig. 1C, 3a). Moreover, Devoto et al. (2002) characterized COI1-interacting proteins and recovered a histone deacytelase (RPD3b) co-immunoprecipitating with COI1. Histone deacetylation is believed to decrease the accessibility of chromatin to the transcriptional machinery and thereby repressing transcription. They now examine whether COI1 regulates the ubiquitination and proteolytic destruction of RPD3b, representing another mechanism of JA-mediated de-repression of JA-responsive genes (Fig. 1C, 3b).

In summary, all steps involved in converting the information content of a gene into a protein appear to be involved in transcriptional control. Hence, a deep knowledge of the mechanisms underlying transcriptional regulation is required to understand how specific environmental stimuli, such as attack from different herbivore species or feeding guilds, are 'recognized' by a plant. Furthermore, this mechanistic understanding enables molecular ecologists to identify targets for the manipulation of herbivore-induced phenotypes, which, in turn, provide the tools required to elucidate the function and significance of herbivore-induced traits and their significance for ecosystem function.

17.4. Insect-Induced Transcriptional Changes

From a plant's perspective, herbivorous insects differ in their feeding apparatus, saliva composition, the type of plant tissue they attack, the amount of tissue they remove or destroy, and feeding rhythms; in short they can be assigned to different feeding guilds (Root 1973), whose damage has different fitness consequences for the plant. Moreover, the plant-insect interaction is not a binary interaction, since other trophic levels can considerably influence its outcome. Herbivorous insects may or may not vector diseases and they may or may not be negatively affected by predacious insects, parasitoids, or intra- and inter-specific competitors. These ancillary interactions can all be influenced by plant traits, and as a consequence the plant-insect interaction is frequently played out on a spatial scale larger than the plant itself and includes components of the plant's community. Consequently, plants may fend off insect attack by eliciting direct and indirect defenses that influence herbivore performance and survival through bottom-up or top-down control, respectively.

Herbivore attack elicits a myriad of plant responses (Karban and Baldwin 1997; Walling 2000) and some of these responses may be tailored to the particular attacker as has been demonstrated on the level of signal molecules (e.g. JA: Schittko et al. 2000, Ziegler et al.

2001, or ethylene: Kahl et al. 2000), secondary metabolites (e.g. nicotine: Winz and Baldwin 2001), protein amounts and activities (Stout et al. 1994, Tamayo et al. 2000), as well as volatile organic compound (VOC) emission (Halitschke et al. 2001, Kahl et al. 2000). Here we review studies that examine insect-induced changes at an earlier stage of phenotypic expression, namely in alterations in transcript abundance. Insect-induced changes in transcript abundance have been measured using the methods summarized in Fig. 2, either singly or in combinations. Real-time quantitative polymerase chain reaction (RTQ-PCR) and reverse transcription PCR (RT-PCR) are based on the ability of a PCR to exponentially amplify initial differences in transcript number even when the transcripts are present in trace quantities. The amplified products are visualized either in real time during the PCR (RTQ-PCR, Fig. 2B) or after (RT-PCR, Fig. 2A) the reaction. In Northern blot analyses (NA), transcript-specific radioactive probes are used to identify (by sequence-specific hybridization) a target mRNA species within an immobilized RNA sample. The generated signal is proportional to the amount of target mRNA in the sample (Fig. 2, C). In a microarray analysis, the hybridization procedure is reversed: many (e.g. frequently more than 8,000 genes) transcript-specific, unlabeled probes are arrayed on glass slides and competitively hybridized to two RNA pools, which originate from samples of a binary comparison (e.g. control and insect-attacked plants) and are labeled with two different fluorescent dyes. The signal intensities stemming from the two dyes are normalized and expressed as a ratio for each gene. Genes with expression ratios that deviate significantly, either positively or negatively, from 1, indicate up- or down-regulation of a gene, respectively (Fig. 2D). Transcript accumulation in response to various environmental stimuli can also be measured indirectly by transforming plants with promoter:glucuronidase (GUS) constructs (fusions of a promoter of a gene of interest with a reporter gene). In comparison to measuring transcript abundance directly by RT-PCR, RTQ-PCR, NA, or microarray procedures, the activity of the reporter gene, GUS, is measured histochemically. In many cases, the reporter gene activity has faithfully mimicked the expression patterns of the endogenous gene from which the promoter was derived. While these ‘reporter plants’ are only able to report the transcriptional activity of a single gene, they allow for detailed spatial and kinetic analyses of transcript accumulation (Fig. 2E).

Since insect herbivory is inevitably accompanied by wounding, many transcriptional studies compared responses to herbivore attack with those to mechanical damage. These studies confirmed the central conclusion from secondary metabolite analyses, namely that

Fig. 2

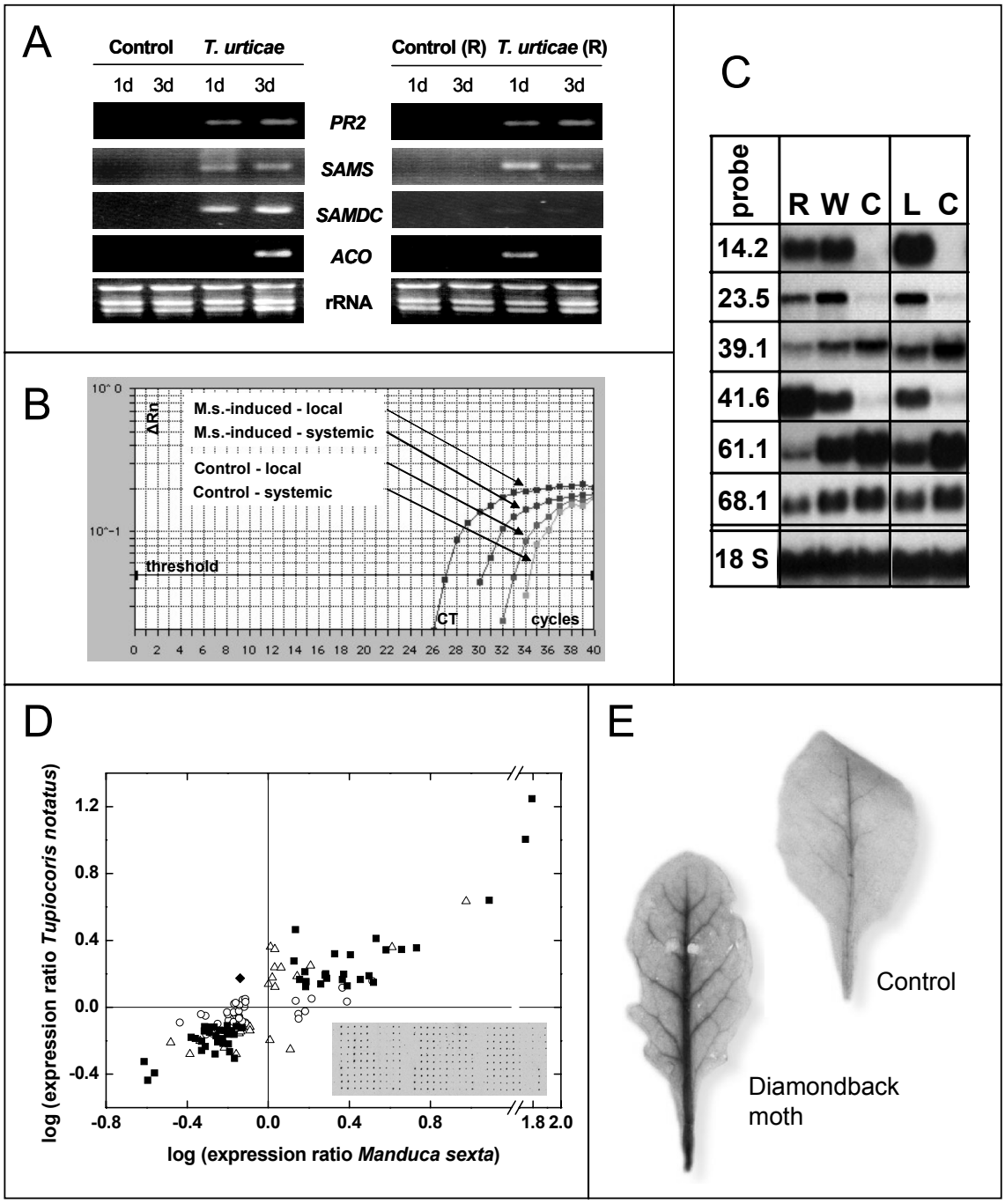

Fig. 2. Examples of commonly applied procedures in gene expression profiling in plant-insect interactions. **A** Reverse transcription-polymerase chain reaction (RT-PCR) analysis of pathogenesis-related protein 2 (PR-2), S-adenosylmethionine synthetase (SAMS), S-adenosylmethionine decarboxylase (SAMDC), and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) transcripts in unattacked Lima bean leaves, leaves infested with the spider mite *Tetranychus urticae*, (receiver-)leaves exposed to volatiles emitted by control leaves (control, R), and (receiver-)leaves exposed to volatiles emitted by *T. urticae*-infested leaves (*T. urticae*, R). After Arimura et al. (2002). **B** Amplification plot, taken from a Real-time Quantitative PCR analysis of trypsin inhibitor (TI) transcripts in *Nicotiana attenuata* plants attacked by *Manduca sexta* larvae, depicting local and systemic induction of TIs. The Cycle Threshold (CT) indicates the number of cycles necessary for an increase

of the reporter signal (Rn) above the background signal (indicated by threshold line) and is inversely proportional to the initial copy number of the transcript. J. Zavala and I.T. Baldwin, unpublished results. **C** Northern blot analysis of threonine deaminase (14.2), α-dioxygenase (41.6), a light harvesting complex II subunit (61.1), and three unknown (23.5, 39.1, 68.1) transcripts comparing expression in wounded+(W)ater-treated and wounded+(R)egurgitate-treated and *M. sexta* 2nd and 3rd instar (L)arvae-attacked tissue with expression in (C)ontrol tissue. Hybridization with an 18 S rRNA probe demonstrates equal loading of samples. After Schittko et al. (2001). **D** Microarray analysis with a cDNA microarray printed with 240 herbivory-related genes comparing changes in *N. attenuata's* transcriptome after infestation with leaf chewers (*M. sexta*) and cell content feeders (*Tupiocoris notatus*). Data evaluation based on statistical significance tests and arbitrary threshold revealed 123 genes to be repressed or induced: 59 were regulated by both species, either similarly (squares, 58) or inversely (diamond, 1), while 40 were regulated only after *Manduca* attack (circles) and 24 only after *Tupiocoris* (triangles) attack. Unresponsive transcripts (117) were omitted; the insert depicts a fluorescence image of the array. C. Voelckel and I.T. Baldwin, unpublished results. **E** Expression of a vegetative storage protein (VSP) as reported by the activity of β-glucuronidase (GUS), which was fused to a *vspB* promoter from soybean and transformed into *Arabidopsis*. Note enhanced GUS expression in the midribs after diamondback moth feeding as compared to control leaves. VSPs are assumed to sequester plant resources during periods of insect attack that will be remobilized when conditions become more favorable. After Berger et al. (2002).

herbivore attack may modify a plant's wound response. Frequently, a stronger and faster induction of a gene is observed after insect herbivory and simulations thereof than after mechanical wounding (Korth and Dixon 1997, Shen et al. 2000, Berger et al. 2002, Table 1). Moreover, wound-inducible genes may only slightly or not at all be induced after insect herbivory (Reymond et al. 2000, Table 1) and there are genes only (Reymond et al. 2000, Table 1) or preferentially (Berger et al. 2002, Table 1) induced by insect feeding as compared to mechanical damage. The wound response of wild tobacco *Nicotiana attenuata* is altered in three different ways when challenged by tobacco hornworm (*Manduca sexta*) larvae. Application of *M. sexta* regurgitant (R) to wounded leaves, which simulates the responses elicited when *M. sexta* larvae attack plants, reduced the accumulation of wound-induced transcripts (e.g. threonine deaminase and two putrescine-methyltransferase genes) or increased both the wound-induced suppression (e.g. a subunit of light harvesting complex II) and wound-induced amplification (e.g. pathogen-induced oxygenase) of many transcripts (Schittko et al. 2001; Winz et al. 2001, Table 1).

Herbivory is associated with a continuum of wounding intensities, which, in turn, is associated with differential gene induction. While leaf chewers, such as lepidopteran larvae or adult beetles, severely wound and remove plant tissues, cell content feeders, such as mites, thrips, or phytophagous bugs, by piercing and sucking out mesophyll cells cause considerable damage but do not substantially decrease a plant's leaf area. Phloem feeders,

such as aphids and whiteflies, must be able to delicately tap into phloem cells without eliciting wound-induced termination of phloem flow and hence feed stealthily with the least amount of damage of all insect herbivores. With their flexible stylets, they move interstitially (fungus-like) before penetrating phloem cells and appear to suppress a plant's wound recognition system. The elicitation of wound-inducible proteinase inhibitor (PI), proteins that interfere with herbivore digestion, reflects this pattern: while PI transcripts increased after herbivory from corn earworm larvae (Fidantsef et al. 1999; Stout et al. 1999), tobacco hornworm larvae (Voelckel and Baldwin 2003), two-spotted spiders (Li et al. 2002), mirids (Voelckel and Baldwin 2003), they do not after whitefly (Walling 2000) and aphid attack (Fidantsef et al. 1999) (Table 1). Interestingly, these stealthy herbivores appear to elicit transcriptional responses characteristic of those elicited by pathogen attack. For example, transcripts of pathogenesis-related (PR) proteins, which are usually involved in conferring systemic acquired resistance (SAR) after pathogen infestation, are induced after feeding of *Macrosiphum euphorbiae*, *Myzus persicae*, and *Brevicoryne brassicae* aphids (Fidantsef et al. 1999; Moran and Thompson 2001; Moran et al. 2002), *Bemisia argentifolii, Trialeurodes vaporariorum* whiteflies (Walling 2000), and *Tetranychus urticae* mites (Arimura et al. 2000a, b). In contrast, in response to attack from grazing herbivores such as *Helicoverpa zea* and *Pieris rapae* larvae, PR transcripts are only weakly elicited (Fidantsef et al. 1999; Reymond et al. 2000, Table 1). Whether this difference in response is due to the more localized and pathogen-like damage resulting from attack by sucking as opposed to chewing insects, or a higher probability of stylet feeders to vector pathogens, remains to be determined.

Evidence is accumulating that plants can distinguish attack from closely related species and even from different stages of the same species. Species-specific changes in transcript accumulation are best exemplified in whitefly-squash and whitefly-tomato interactions. Trancripts coding for a M20B metallopeptidase-like protein were elicited to a much higher level in silverleaf whitefly-infested than in sweet potato whitefly-infested leaves, and for transcripts of a β-glucosidase-like protein, which are induced by both whitefly species in infested leaves, systemic induction was only observed after silverleaf whitefly attack (van de Ven et al. 2000). Transcripts for a subunit of NADPH oxidase (Wfi1) accumulate in local and systemic tomato leaves only after whitefly- but not pink potato aphid feeding (Walling 2000). Interestingly, only whitefly nymphs, but not adults, are responsible for these changes in transcript accumulation (van de Ven et al. 2000; Walling 2000). In contrast, plants appear not to distinguish attack from different instars of the same

lepidopteran species by differences in larval oral secretion chemistry, because R collected from 3rd, 4th, and 5th instars of *M. sexta* was equally active in causing R-induced changes in gene expression (Schittko et al. 2001, Table 1).

By monitoring changes in gene expression for hundreds of genes simultaneously, microarray techniques (Fig. 2D) have allowed plant-insect interactions to be explored at the level of the plant's transcriptome, which has led to important new insights. First, the transcriptional signatures of various stressors may overlap substantially. To paraphrase Shakespeare, stresses in nature, not as single spies but in battalions come (e.g. wounding is often associated with water stress; pathogen attack leads to oxidative stress). As a consequence, plants have not been under selection to perceive well-defined stresses individually. For example, Moran et al. (2002) found some oxidative stress genes, which are usually expressed after ozone fumigation (Sharma and Davis 1994), such as glutathione S-transferases and one form of a superoxide dismutase (SOD) to increase, but others such as another form of SOD and a peroxidase gene to decrease upon aphid infestation. Moreover, Reymond et al. (2000) found that the transcriptional signature of wounding was more similar to that of dehydration than that of *Pieris rapae* feeding. Presumably, *P. rapae* has adopted a feeding strategy that minimizes the effects of water stress on gene expression. Second, a plant's response to herbivory is embedded in a large transcriptional reconfiguration of metabolism *sensu lato.* Hence herbivore attack not only elicits increases in the expression of defense genes (see PRs, alkaloids, PIs, octadecanoids), but also of genes involved in (post-) transcriptional and (post-) translational processes, protein folding- and degradation, membrane transports, hormone- and second messenger synthesis, cell wall modulation, and carbohydrate metabolism and photosynthesis (Reymond et al. 2000; Arimura et al. 2000b; Hermsmeier et al. 2001; Falco et al. 2001; Moran et al. 2002; Hui et al. 2003). Techniques designed to monitor changes in transcription factor abundance hold the potential of elucidating signal pathways, examining aspects of specificity, and investigating large-scale transcriptional changes in the context of plant-insect interactions.

Table 1. Summary of studies examining insect-induced transcriptional changes; plant system, herbivorous elicitors (=insects), investigated genes, and profiling tools are listed for each reference. According to the standards adopted by the *Arabidopsis* community, gene symbols are italicized (lower case letters for mutant genes, capital letters for wild type alleles), while protein products of genes are written in capital letters without italics (http://mutant.lse.okstate.edu/genepage/namerule.html, Meinke and Koornneef 1997). All gene names appear as in the original reference; references are organized by plant taxa. Since the characterization of many of these genes is still in its infancy, classification into functional groups is at present not possible.

Table 1

Plant	Herbivore/Treatment	Induced transcripts	Method	Reference
Phaseolus lunatus (Lima bean)	*Tetranychus urticae* (two-spotted spider mite) and *T. urticae*-induced volatiles	β-1,3-glucanase (PR-2), chitinases (PR-3, PR-4), lipoxygenase (LOX), phenylalanine ammonia-lyase (PAL), farnesyl-pyrophosphate synthetase (FPS), S-adenosylmethionine (SAM) synthetase (SAMS), 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (ACO), SAM decarboxylase (SAMDC)	Polymerase chain reaction after reverse transcription of RNA (RT-PCR)	Arimura et al. 2000a; Arimura et al. 2002
P. lunatus	*T. urticae* and *T. urticae*-induced volatiles	ca. 80 genes related to: pathogenesis/wounding (52%), hormones (3.6%), ethylene biosynthesis (7.7%), flavanoid biosynthesis (1%), transcriptional modifications (2%), translations (13.3%), chaperons (2%), secondary signaling messengers (1.5%), membrane transports 3.6%), protein/peptide degradations (2%), photosynthesis (1.5%)	Microarray with 2032 cDNAs derived from a cDNA library of *T. urticae* and *Spodoptera exigua*-treated *P. lunatus* plants	Arimura et al. 2000b
Lycopersicon esculentum (tomato)	*T. urticae*	Serin PI-II, Serin PI-I, cathepsin D inhibitor (strong increase) LoxD, AOS1 (modest and gradual increase)	Northern blot analysis (NA)	Li et al. 2002
L. esculentum	*Macrosiphum euphorbiae* (potato aphid)/ *Myzus persicae* (green peach aphid)	LOX (modest), proteinase inhibitor II (PINII) (no effect), PR-4 (strong)	NA	Fidantsef et al. 1999
L. esculentum	Whitefly Pink potato aphid	Wfi (NADPH oxidase subunit) RNAs accumulate locally and systemically by whitefly nymphs but not by pink potato aphids or whitefly adults	?	Walling 2000
L. esculentum	*Bemisia argentifolii* (silverleaf whitefly) *Trialeurodes vaporariorum* (green house whitefly)	no accumulation of wound-inducible leucine aminopeptidase (LapA) and pin2, PR (pathogenesis related) transcripts regulated by JA and/or ethylene (basic β-1,3-glucanase, basic chitinase , PR-1) accumulate to higher levels than SA-regulated PR mRNAs (acidic chitinase, acidic β-1,3-glucanase)	?	Walling 2000
L. esculentum	*Helicoverpa zea* (corn earworm)	LOX (weak), proteinase inhibitor II (PINII) (strong) (locally and systemically), PR-4 (weak) (locally and systemically)	NA	Fidantsef et al. 1999 Stout et al. 1999
L. esculentum	*Manduca sexta* (tobacco hornworm)	Earlier and higher accumulation of prosystemin mRNA levels in wild type than in prosystemin antisense plants after herbivory	NA	Orozco-Cardenas et al. 1993
L. esculentum	*Spodoptera littoralis*	Increase in LAP transcripts	NA	Pautot et al. 1993
Solanum tuberosum (potato)	*M. sexta*	PI-II- and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) transcripts accumulate faster after simulated and real herbivory than after mechanical wounding	NA	Korth and Dixon 1997
S. tuberosum	*Leptinotarsa decemlineata* (Colorado potato beetle) *Spodoptera exigua* (beet armyworm)	Both insects induce isoforms of lipoxygenase (H1, H3) and proteinase inhibitor 2 transcripts while feeding on wild-type but not feeding on LOX-H3 depleted plants	NA	Royo et al. 1999
Zea mays (maize)	*S. exigua*	Stronger and earlier stc1 (sesquiterpene cyclase gene 1) transcript induction after real and simulated herbivory than after mechanical damage	NA	Shen et al. 2000
Cucurbita pepo cv. Chefini (squash)	*B. argentifolii* *Bemisia tabaci* (sweet potato whitefly)	M20B metallo-peptidase like protein (SLW1) and β-glucosidase (SLW3) transcripts were expressed locally in response to both species, only after silverleaf whitefly feeding in apical leaves, and only after nymph but not adult feeding	Differential Display (DDRT-PCR), NA	van de Ven et al. 2000
Arabidopsis thaliana	*Plutella xylostella* (diamondback moth)	increased levels of LOX2, a vegetative storage protein (VSP), β-glucosidase 1 (BGL1), glutathione S-transferase 2 (GST2), GST6, a putative calcium-binding elongation factor hand protein (CaEF) in infested rosette tissue	DDRT-PCR, NA	Stotz et al. 2000

Table 1 continued

Plant	Herbivore/Treatment	Induced transcripts	Method	Reference
A. thaliana	*Pieris rapae* (cabage butterfly) *(Pieris brassicae)*	many genes strongly induced by mechanical damage were less or not at all induced after herbivore-attack (e.g. PR-2, phenylalanine ammonia lyase (PAL), cinnamoyl-coA reductase (CCR), o-methyltransferase (COMT)), hevein-like protein gene (HEL) was induced by *P. rapae* larvae but not by mechanical wounding no preferential activation of either jasmonate-dependent or –independent genes by *P. rapae,* no activation of water stress-inducible genes by *P. rapae*	cDNA microarray consisting of 150 expressed sequence tags (ESTs) of defense related genes	Reymond et al. 2000
A. thaliana	*P. xylostella* *Spodoptera littoralis* (Egyption cotton worm)	*S. littoralis* regurgitate accelerates the accumulation of *vsp1* and *vsp 2* preferential induction of *vsp1* relative to *vsp2* by both insect species in contrast to wounding, apparent suppression of systemic *vsp* expression after *S. littoralis* feeding relative to *P. xylostella* herbivory	Quantitative real-time PCR, *vspB*:GUS fusion assays	Berger et. al. 2002
A. thaliana	*M. persicae*	PR-1, an apoplastic form of β-1,3-glucanase (BGL2), a defensin (PDF1.2), LOX2, PAL1, and a sugar transport protein gene (STP4) are induced in aphid-infested rosette leaves and BGL2 also in inflorescence bolts	NA	Moran and Thompson 2001
A. thaliana	*M. persicae*	response profiles at 72 h (micorarray-analysis) and 96 h (macroarray-analysis) contain induced and repressed genes related to oxidative stress, Ca^{2+}/calmodulin signaling, tryptophan-, ethylene-, and aromatic biosynthesis, a hevein-like protein, α-dioxygenase, and endo-transglycosylase	cDNA microarray consisting of 105 ESTs of defense related genes, macroarray consisting of differential ESTs from the preceding microarray, NA	Moran et al. 2002
	Brevicoryne brassicae (cabbage aphid)	Increased levels of PR-1, BGL2, PDF1.2, and STP4 transcripts in infested leaves		
Nicotiana attenuata	*M. sexta*	up- and down-regulation of 27 genes related to photosynthesis (e.g. chelatase), electron transport (cytochrome c-type protein), cytoskeleton (β-tubulin), carbon- (threonine deaminase) and nitrogen- (ferredoxin-dependent Glu synthase) metabolism, signaling (importin α, kinase cofactor GAL 83), and stress, wounding, or pathogen invasion (e.g. Sn-1 gene, dehydration responsive protein, germin, retrotransposon, α-dioxygenase, luminal binding protein)	DDRT-PCR, NA	Hermsmeier et al. 2001, Schittko et al. 2001
N. attenuata	*M. sexta*	increase in putrescine N-methlytransferase I and II transcripts after pre-treatments with ethylene perception inhibitor 1-MCP	NA	Winz and Baldwin 2001
N. attenuata	*M. sexta*	increase of allene oxide synthase (AOS) levels in attacked leaves	NA	Ziegler et al . 2001
N. attenuata	*M. sexta*	73 differentially expressed transcripts: decrease in photosynthesis related transcripts, increase in transcripts associated with protein and nucleotide turnover and carbohydrate metabolism, altered transcript levels for RNA binding proteins, putative ADP/ATP translocators, chaperonins, histones, water channel proteins, terpenoid-, cell wall-related, and oxylipin-related transcripts	DDRT-PCR, subtractive library (SHMB), c-DNA microarray with genes related to real- and simulated *M. sexta*-herbivory	Hui et al. 2003
N. attenuata	*M. sexta/Tupiocoros notatus* (suckfly)	altered expression of photosynthesis-related transcripts (small subunit of RuBPCase, RuBPCase activase, cytochrome f, phosphoglycerate kinase, photosystem IE, plastidic aldolase), rhamnosyl-transferase, thionin, SAM-decarboxylase, phospholipase C, trypsin inhibitors, and others after mirid attack	DDRT-PCR, SHMB, NA	Voelckel and Baldwin 2003
Saccharum officinarum (sugarcane)	Potential insect pests of sugarcane	transcripts related to signal perception, signaling pathways, defense, and proteolysis	*In silico* gene expression analysis from cDNA libraries of different sugarcane tissues	Falco et al. 2001

17.5. How a Molecular Understanding of Plant-Insect Interactions Can Help Elucidate Ecosystem Function

We are just beginning to appreciate the extent to which biotic interactions influence ecosystem function through the reconfiguration of plant metabolism and the bottom-up ecosystem responses that arise from reconfigured plant metabolism. While ecosystem scientists have tried to measure and manipulate these biotic interactions, the techniques employed have been necessarily coarse-grained and lack the precision with which the interactions are known to occur (Hjältén - chapter 12; Lehtilä and Boalt - chapter 13; Schmitz - chapter 14). Here we provide examples of how molecular tools can be applied to monitor and eventually manipulate these elusive ecosystem functions.

To examine how plant-insect interactions affect ecosystem function requires first and foremost an understanding of the frequency and duration of these interactions. Herbivore-induced responses are a phenomenon largely studied in laboratory experiments and while it is known to be widespread among plant taxa, it is not known whether plants growing in nature are induced most of the time. Inducible reporter systems, i.e. plants transformed with constructs, consisting of insect-inducible promoters and easy-to-measure reporter genes such as β-glucuronidase, could monitor how often particular species or guilds of insects attack plants by indicating how often their attack is 'recognized' as a transcriptional response. Promoters of genes specifically expressed in certain tissues (e.g. roots, trichomes) or catalyzing committed steps in secondary metabolite biosyntheses could report the frequency of below-ground or plant-surface interactions as well as the production of certain metabolites. Promoters for genes active in early stages in signal-transduction cascades that respond to insect attack, such as the early stages in JA biosynthesis (Fig 1C), could be used to monitor the frequency of attack from herbivores which cause extensive wounding, in a manner similar to the approaches used to create reporter plants that monitor bioavailability of a specific contaminant in either soil or water.

Krizek et al. (2003) have recently developed an *Arabidopsis* plant that 'reports' the bioavailability of Ni in a dose-dependent manner. The research team used the Affimetrix *Arabidopsis* GeneChip™ microarrays to analyze the transcriptome of seedlings exposed to Cd, Cu, or Ni and identified *AHB1* (nonsymbiotic hemoglobin), which was strongly up-regulated by Ni. The gene is neither induced by other metals nor by other stresses including cold, dehydration, heat shock, oxidative stress, or wounding. Transgenic plants expressing GUS under the *AHB1* promoter reported on the presence and concentrations of Ni in plant growth media. Although plant-based bioindicators are not as sensitive as microbial

biosensors, they could serve as cheap and effective monitors of plant-available heavy metal contaminations in soils and sediments (Krizek et al. 2003).

A determination of recognition frequencies however does not reveal how often the perception of insect attack results in alterations of plant function, which, in turn, may lead to alterations in ecosystem function. Therefore we would need to monitor genes whose expression reflects commitments to metabolic reconfiguration, e.g. up-regulation of defense and down-regulation of growth processes, after insect attack. Transcription factors, such as ORCAs that regulate genes of primary and secondary metabolism (Fig 1C) are candidate genes and microarray technology will likely identify more of these major 'metabolism switch' genes. In a similar fashion, transgene-based systems have been used to monitor the consequences of exposure to environmental mutagens: transgenic *Arabidopsis* plants, which had been designed to analyze point mutations and homologous recombination events in a GUS transgene, have been specifically applied to evaluate the mutagenicity of ionizing and UV radiation and the toxicity of heavy metal ions (Kovalchuk et al. 2001).

In addition to providing the tools to monitor the frequencies and consequences of plant-insect interactions, transgenic technology will also allow for a manipulation of these interactions. To date, ecosystem consequences of plant-insect interactions have been studied experimentally by the application of insecticides (Siemann and Weisser - chapter 18) and caging (Schmitz - chapter 14) in order to exclude insects; by mechanical damage and leaf removal treatments (Hjältén - chapter 12; Lehtilä and Boalt - chapter 13) to simulate the feeding activity of insects; and by the introduction of insects to increase herbivore loads and in some cases, create experimental insect outbreaks. While these approaches relay mainly on top-down control of insect populations, bottom-up manipulations, which are mediated by the plant, may provide a powerful manipulation that could be exploited to study ecosystem function. Three examples, in which plants have been transformed with novel genes that affect 'down-stream' resistance traits, serve to illustrate the procedure.

Genes for proteinaceous toxins from *Bacillus thuringiensis* subspecies (BT toxins), which are specifically effective against lepidopteran, dipteran, and coleopteran insects, have been inserted into various crop species. These transgenic crops are widely cultivated in USA, Australia, and China and enjoy greatly reduced herbivore loads (Sharma et al. 2000). Since BT toxins are direct gene products that plants can produce without a measurable metabolic load and since BT toxins are specific to particular insect taxa, one could imagine creating ecosystems of plants transformed with different BT toxins in which herbivory from lepidopteran and coleopteran herbivores was independently manipulated. In contrast, BT-

containing insecticides require repeated applications and are difficult to target to a single plant species.

At an additional level of complexity, plants have been transformed to introduce the entire pathway of a novel secondary metabolite. For example, the biosynthetic pathway of the tyrosine-derived cyanogenic glycoside dhurrin from *Sorghum bicolor* has been engineered into acyanogenic *A. thaliana*, which rendered the transgenic *Arabidopsis* plants more resistant to the flea beetle *Phyllotreta nemorum*. This beetle specifically attacks crucifers (e.g. *Arabidopsis*) and has not evolved mechanisms to detoxify or sequester cyanogenic glycosides. To transfer the dhurrin pathway, plants were transformed with the two multifunctional cytochromes P450 (CYP79A1 and CYP71E1) and a soluble UDPG-glucosyltransferase and were substantially more resistant to flea beetles as compared to plants expressing the cytochrome P450 genes only, the glucosyltransferase only, or plants carrying the empty expression vectors. Thus, increased resistance could directly be attributed to the presence of dhurrin (Tattersall et al. 2001).

Herbivore resistance can also be increased by constitutively expressing the signals that elicit resistance as illustrated in *Zea mays* plants transformed to express a wheat gene that produces hydrogen peroxide (H_2O_2). H_2O_2 contributes to cell wall fortification, elicits pathogen-induced defensive proteins, inhibits pathogen growth, and through lipid peroxidation harms insect development and reproduction. Ramputh et al. (2002) overexpressed a cell wall-localized H_2O_2-generating enzyme (germin) and produced plants less susceptible to attack by the European corn borer *Ostinia nubilalis* (ECB). In addition to a reduction in ECB feeding and growth, stalk tunneling damage was reduced by 50% at plant harvest in all transgenic lines, which should decrease crop losses resulting from plant lodging caused by heavy ECB infestations.

The above three examples provide a means of excluding herbivores from particular feeding guilds (BT toxins) or with particular host-specificity (cyanogenic glycoside intolerant insect species), or tissue specificity (stem miners) by introducing novel genes from other species. Genetic tools can also be used to increase the susceptibility of plants to particular groups of insects by antisense expression-mediated silencing of endogenous genes, which is illustrated by four examples. In 1993, Orozco-Cardenas et al. demonstrated that resistance towards insects could be modulated by genetically engineering a gene encoding a component of the inducible systemic signaling system (prosystemin) that regulates a plant defense response (proteinase inhibitors). *M. sexta* larvae grew much faster on tomato plants constitutively expressing a prosystemin antisense gene. This enhanced

growth was correlated with a severe delay in prosystemin mRNA as well as proteinase inhibitor accumulation in transgenic as opposed to wild type plants. Similarly, antisense-mediated depletion of hydroperoxide lyase (HPL) has identified this enzyme as a major route of 13-fatty acid hydroperoxide degradation, since both of its products, hexanal and 3-hexenal, have highly reduced levels in transgenic potato plants. Although, transgenic and wild type plants did not significantly differ in the expression of wound-induced genes, *Myzus persicae* aphids feeding on the HPL-depleted plants displayed a two-fold increase in fecundity above those feeding on non-transformed plants. Thus, HPL-catalyzed production of C6 volatile aldehydes may be part of a constitutive resistance mechanism against some sucking insect pests (Vancanneyt et al. 2001). The Colorado potato beetle, a specialist leaf feeder on solanaceous plants, and the beet armyworm, a generalist feeder, have greater rates of weight gain on transgenic potato plants devoid of a specific 13-lipoxygenase isoform (LOX-H3), an enzyme involved in JA production (Royo et al. 1999). Suppressing the activity of a trichome gland-specific P450 hydroxylase, Wang et al. (2001) found a decrease in the predominant exudate component, cembatriene-diol, and an increase in its precursor, cembatriene-ol, which is particularly toxic to *Myzus nicotianae* aphids and greatly diminished aphid colonization responses. In summary, by silencing endogenous resistance genes or by expressing novel resistance genes from other species, ecologists could experimentally manipulate the species composition and the feeding behavior of the herbivore community with a degree of specificity not possible with current techniques.

In all of the above-mentioned studies the respective transgenes have been under the control of constitutive promoters. Constitutive gene expression leads to changes in plant metabolism throughout the life cycle of the plant. For ecosystem studies conditional manipulation of biotic interactions may be advantageous. For example, an inducible BT production would allow researchers to time insect removal with a high degree of spatial and temporal precision. Several chemically inducible systems have been developed that enable a precise control over gene expression (Padidam 2003 and references therein). These systems usually contain two transcription units. The first unit encodes a transcription factor that responds to a chemical signal, while the second unit contains a response element (*cis*-element) that binds the activated transcription factor and is fused to the gene of interest. Ideally, these inducible expression systems should have a low basal, but a high induced level of expression and respond rapidly to the addition as well as the removal of the inducer. The inducer itself should be non-toxic to plants, highly specific, and, if intended for field use, environmentally friendly. A recent review of pros and cons of chemically inducible

expression systems highlights ones with attributes conducive to the study of ecosystem processes (Padidam 2003). With the identification of regulatory promoter elements responsive to plant secondary metabolites (e.g. the diterpenoid sclareol, Grec et al. 2003), new, plant-derived inducible expression systems are imaginable. Given the recent discoveries of insect-specific gene activation (Table 1), the genomes of native plants are likely to harbor insect-specific promoters, which, when fused to BT genes, could allow BT expression to be triggered by attack from particular herbivores. Similar constructs for the silencing of endogenous genes would enable insect-activated gene knockouts.

Moreover, artificial transcription factors that allow the activation or suppression of endogenous genes and thereby represent an alternative to antisense mRNA-mediated gene silencing, are being developed. These artificial transcription factors are based on predefined zinc-finger modules of which each recognizes a unique 3 base pair (bp) sequence of DNA (Segal et al. 2003). Six of these modules can identify an 18 bp sequence in the promoter of interest and when fused to activation or repression domains, regulate the expression of the corresponding gene. This new technique is especially valuable for genes for which the relevant endogenous transcription factors are yet unknown.

The utility for ecosystem scientists of these potential monitoring and manipulation tools depends in large part on the discovery of candidate genes and the establishment of efficient transformation protocols for plants with natural history characteristics that are relevant for ecosystem scientists. Both requirements are within grasp, as is suggested by the increasing number of array and other transcriptional studies that are identifying insect-specific genes, as well as the development of transformation procedures for non-model plants from different functional groups (e.g. trees, herbs, grasses, nitrogen fixing plants, etc.) in addition to plants of commercial interests, such as cereals, fruits, vegetables, ornamental, aromatic, and medicinal plants (Bajaj 1999, 2000, 2001a, 2001b). In concert with the recent developments of inducible expression systems and artificial promoters, the biotechnological preconditions for the use of molecular tools in ecosystem studies are gradually being met. Although these molecular techniques come at a substantial price, their potential to precisely monitor and manipulate plant-insect interactions may justify their costs.

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Manuscript II

Detecting Herbivore-Specific Transcriptional Responses in Plants with Multiple DDRT-PCR and Subtractive Library Procedures

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Abstract

Differential display-reverse transcriptase PCR (DDRT-PCR) and subtractive hybridization with magnetic beads (SHMB) procedures were modified to compare the transcriptional responses of the post-fire desert annual, *Nicotiana attenuata* when it is attacked by its two most abundant herbivores: the voracious lepidopteran caterpillars of *Manduca sexta* and the piercing/sucking nymphs and adults of the mirid bug *Tupiocoris notatus*. We compare the relative merits of the two procedures. DDRT-PCR requires less starting material, allows for comparisons of multiple herbivores, and identifies both down- and up-regulated responses, but is more laborious than SHMB. SHMB produced a greater proportion of known sequences (43.8 vs. 35.6%), but the sequences were not significantly longer than those obtained by DDRT-PCR, suggesting that the SMART (switching mechanism at 5' end of RNA transcript) modification of the SHMB procedure did not produce the desired result. Both procedures produced apparent false positives and microarray-based verification of differential expression would be a powerful approach to identify novel genes involved in ecological interactions. During the *N. attenuata*-*T. notatus* interaction, the expression of several photosynthesis-related genes (cytochrome f, psaE, rubisco, rubisco activase, two aldolases, and phosphoglycerate kinase) was altered, which suggests that reprogramming of photosynthesis contributes to the mechanisms mediating tolerance to mirid damage. In addition, *T. notatus*-induced changes in the expression of transcripts coding for a rhamnosyl transferase, a thionin, and an S-adenosylmethionine-decarboxylase are particularly interesting in the context of what is known about defense against herbivores in *N. attenuata*.

Keywords: DDRT-PCR, subtractive hybridization, *Nicotiana attenuata*, *Manduca sexta*, *Tupiocoris notatus*, herbivory, plant transcriptional responses

Abbreviations: A, anchor primer; BiP, luminal binding protein; DDRT-PCR, differential display reverse transcriptase polymerase chain reaction; ER, endoplasmatic reticulum; GS-2, glutamine synthetase 2; HCAs, hydroxycinnamic acid amids; HMGR, 3-hydroy-3-methylglutaryl-coenzyme A reductase; LD PCR, long-distance PCR; MeJA, methyl jasmonate; NA, Northern blot analysis; Pftf, plastid fusion and/or translation factor; PIs, proteinase inhibitors; PR, pathogenesis-related; psaE, photosystem I subunit E; R, arbitrary primer; Rca, rubisco activase; RD22, responsive to dehydration 22; RT, reverse transcription; SAM, S-adenosylmethionine; SAMDC, S-adenosylmethionine decarboxylase; SMART, switching mechanism at 5' end of RNA transcript; ss, single-stranded; SHMB, subtractive hybridization based on magnetic beads; TMV, tobacco mosaic virus

Introduction

Expression profiling of non-model systems is playing an increasingly important role in attempts to identify genes involved in ecological interactions. For example, differential display reverse transcriptase-PCR (DDRT-PCR) was used by Kaijalainen et al. (2002) to study genes responsible for nodulation in the nitrogen-fixing forage legume *Galega orientalis;* by Jones et al. (2002) to identify molecular markers associated with sea louse resistance in Atlantic salmon; by Yoda et al. (2002) to identify WRKY-type transcription factors associated with the hypersensitive responses to tobacco mosaic virus (TMV); by Malatrasi et al. (2002) to identify a putative drought-induced transcription factor in barley; and by Banzai et al. (2002) to characterize differentially expressed mRNAs in response to high salinity in the mangrove *Bruguiera gymnorrhiza*. Additionally, Caturla et al. (2002) used a suppression subtractive hybridization approach to identify genes associated with the release of dormancy after submergence in root primordia.

Nicotiana attenuata, an annual plant of the Great Basin Desert of the Southwestern USA, is attacked by a variety of insect and mammalian herbivores as it germinates from long-lived seed banks after fires (Baldwin 2001). Larvae of *Manduca sexta* and *M. quinquemaculata* and the nymphs and adults of the mirid bug *Tupiocoris notatus* are its most abundant insect herbivores (Kessler and Baldwin 2001, 2002). Whereas *Manduca* larvae are free-living leaf chewers that may defoliate several plants before pupation, mirids, which pierce and suck out mesophyll cells, cause leaf chlorosis, reduce weight and thickness of leaves, but do not remove large fractions of a plant's canopy. Hermsmeier et al. (2001) initiated a DDRT-PCR-based global characterization of the *Manduca*-responsive transcriptome of *N. attenuata* and found 27 transcripts related to photosynthesis, electron transport, cytoskeleton, carbon metabolism, nitrogen metabolism, signaling, stress, wounding, and pathogen invasion or with an unknown function to be either up- or down-regulated after *Manduca* attack. This characterization is ongoing with additional DDRT-PCR and subtractive hybridization experiments (Hui et al. 2003). Herein we examine the transcriptional changes associated with mirid attack to understand the responses elicited by a cell content feeder.

Only recently have researchers investigated plant molecular responses to non-chewing insects. Employing a differential display approach, van de Ven et al. (2000) identified a M20b peptidase (*SLW1*) and a ß-glucosidase like protein (*SLW3*), which are elicited in response to attack from two phloem feeding whitefly species in squash. Nymphs (but not adults) induce these genes, and systemic responses occur only after silverleaf whitefly- (but

not after sweet potato whitefly) attack, suggesting ontogenetic and species-specific elicitation. *Wfi1* (*gp91-phox*), coding for the large subunit of NADPH oxidase, accumulates only after whitefly but not pink potato aphid feeding in tomato (Walling 2000), suggesting distinct elicitation within the phloem-feeding guild. Pathogenesis related (PR) gene expression and protein activities are influenced by aphid attack in *Arabidposis thaliana* (*Myzus persicae*, Moran and Thompson 2001), *Lycopersicum esculentum* (*Macrosiphum euphorbiae, M. persicae,* Stout et al. 1998*,* Fidantsef et al. 1999), *Triticum astivum* cultivars (*Diuraphis noxia*, van der Westhuizen et al. 1998a, b), and redlegged earth mite attack in *Trifolium subterraneum* (Broderick et al. 1997) suggesting similarities in plant responses to phloem- and cell content-feeding insects, and those elicited by pathogen attack. Moreover, a plant's wound response when elicited by chewing insects (e.g. lepidopteran larvae), is frequently altered by factors in the oral secretions and regurgitants of those insects (*M. sexta-L. esculentum*, Korth and Dixon 1997; *M. sexta-N. attenuata*, Schittko et al. 2001, Winz and Baldwin 2001). Taken together, these studies demonstrate that plants perceive and respond to the different amounts of wounding associated with different insect feeding modes and react to species-, guild-, and ontogenetic-specific elicitors to modify their wound responses (Walling 2000).

To initiate a characterization of the mirid-responsive transcriptome in *N. attenuata* and to evaluate its degree of overlap with the *Manduca*-elicited transcriptional signature, we applied two widely used protocols: DDRT-PCR and subtractive hybridization based on magnetic beads (SHMB). Initially introduced by Liang and Pardee (1992), DDRT-PCR has become the preferred method for identifying and cloning differentially expressed genes, as a majority (67%) of gene expression studies filed in Medline databases used this method (GenHunter Corporation, Nashville, TN, USA, 1999). Usually, mRNA populations from two treatments of interest are divided into subpopulations by reverse transcription using either 12 two base-anchored primers (T_nMN, where M=dA/dC/dG and N= dA/dC/dG/dT: Liang and Pardee 1992) or three one base-anchored primers (T_nM: Liang et al. 1994). Subsequently, cDNA subpopulations are PCR-amplified and further divided using the same set of anchor primers (A) in combination with short, arbitrary 5' primers (R), with the PCR products separated on denaturing polyacrylamide gels. Differential bands on autoradiograms are eluted, re-amplified, cloned, and further analyzed. Here, we comparatively display PCR products originating from untreated, *M. sexta*-, and *T. notatus*-attacked *N. attenuata* plants, based on the protocols of Hermsmeier et al. (1998, 2001). This analysis was complemented with a subtractive hybridization of mRNA populations derived

from mirid-attacked and unattacked plants according to a DYNAL SHMB protocol (from procedures developed by Aasheim et al. (1994, 1996) and Sharma et al. (1993)) that uses oligo(dT)coupled paramagnetic beads, in order to clone genes up-regulated by mirid attack.

We present putative differentials obtained with both protocols, discuss their potential role in plant defense, and identify targets for future research. Furthermore, we evaluate both methods with regard to their yield of known and unknown mRNAs, overlap in results, starting material, inherent information content and drawbacks. With Northern blot analysis (NA) we examine the differential expression of 10 DDRT-PCR- and SHMB-derived fragments.

Material and Methods

Plant Growth, Insects Rearing, and Plant Treatments

An inbred line of *Nicotiana attenuata* Torr. ex Wats., originally collected from Southwestern Utah in 1988, was used for all experiments. Seed germination and hydroponic plant growth were conducted as described by Hermsmeier et al. (2001). In separate experiments, 12 of the most similar looking plants in the rosette stage of growth were chosen for DDRT-PCR; whereas 32 plants were selected for SHMB and NA.

The eggs of *Manduca sexta* (Lepidoptera, Sphingidae) from Carolina Biological Supply (Burlington, NC, USA) were hatched at 28 °C. Nymphs and adults of *Tupiocoris notatus* (Hemiptera, Miridae) were taken from a colony started in summer 2000 with individuals from our Utah Apex mine field site (Kessler and Baldwin 2001). For all experiments, 20-25 first-instar larvae were placed on each of four (DDRT-PCR) or eight (SHMB, NA) plants, with one to three larvae per leaf, depending on the leaf size. Similarly, another set of plants was infested with 20-25 *T. notatus* individuals of all stages, and a third set was left untreated. All plants were kept in wood insect cages (30 x 30 x 60 cm, each cage accommodating four plants) to avoid cross-infection. After 24 hours of feeding, the herbivores and their frass were removed, and shoots of plants were harvested, immediately placed in liquid nitrogen, and stored at –80 °C until used for DDRT-PCR, SHMB, and NA.

Multiple DDRT-PCR

Total shoot RNAs from four control plants and four plants exposed to either *M. sexta* or *T. notatus* for 24 hours, respectively, were isolated according to the methods of Pawlowski

Fig. 1

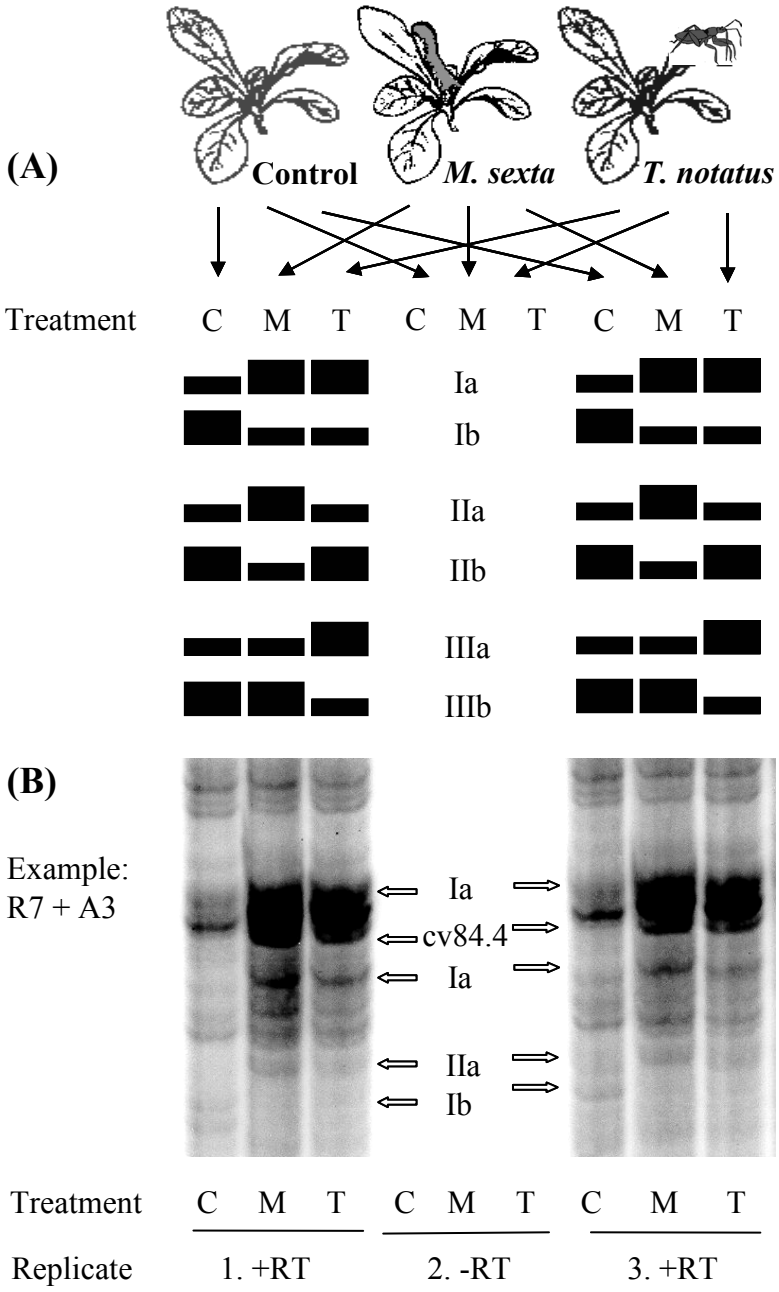

Fig. 1. (A) Scheme for loading PCR products on Differential Display gels when comparing transcriptional responses of unattacked *Nicotiana attenuata* plants to those attacked by two different herbivores (*Manduca sexta* and *Tupiocoris notatus*). A total of nine PCR's (three for each treatment) were performed for each arbitrary primer x anchor primer combination. The sample sequence: - control*, M. sexta, T. notatus* - was loaded in three replicates: 1. +RT-enzyme, 2. -RT-enzyme, 3. +RT-enzyme. Patterns of regulation were categorized as: **Ia,** up-regulated by attack from both herbivores; **Ib,** down-regulated by attack from both herbivores; **IIa,** up-regulated by *M. sexta* attack; **IIb,** down-regulated by *M. sexta* attack; **IIIa,** up-regulated by *T. notatus* attack; and **IIIb,** down-regulated by *T. notatus* attack. **(B)** Example from autoradiograph of primer combination anchor primer 3 x random primer 7 depicting fragments with Ia-, Ib, and IIa-patterns of expression and the Ia fragment cv84.4, which was later identified as a Solanaceae-specific thionin. (Category labels were placed in the middle panel for convenience.)

et al. (1994). The DDRT-PCR procedure was applied as described in Hermsmeier et al. (1998, 2001) and modified as outlined below.

First strand cDNAs were synthesized from 400 ng of purified, DNAse-treated, total RNAs. A total of 90 reactions was performed, since each of the three samples was reverse-transcribed in two replicates and with 10 anchor primers (A1, T_{12}AA, A2, T_{12}AC, A3, T_{12}AG, A4, T_{12}CA, A5, T_{12}CC, A6, T_{12}CG, A7, T_{12}GA, A8, T_{12}GC, A9, T_{12}GG, A10, T_{12}GT, Hermsmeier et al. 2001) (60), and for each treatment and anchor primer combination (30) a quality control without reverse transcriptase was added to monitor for potential RNA contamination by residual genomic DNA, which could be amplified during subsequent PCR. Two arbitrary primers (R1, TACAACGAGG, R7, TCGATACAGG, Bauer et al. 1993) were used for PCR. A total of 180 PCRs (90 reverse transcriptions x two arbitrary primers) were separated on four (45 reactions each) vertical, denaturing gels as outlined in Fig. 1. Using gel-derived autoradiographs as templates, both amplified and suppressed bands were excised, as long as the differential pattern appeared in both replicates. After re-amplification of eluted cDNAs, PCR-products were gel-purified (NucleoSpin® Extract kit, Machery-Nagel, Dueren, Germany) and cloned employing the TOPO TA Cloning® kit (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions (except using only 0.5 instead of 1 µl of the pCR®2.1-TOPO® vector for ligation). Plasmid DNA from at least four clones per transformation was isolated (NucleoSpin® plasmid kit, Macherey-Nagel, Dueren, Germany), EcoRI-digested, and separated on agarose gels to reveal insert size. Inserts of at least two (when both were of equal size) or all clones (when size of inserts varied) were unidirectionally sequenced on an ABI Prism 377 XL DNA sequencer using the M13Forward (-20) primer (supplied with TOPO®) and the BigDye terminator kit (PE-Applied Biosystems, Weiterstadt, Germany). Sequence data were analyzed using the Lasergene software package (DNAstar, Madison, WI, USA) and sequence similarity searches in the GenBank were performed using the BLAST algorithms (Altschul et al. 1997).

Subtractive Hybridization Using Magnetic Beads (SHMB)

mRNA-isolation and preparation of immobilized subtractor cDNA: Total shoot RNA from eight plants, either exposed to 24 hours of attack by *T. notatus* (tester) or unexposed (driver), was isolated. Both polyA-RNA's were captured with paramagnetic Oligo$(dT)_{25}$ beads (Dynabeads: Dynal Biotech., Hamburg, Germany) - using a fourfold excess of driver

over tester RNA (600 and 150 µg, respectively) - according to the manufacturer's instructions. Tester mRNA was eluted from the beads (in 20 µl Tris-HCL, 2 min at 65 °C) and resuspended in 200 µl of hybridization solution (4.5 x SSPE and 0.1 % SDS). Driver mRNA on the beads was directly converted to the complementary first-strand cDNA: with an estimated amount of 12 µg starting material (2 % of 600 µg), the reaction parameters given by the manufacturer's reverse transcription (RT) protocol for SuperScript-II reverse transcriptase (Gibco BRL, Life Technologies, Karlsruhe, Germany, 20 µl reaction volume for an average of 0.3 µg mRNA) were scaled up to a reaction volume of 400 µl. Two 400 µl RT reactions were performed (50 min at 42 °C in a hybridization oven with continuous rolling agitation), then the RT-Mix was removed, the beads resuspended in 20 µl 2 mM EDTA, heated to 95°C for 3 min to melt away the template RNA (which was retained for agarose gel electrophoresis), washed 3 x in TE pH8, re-suspended again in 100 µl TE and combined into one tube. With an estimated 25 % efficiency of cDNA synthesis, a potential yield of 3 µg cDNA (from 12 µg immobilized driver-mRNA) was then hybridized (see next section) to 3 µg of tester-mRNA (2 % of 150 µg tester-RNA).

Subtractive hybridization: After simultaneous heating of both bead-immobilized cDNA pools in TE-buffer and tester-mRNA in hybridization buffer (3 min at 68 °C) and subsequent TE removal, subtractor-beads were resuspended in the 200 µl tester-mRNA containing hybridization solution, and incubated 24 hours at 68 °C in a hybridization oven facilitating the complete mixing of tester and driver molecules. After the first round of hybridization subtractor-beads with cDNA/mRNA hybrids were separated from the hybridization solution, regenerated by eluting mRNA (in 20 µl DEPC-water, 3 min at 95 °C, which was retained for agarose gel electrophoresis) and adding 200 µl TE. Evaporative losses of hybridization solution were adjusted with fresh solution to 200 µl and the entire hybridization procedure was repeated twice. A total of four mRNA-samples (1: eluted driver-mRNA after cDNA synthesis, 2-4: eluted tester-mRNA after three rounds of subtractive hybridization) were separated on a 1.6 % agarose gel on which the expected thinning was observed (DYNAL, 1998). Tester-specific mRNA was captured from the hybridization solution with 20 µl fresh Dynabeads (5-10 min at room temperature). Finally, the target mRNA was eluted in 50 µl DEPC-water (2 min at 65 °C), and after spectrophotometric quantitation, concentrated in a speed-vac to 3 µl for subsequent RT-reaction.

Reverse transcription and Long-Distance PCR (LD-PCR) with target mRNA: The SMART™ (Switching Mechanism At 5' End of RNA Transcript) cDNA Library Construction Kit (Clontech, Heidelberg, Germany) was used to produce full-length cDNA from a limited amount of starting material. A modified oligo(dT) primer (CDSIII 3'PCR Primer, with an Sfi 1B restriction site) primed the first-strand synthesis reaction. The SMART IV Oligo, which has an oligo(G) sequence at its 3'end (before the Sfi 1A site), anneals with the deoxycytidine stretch, which is produced by the reverse transcriptase's terminal transferase activity, thereby creating an extended template. The enzyme then switches templates and continues replicating to the end of the oligonucleotide. The resulting full-length single-stranded (ss) cDNAs contain the CDSIII and the complementary SMART IV sequences, which provide priming sites for the subsequent LD-PCR. Incomplete ss cDNAs will lack the SMART anchor and thus will not be amplified. The incorporation of the asymmetrical Sfi restriction sites facilitates directional cloning into a Sfi 1-digested vector. For RT, LD-PCR, and Proteinase K treatment we exactly followed the manufacturer's protocol.

Sfi 1 digestion, ligation, and cDNA cloning: The plasmid vector pTriplEx2, derived from the phage λTriplEx2, and the cDNA were both subjected to Sfi 1-digestion (final: 1 x BSA, 1 x Sfi 1-buffer, 2 u/µl Sfi 1), before the linearized plasmid was gel-purified (Nucleotrap, Machery-Nagel) and Sfi 1 fragments were removed from the digested cDNA (QIAquick PCR Purification Kit, QIAGEN GmbH, Hilden, Germany). After an overnight ligation (final: 3 u/µl T4 ligase, 1 x T4 buffer), the recombinant plasmids were electroporated into *E. coli* Top10F' cells. Plasmid DNA from the resultant clones was isolated with the NucleoSpin® plasmid kit, Sfi 1-digested, and separated on agarose gels to reveal insert size. From a total of 92 cloned cDNAs the longest 37 (estimated insert size ≥150 bp) were sequenced and sequence data were analyzed as described above.

Northern Blot Analysis (NA)

Total RNA was prepared as described in the SHMB section. Gel electrophoresis of RNA, Northern blotting, probe labeling, hybridizations, and recording of autoradiographs were performed as described in Hermsmeier et al. (2001).

Probe source and amplification primers: Out of the 30 transcripts with homologies (Table 1, 2) 10 were randomly chosen to verify their expression pattern. Three DDRT-PCR- (cv57.4, cv84.4, cv46.1), and seven SHMB- (cvs80, cvs13, cvs52, cvs45, cvs50, cvs53,

cvs43) derived cDNA inserts served as templates for radio-labeling after being PCR-amplified from the pCR2.1-TOPO- and the pTriplEx2-vectors, respectively. Additionally, trypsin inhibitor (repeat region: Glawe et al. 2003) and 18S rRNA fragments from the pUC19 and pCR2.1-TOPO vectors, respectively, were PCR-amplified and radio-labeled for control. Primers used in the radiolabeling PCR were TOPO-F primer (5' CTCATCGATGGATATCTGCAGAATTCGCCC 3') and TOPO-R primer (5' CTCATCGATAGTGTGCTGGAATTCGCCC 3') for the pCR2.1-TOPO-inserts, TEXF primer (5' GTACCCGGGAATTCGGCCAT 3') and TEXR primer (5' AGCTTGCTCGAGTCTAGAGTC 3') for the pTriplEx2-inserts, and Sma 1 primer (5' GAATTCGAGCTCGGTACCC 3') and Sma 2 primer (5' GTCGACTCTAGAGGATCCCC 3') for the pUC19 PI insert (Glawe et al. 2003).

Results

Multiple DDRT-PCR

The procedure aimed at comparing transcriptional changes in *N. attenuata* after 24h *M. sexta* and 24h *T. notatus* attack with those of non-attacked, control plants. With the combination of two single arbitrary primers (R1, R7, selected from a complete set of 26 primers, Bauer et al. 1993) and 10 anchor primers (A1-A10), we investigated $1/13^{th}$ of the plant's insect-responsive transcriptome and found 96 cDNA (R1: 79, R7: 17) fragments to exhibit differential expression on the display gel. Eighty-eight cDNAs (R1: 72, R7: 16) were re-amplified by PCR, subcloned, and sequenced in sets of 2-5 per transformation. After analyzing 222 single-sequence runs, the 88 cDNAs could be assigned to 45 contigs (R1: 29, R7: 16), due to repeated priming of some cDNAs. Sequence similarity searches in the NCBI databases revealed the potential identity of 16 cDNAs (35.6 % of 45, R1: 12, R7: 4; Table 1) while no similarity was found for 29 cDNAs (64.4 % of 45, R1: 17, R7: 12).

Patterns of regulation were categorized as: Ia, up-regulated by attack from both herbivores; Ib, down-regulated by attack from both herbivores; IIa, up-regulated by *M. sexta* attack; IIb, down-regulated by *M. sexta* attack; IIIa, up-regulated by *T. notatus* attack; and IIIb, down-regulated by *T. notatus* attack (Fig 1A). Sixteen cDNAs were similarly up- (Ia:eight) or down- (Ib: eight) regulated in response to attack from both herbivores, 14 cDNAs responded only to *M. sexta* attack (increase IIa: seven, decrease IIb: seven), whereas two cDNAs responded only to *T. notatus* attack (both increased, IIIa). Single transcripts had been amplified with different anchor primers or in different lengths (within the same anchor

Table 1. Summary of 16 "differentials" from multiple DDRT-PCR analyses to which homologies to genes from databases could be assigned. Listed for each transcript are clone-ID, -size, and -accession no, putative ID, functional type (see Discussion), E-value from BLAST alignments, arbitrary primer, number of times this band was cloned from the gel, and category of putative patterns (see caption Fig. 1 for codes, m = multiple, i.e. no prevailing pattern).

Clone	Size (bp)	Accession no.	Sequence similarity	Functional type	E value	Primer	Times cloned	Pattern
cv57.4	444	BU494500	*N. attenuata* pathogen-inducible α-dioxygenase mRNA (AF229926)	Signaling	0.0	R1	25	Ia
cv84.4	306	BU494528	Solanaceae mRNA for flower-specific thionin (Z11748.1)	Stress, Wounding, Pathogens	2E-96	R7	2	Ia
cv14.2	485	BU494506	*G. hirsutum* dehydration-induced RD22-like protein (AAL67991)		6E-29	R1	2	m
cv52.1	258	BU494509	*C. annuum* wound-stimulated protein, Sn-1 gene, vacuolar membrane (S65081)		3E-17	R1	4	m
cv46.1	314	BU494503	*N. tabacum* blp5 luminal binding protein protein (BiP) (X60058)		2E-55	R1	3	IIa
cv63.1	401	BU494502	*N. tabacum* cytochrome f (NP_054512)	Photosynthesis	2E-68	R1	1	Ib
cv95.1	312	BU494530	*P. hybrida* mRNA for UDP rhamnose:anthocyanidin-3-glucoside rhamnosyltransferase (Z25802.1)	Secondary metabolism	4E-33	R7	4	Ia
cv53.3	257	BU494511	*A. thaliana* 3-hydroxy-3-methylglutaryl-coenzyme A reductase 2 (AAF63224)		2E-08	R1	1	Ib
			A. thaliana homoserine kinase (AAD33097)	Miscellaneous	1E-08			
cv8.4	216	BU494501	*N. tabacum* FtsH-like protein Pftf precursor mRNA, nuclear gene encoding chloroplast protein (AF117339)		4E-91	R1	1	IIb
cv12.2	513	BU494505	*A. thaliana* gene_id:K15M2.4 unknown protein (BAA97053)		5E-30	R1	2	m
cv2.4	226	BU494507	*A. thaliana* oxysterol-binding protein (BAA97478)		6E-29	R1	1	IIa
cv15.3	454	BU494508	*A. thaliana* putative mutT protein (AAG52038)		4E-26	R1	1	Ib
cv1.3	369	BU494510	*Z. mays* CL8986-1 mRNA sequence (AY110305)		1E-10	R1	1	IIa
cv96.2	160	BU494531	*A. thaliana* unknown protein (NP_172112.1)		2E-20	R7	1	IIIa
			A. thaliana translation elongation factor 2-like protein (NP_197905.1)	Protein translation	5E-20			
cv11.3	153	BU494504	*N. tabacum* NeIF-4A15 mRNA (X79138)		7E-55	R1	1	Ib
cv81.4	122	BU494529	*N. tabacum* chloroplast 16S-23S rRNA intergenic spacer region + 23S rRNA gene (AY123764)		2E-43	R7	1	IIa

Table 2. Summary of 14 "differentials" from SHMB-analysis to which homologies to genes from databases could be assigned. Listed for each transcript are clone-ID, -size, and -accession no, putative ID, functional type (see Discussion), E-value from BLAST alignments, and category of putative pattern (see caption Fig. 1 for code).

Clone	Size (bp)	Accession no.	Sequence similarity	Functional type	E value	Pattern
cvs43	130	BU494557	*N. tabacum* phospholipase C2 mRNA (AF223573.1)	Signaling	6E-24	IIIa
cvs44	162	BU494556	*N. tabacum* mRNA for TMV response-related gene product (AB024512.2)	Stress, Wounding, Pathogens	1E-28	IIIa
cvs47	372	BU494544	*N. sylvestris* mRNA for the small subunit of rubisco (X01722.1)	Photosynthesis	1E-156	IIIa
cvs60	268	BU494545	*N. tabacum* rca mRNA for rubisco activase (Z14981.1)		1E-127	IIIa
	210	BU494546			1E-100	
cvs80	465	BU494547	*N. sylvestris* psaEa=photosystem I subunit PSI-E (S72356.1)		1E-109	IIIa
cvs53	204	BU494553	*N. paniculata* mRNA for plastidic aldolase (AB027001)		3E-36	IIIa
cvs78	225	BU494549	*N. paniculata* mRNA for plastidic aldolase (AB027002)		1E-60	IIIa
cvs50	147	BU494552	*N. tabacum* mRNA for phosphoglycerate kinase (Z48977.1)		2E-39	IIIa
cvs52	146	BU494555	*N. sylvestris* mRNA for S-adenosylmethionine decarboxylase (AB015609.1)	Secondary metabolism	1E-31	IIIa
cvs45	215	BU494550	*N. sylvestris* GS-2 mRNA encoding glutamine synthetase (X66940.1)	Miscellaneous	1E-47	IIIa
cvs30	85	BU494554	*N. tabacum* BYtuba mRNA for alpha tubulin (AB052822.1)		2E-32	IIIa
cvs25	476	BU494551	*A. thaliana* probable RNA helicase F21E10.1 (T01202)	Protein translation	8E-47	IIIa
cvs13	386	BU494548	*N. tabacum* partial mRNA for elongation factor 2 (AJ299248.1)		3E-74	IIIa
cvs49	239	BU494558	*O. sativa* mRNA, partial homologous to ribosomal protein L7 (D29720)		9E-20	IIIa

primer) and consequently were excised from the gel several times (up to 25 for cv57.4). Therefore, 13 genes (28.9 % of 45) had inconsistent patterns of expression and were not considered to be differentially expressed. However, for repeatedly primed transcripts, tendencies for particular expression patterns could be discerned as in the case of cv57.4 for which 52 % of 25 gel-excised transcripts revealed a Ia expression pattern. Expression patterns were examined with Northern gel blot analysis for three transcripts (cv57.4, cv84.4, cv46.1) in separate experiments of *M. sexta*- and *T. notatus*-attacked plants. A probe for proteinase inhibitors (PIs) was included as a check on the verification procedure, since previous work had established that PI proteins accumulate dramatically after *M. sexta* attack (van Dam et al. 2001). For transcripts of cv57.4 and cv84.4, the Northern analysis revealed a pattern consistent with that of the DDRT-PCR, whereas for cv46.1, a Ia rather than a IIa expression pattern was found (Fig. 2; Table 1). Consistent with previous work, PI transcripts were induced after *M. sexta* attack, and additionally after *T. notatus* attack.

The DDRT-PCR analysis also provided a test of the repeatability of the procedure. Arbitrary primer R1 had been previously used in an experiment with *N. attenuata* plants attacked under the same conditions (24 hours of attack by first instar *M. sexta* larvae) by Hermsmeier et al. (2001). In this study, 53 contigs were obtained from an analysis of an equal blend of root and shoot RNA while in the current study, 29 contigs were obtained from an analysis of only shoot RNA. Approximately half (13) of the contigs from the current analysis had been previously found in the Hermsmeier et al. (2001) study. This overlap might have been even higher had identical starting material been used and demonstrates that the DDRT-PCR produces repeatable results.

SHMB

For the SHMB procedure, we used *T. notatus*-attacked material as the ‘tester’ and unattacked material as the ‘driver’ and therefore selected for genes up-regulated in mirid-attacked plants. From 92 clones, we sequenced 37 ≥150 bp clones, which could be assembled into 32 contigs. BLAST queries (Table 2) revealed the potential identity of 14 cDNAs (43.8 % of 32) while no similarity was found for 18 cDNAs (56.2 % of 32). Expression patterns were examined with Northern gel blot analysis for seven transcripts and up-regulation was confirmed for cvs13 and cvs52 (Fig. 2). Surprisingly, cvs45 and cvs80 were found to be down-regulated (Fig. 2), and cvs50, cvs53, and cvs43 were below detection limits of the Northern analysis.

Fig. 2

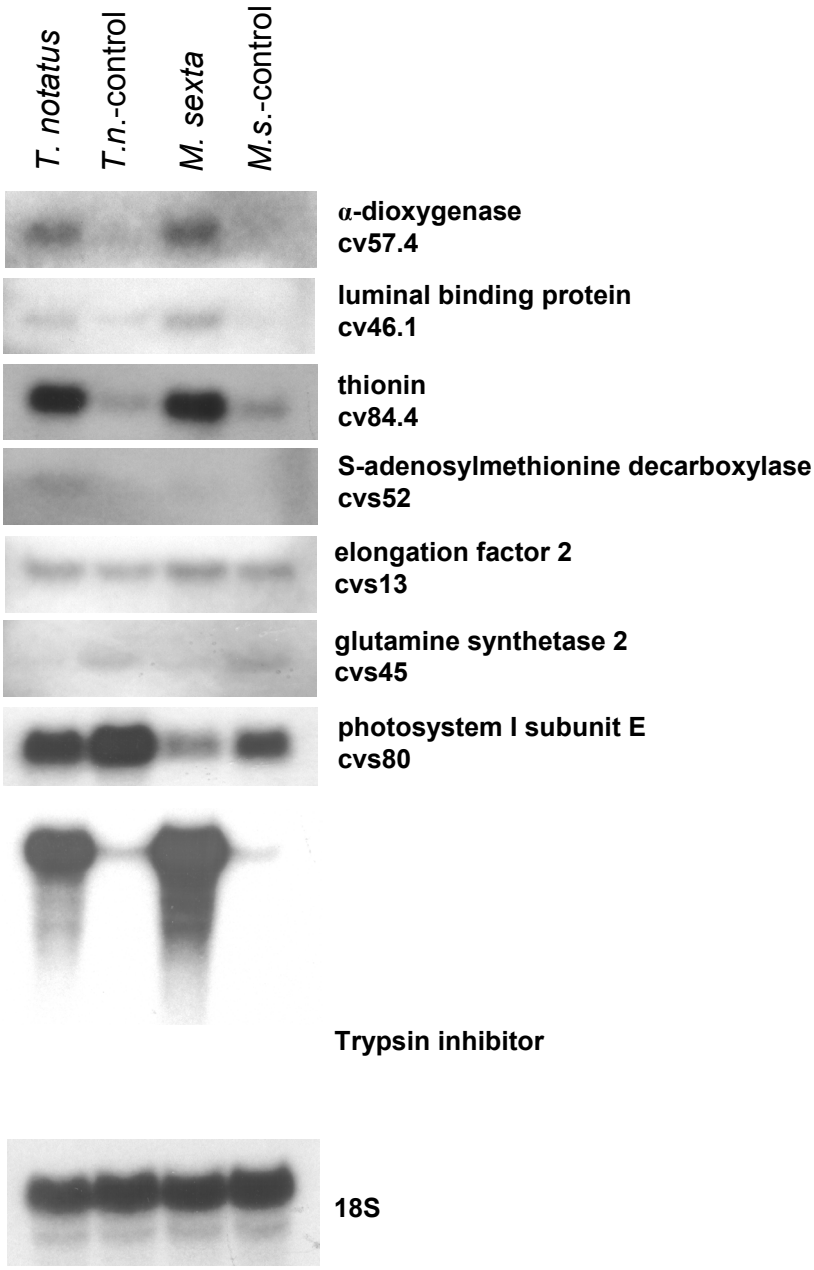

Fig. 2. Verification of transcript accumulation in *Nicotiana attenuata* plants in response to attack from *Tupiocoris notatus* and *Manduca sexta* as compared to their respective controls with Northern gel blots hybridized with probes from 3 DDRT-PCR clones (cv), 4 SHMB clones (cvs), and the *N. attenuata* trypsin PI repeat domain cDNA (Glawe et al. 2002). Hybridization with the 18S rRNA probe served as a loading control.

Discussion

Comparing DDRT-PCR and SHMB Procedures

Multiple DDRT-PCR procedure: An important advantage of DDRT-PCR is that it requires smaller amounts of total RNA (400 ng per RT-PCR, *30=12 μg per treatment) in comparison to the SHMB procedure (see below). Arbitrary primers select for the part of the transcriptome to be displayed, and a full coverage is laborious (requiring 26*90 RT-PCRs and 52 gels). To date, we have only investigated 4/13th (R1-7, R14, Hermsmeier et al. 2001, Hui et al. 2003, this study) of the *M. sexta*-responsive transcriptome. The method reveals all aspects of regulation (up and down), as well as the absence/presence of bands suggesting qualitative differences, and signals with varying intensity suggesting quantitative differences. Repetitive PCR reactions for each primer combination and treatment allow a more strict evaluation of the differential pattern and control for residual genomic DNA contamination, which reduces the chance of cloning false positives. A distinct advantage of the procedure is that multiple treatments can be compared. However, the more arbitrary primers tested, the more repetitive PCRs added, and the more treatments compared, the more costly the method becomes. A disadvantage of the procedure is the high degree of redundancy that results from unspecific anchor-priming and the different-length excisions of the same transcript. This redundancy can cause inconsistent expression patterns. Due to the separation limits of the DD gel, re-amplification of gel-excised fragments can produce multiple clones with only some being differential, thus representing a source of false positives. In short, all transcripts derived from DDRT-PCR require verification before they can be considered differentially expressed.

SHMB procedure: With a fourfold excess of driver (600 μg) over tester (150 μg) total RNA, the procedure requires comparatively large amounts of starting material. However, in contrast to the DDRT-PCR, the full transciptome is queried in one step, but the directionality of regulation (up or down) is determined by the choice of tester and driver. An additional limitation of the procedure is that only pair-wise comparisons are possible. Moreover, decisions about the magnitude of quantitatively expressed transcripts to be detected must be made *a priori*. Here we chose parameters so as to detect a 3 x increase in transcript levels (by using a 4 x excess of driver mRNA, with an estimated cDNA translation efficiency of 25 % and three rounds of highly efficient hybridizations). One of the drawbacks of the DDRT-PCR procedure is that it delivers differentials from the highly gene- and species-specific 3'UTR, which decreases the probability of finding similarities in

the data bases. To increase the probability of cloning ORFs, we modified the SHMB procedure by using the SMART principle (see methods) in order to obtain longer cDNAs comprising 3'UTR+ORFs when amplifying tester-specific mRNA. Interestingly, this goal was not accomplished (note similar size range of clones in Tables 1 and 2). From the presence of GGG residues at 5' end of cloned fragments, we infer that the SMART procedure worked. Therefore the shortness of the fragments obtained suggests that the fragments were already short before amplification (e.g. perhaps due to degradation during hybridization or RNA extraction), or that there was a bias towards shorter fragments either during LD-PCR, ligation, or cloning. Finally, SHMB-derived transcripts also require confirmation before they can be considered to be differentials.

We were surprised to find no overlap in clones obtained from the two procedures and consider the following explanations: the 10 *T. notatus*-up regulated genes from DDRT-PCR (2 x IIIa, 8 x Ia) may not have been found with SHMB because some were up-regulated less than threefold, and others were false positives. On the other hand the 32 *T. notatus*-up regulated genes from SHMB may not have been found with DDRT-PCR, because some of them could not be amplified by the two arbitrary primers tested (but by one of the remaining 24), and others could be false positives. To characterize transcriptional changes in response to two different treatments, the multiple DDRT-PCR with more arbitrary and fewer anchor primers (e.g. three, Liang et al. 1994; to increase coverage with less redundancy) may be comparable to the effort required for four pair-wise comparisons (up- and down-scenarios for each treatment) with SHMB. SHMB is likely to yield slightly more transcripts with similarities to known genes (43.8 %) than DDRT-PCR (35.6 %). However, the drawbacks of SHMB emerging from this study, such as the rate of false positives, and the lack of control over quantification, outweigh its advantage of analyzing the transcriptome in a single step. Given the repeatability of the DDRT-PCR and taking into account its advantages and labor-saving modifications, we consider DDRT-PCR the procedure of choice for analyzing herbivore-specific transcriptional responses in plants.

Both DDRT-PCR and SHMB are 'ask the organism' approaches, which allow researchers to query the transcriptome for an understanding of how an organism responds to an environmental challenge. This approach has the enormous advantage of removing any researcher-imposed bias in identifying target genes, but also highlights the importance of verifying differential expression. Verification by Northern blot analysis is too time consuming when working with a large number of fragments. Indeed, cumbersome verification procedures and a high rate of false positives are perceived as the major

drawbacks of DDRT-PCR (Appel et al 1999). Reverse Northerns (DNA macroarrays) allow for simultaneous hybridization of a suite of target genes immobilized on Nylon membranes with cDNA probes originating from the experimental conditions of interest. Therefore the original (Mou et al. 1994) as well as modified protocols (Voegeli-Lange et al. 1996, Poirier et al. 1997, Poirier and Erlander 1998) are frequently used to verify expression of fragments derived from DDRT-PCR. DNA microarrays enable a simultaneous monitoring of changes in gene expression for a large number of genes immobilized on glass slides, with a sufficient replication (Ramsay 1998, Marshall and Hodgson 1998), thus representing a reliable alternative to Northern and reverse Northern blots. The fabrication of such a cDNA microarray, spotted with clones from several herbivore-induction experiments (including those from this study) is in progress.

Gene Functions

Herein we summarize established functions of the genes identified by both procedures and categorize them according to functional types while in the next section we discuss candidate genes which due to their putative ecological roles will be the subject of future study.

Signaling: a-dioxygenase (cv57.4) produces 2-hydroperoxy fatty acids from C_{18}-unsaturated fatty acids, is induced by pathogens, methyl jasmonate (MeJA), wounding, and herbivory (Sanz et al. 1998, Hamberg et al. 1999, Hermsmeier et al. 2001, this study), but its role in defense is still unclear. Phospholipase C (cvs43) catalyzes the formation of diacylglycerol, an activator of protein kinase C, which, by stimulating protein phosphorylation, may mediate transcription factor binding to elicitor responsive elements and has been shown to be involved in PR-10a expression in tomato (Subramaniam et al. 1997). Isoforms of phopholipase C have also been cloned from potato (Kopka et al. 1998) and *Nicotiana rustica* (Pical et al. 1997).

Secondary metabolism: By decarboxylating S-adenosylmethionine (SAM), SAM decarboxylase (SAMDC, cvs52) provides a precursor for higher polyamines synthesis. UDP rhamnose:anthocyanidine-3-glucoside rhamnosyltransferase (cv95.1) attaches rhamnose to flavanoid-glucosides resulting in runtinoside formation. Cv53.3 shares similarity with *A. thaliana* 3-hydroxy-3-methylglutaryl-coenzyme A reductase 2 (HMGR). This enzyme catalyzes the rate-limiting step in mevalonate synthesis, a precursor for isoprenoid

biosynthesis, and therefore mediates the production of all isoprenoids that arise from mevalonate (Yang et al. 1991).

Stress, Wounding, Pathogens: The Sn-1 protein from bell pepper (cv52.1) is wound-inducible and suggested to be a secretory protein that participates in the early disease resistance response (Pozueta-Romero et al. 1995). Hermsmeier et al. (2001) found the *N. attenuata* homolog (identical to cv52.1) to be down-regulated by *M. sexta*-attack. Luminal binding proteins (BiPs, cv46.1) mediate import and maturation of secretory proteins in the endoplasmatic reticulum (ER), but also remove malfolded proteins during ER stress (Jelitto-van Dooren et al. 1999; Leborgne-Castel et al. 1999). Increased expression of BiPs or BiP-homologs has been reported in white fly-attacked soybean (Kalinski et al. 1995) and in *M. sexta*-attacked *N. attenuata* (Hermsmeier et al. 2001). Thionins (cv84.4) are plant peptides with antimicrobial properties that are increasingly found to be involved in plant-insect interactions (see next section). Cv14.2 encodes a paralog of the cotton responsive to dehydration protein RD22. In *A. thaliana* RD22 transcripts are elicited in vegetative tissue during drought, salt stress, or absicic acid treatment (Yamaguchi-Shinozaki and Shinozaki 1993, Shinozaki et al. 1998). Surprisingly, pDH19.3 (=cv14.2), that exhibited root-restricted expression in a previous study (Hermsmeier et al. 2001), was cloned in this study from a shoot mRNA sample. A transcript with similarity to a TMV response-related gene product (cvs44) was up-regulated after mirid-attack. Another species of mirid (*Cyrtopeltis nicotianae*) is known to vector velvet tobacco mottle virus when feeding on *Nicotiana velutina* (Gibb and Randles 1988). TMV is not vectored by insects, but whether the increase of cvs44 is a response to the insect rather than insect-transmitted viruses, remains unclear.

Protein translation: Cvs 25 and cv11.3 have similarity to a DEAD box RNA helicase of *A. thaliana* and to the translation initiation factor eIF-4A from *N. tabacum*, which represents the prototype and the best biochemically-characterized member of the DEAD box family (Pause et al. 1993, Owttrim et al. 1994, Aubourg et al. 1999, Linder 2000). These helicases promote RNA unwinding and are involved in different molecular mechanisms, such as RNA splicing, ribosome assembly, and initiation of translation (Schmid and Linder 1992, Pause and Sonenberg 1993). Elongation factors (cv96.2, cvs13) aid in the addition of amino acids to the growing polypeptide. L7 proteins (cvs49) are part of the large subunit of eukaryotic ribosomes and 23S rRNA (cv81.4) constitutes the rRNA component of the big subunit of plastid ribosomes.

*Miscellaneou*s: For transcripts related to photosynthesis see next section. Oxysterols and their binding proteins (cv2.4), widely characterized in mammals, also occur in plants

(Lin et. al 1999), where their function remains to be elucidated. Cvs30 encodes a cytoskeletal compound, which is similar to a tobacco α-tubulin. Other cytoskeletal constituents like β-tubulin decrease after herbivory (Hermsmeier et al. 2001), elicitor treatment (Gianfagna and Lawton 1995), or fungal infection (Gross et al. 1993). Cvs45 encodes a plastidic glutamine synthetase (GS-2), which is down-regulated by both herbivores (Fig. 2), in contrast to its discovery by SHMB, which should only yield up-regulated transcripts. GS-2 in combination with ferredoxin-dependent glutamate synthase is involved in the assimilation of ammonium produced by nitrate reduction and photorespiration (Lea 1997). Tobacco seedlings overexpressing GS-2 grew faster than wild type plants, suggesting a key role for GS-2 in biomass production (Migge et al. 2000). Cv53.3 shares similarity to both HMGR and an *Arabidopsis* homoserine kinase, whereby the latter enzyme provides the precursor for methionine, threonine, and isoleucine synthesis in plants (Lee and Leustek 1999, Azevedo et al. 1997). Cv8.4 encodes a plastid fusion and/or translation factor protein (Pftf) homolog with similarity to a Pftf from tobacco, which is an ATP-dependent metalloprotease localized in thylakoid membranes with unknown function (Summer and Cline 1999). MutT proteins (cv15.3) are known from humans and human pathogens and encode phosphohydrolases, which are believed to eliminate toxic nucleotide derivatives from cells and regulate levels of important signaling nucleotides (McLennan 1999). Lastly, cv12.2 and cv1.3 have similarities with unknown transcripts from *Arabidopsis* and maize.

Target Genes for Further Research

In response to herbivore attack, a plant may reduce its conspicuousness, employ direct and/or indirect defenses, or tolerate the attack and increase its growth rates. The display procedures used in this study provide insights into both defense and tolerance mechanisms.

Glandular trichomes on the leaf surface constitute a first barrier to herbivores in *N. attenuata*. With specialized pretarsi, *T. notatus* is able to keep its body away from their sticky exudates and avoids being trapped (Southwood 1986, Schuh and Slater 1995). Mirids will actively ingest exudate droplets from trichomes, potentially to exploit an additional, easy-access, nutritional source (van Dam and Hare 1998) and/or to sequester defensive compounds for their own defense. Additionally, recent bioassay data suggest that mirids are attracted to quercetin, a flavanoid excreted onto the plant surface (Roda et al. 2003). It is in this context that the DDRT-PCR derived transcript for UDP rhamnose-anthocyanidine-3-

glucoside rhamnosyltransferase may function. The gene has been characterized in citrus and *Petunia hybrida* where it catalyzes the formation of the bitter flavanone-glucosides (Bar-Peled et al. 1991) and the color-influencing anthocyanidin-3-rutinoside (Yamazaki et al. 2002, Kroon et al 1994, Brugliera et al. 1999), respectively. In *N. attenuata*, rutin, the rutinoside of quercetin-3-glucoside is stored in the vacuole, whereas the aglycon, quercetin, is excreted onto the leaf surface, where it functions as a phagostimulant for mirids. Thus, the elicitation of glycosyltransferase transcripts by mirid attack may represent a means of reducing a plant's attractiveness to the bugs, by channeling quercetin to the central vacuole, rather than the plant surface.

M. sexta larvae grow slower on *N. attenuata* foliage previously infested with mirids and both herbivores rarely co-occur on plants in the field (A. Kessler and I.T. Baldwin, unpublished data), suggesting that *N. attenuata* mediates a competitive interaction between the two species. Chemical alterations elicited by mirid feeding, such as increased PI and thionin titers, may contribute to this cross-resistance phenomenon. Thionins are small, cysteine-rich plant peptides whose toxicity is due to electrostatic interactions with the negatively charged lipids of the outer leaflet of microbial membranes, which results in pore formation and eventually, membrane collapse (Florack and Stiekema 1994, Zasloff 2002). Furthermore, thionin-mediated inhibitions of enzymes such as β-glucuronidase, insect α-amlyase (sorghum thionin, Bloch and Richardson 1991) and bovine pancreatic trypsin (cowpea thionin, Melo et al. 2002) have been reported. Thionins are predominantly induced after bacterial (Jung and Hwang 2000) or fungal (Oh et al. 1999) infection, but also after wounding and MeJA elicitation (Bohlmann et al. 1998). The insect-responsiveness of thionin transcripts presented here clearly represents an overlap between pathogen-, wounding-, and herbivore-induced plant responses.

The results from the SHMB and NA demonstrated that SAMDC, a key enzyme in the biosynthesis of spermidine and spermine from putrescine (Slocum 1991), is mirid-induced. Interestingly, SAMDC up-regulation was found after infestation of lima bean leaves with the spider mite *Tetranychus urticae* (Arimura et al. 2002), an herbivore with a feeding mode identical to that of *T. notatus*. Polyamines conjugated to hydroxycinnamic acids (hydroxycinnamic acid amides, HCAs) are wide-spread in plants (Flores and Martin-Tanguy 1991), have been implicated in plant defense against viral and fungal pathogens (Torrigiani et al. 1997, Walters 2000, Walters et al. 2001, Walters et al. 2002), are inducible by methyl jasmonate (Lee et al. 1997, Mader 1999, Biondi et al. 2000, Biondi et al. 2001, Keinaenen et al. 2001), and accumulate in *N. attenuata* after *T. notatus* infestation (2.2 fold

increase in caffeoyl putrescine, A. Kessler and I.T. Baldwin, unpublished data). Free polyamines have been implicated in defense against pathogens, as shown by high spermine titers in necrotic lesion forming, TMV-infected tobacco leaves, and spermine-mediated induction of PR-proteins and TMV resistance (Yamakawa et al. 1998). PA levels also increased in response to fungal pathogens in barley (Greenland and Lewis 1984) and tomato (Stroinski and Szczotka 1989). Taken together, SAMDC's products in either free or conjugated forms appear to be involved in plant defense responses.

The ability to compensate for herbivory is more pronounced in some plant species than in others. *N. attenuata* plants when attacked by mirids in the field, suffer almost no reduction in fitness as compared to *M. sexta*-attacked plants (A. Kessler and I.T. Baldwin, unpublished data). The mechanisms mediating such tolerance are only poorly understood (Strauss and Agrawal 1999). Our results implicate photosynthetic regulation (decrease: cv63.1, cvs80; increase: cvs47, cvs60, cvs50, cvs78, cvs53) after mirid attack (Tables 1, 2, Fig. 2), thus we suggest that an adjustment of photosynthesis is involved in *N. attenuata's* ability to tolerate mirid attack. The regulation of photosynthesis is complex and the obtained clones suggest that the targets of regulation may range from proteins involved in photosynthetic electron transport (cytochrome f, psaE) to CO_2 fixation and ribulose bisphosphate regeneration (rubisco, rubisco activase, phosphoglycerate kinase, and two isoforms of a plastidic aldolase). Rubisco activase (Rca) is a stromal, regulatory protein catalyzing the dissociation of inhibitory sugar bisphosphates from uncarbamylated and carbamylated rubisco in a process that requires ATP hydrolysis (Robinson and Portis 1989, Portis 1995), and strongly regulates activity of rubisco, the key enzyme in CO_2 assimilation. To date, Rca has not been shown to be insect-regulated, but represents an important regulation target, worthy of further investigation.

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Manuscript III

Herbivore-Induced Plant Vaccination. Part II. Array-Studies Reveal the Transience of Herbivore-Specific Transcriptional Imprints and a Distinct Imprint from Stress Combinations

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Front cover. Plant-herbivore interactions are played out on many spatial scales from the cellular to the whole-plant and community levels and the vaccination of plants against herbivore attack is a case in point. A plant's induced defenses against attack from a particular herbivore or pathogen species may not only result in resistance to subsequent attack from the same species but also from different species. In a combination of field (left background) and laboratory experiments Kessler and Baldwin (pp 639-649) and Voelckel and Baldwin (pp 650-663) describe how the mirid bug, *Tupiocoris notatus* (right) vaccinates *Nicotiana attenuata* plants (left) against subsequent attack by the more severely damaging hornworm, *Manduca quinquemaculata* (caterpillar in the center). The vaccination effect results from the activation of growth-slowing direct defenses and predator-attracting indirect defenses (which attract the voracious generalist predator, *Geocoris pallens*, shown in the middle between the two herbivores). A detailed microarray analysis (right background) revealed that attack from the different herbivore species elicited different transcriptional imprints, but a similar suite of up-regulated defense-related transcripts and down-regulated growth-related transcripts. The analysis of defense metabolites demonstrated that rather similar plant responses to attack from different herbivores can result in dramatic differences for the plant's fitness in nature, which highlights the value of studying plant-herbivore interactions in the complexity of the natural environment. Picture by Andre Kessler.

Summary

Microarray technology has given plant biologists the ability to simultaneously monitor changes in the expression of hundreds of genes, and yet, to date, this technology has not been applied to ecological phenomena. In native tobacco (*Nicotiana attenuata),* prior attack of sap-feeding mirids (*Tupiocoris notatus*) results in vaccination of the plant against subsequent attack by chewing hornworms (*Manduca sexta).* This vaccination is mediated by a combination of direct and indirect defenses and tolerance responses, which act in concert with the attack preferences of a generalist predator. Here, we use microarrays enriched in herbivore-elicited genes with a principal components analysis (PCA) to characterize transcriptional 'imprints' of single, sequential, or simultaneous attack by these two main herbivores of *N. attenuata*. The PCA identified distinctly different imprints left by individual attack from the two species after 24 hours, but not after 5 days. Moreover, imprints of sequential or simultaneous attack differed significantly from those of single attack, suggesting the existence of a distinct gene expression program responsive to the combination of biological stressors. A dissection of the transcriptional imprints revealed responses in direct and indirect defense genes that were well correlated with observed increases in defense metabolites. Attack from both herbivores elicits a switch from growth- to defense-related transcriptional processes, and herbivore-specific changes occur largely in primary metabolism and signaling cascades. PCA of these polygenic transcriptional imprints characterizes the ephemeral changes in the transcriptome that occur during the maturation of ecologically relevant phenotypic responses.

Additional keywords: *Tupiocoris notatus*, *Manduca sexta*, *Nicotiana attenuata*, microarray, PCA, erasability

Introduction

Sessile organisms, such as plants, respond and adjust their phenotype to their ever-changing environment so as to maximize their fitness. Thus they compensate for their immobility with adaptive phenotypic plasticity, an alternative to coping with environmental variability by moving to more suitable environments (Agrawal, 2001). While mobility is easy to quantify, the subtle metabolic and transcriptional changes that mediate environmentally-elicited phenotypic adjustments are difficult to discern without special techniques. Array technology has provided plant biologists with the ability to monitor changes in transcript abundance of hundreds of genes simultaneously, and these techniques have been used to study, among other things, stress responses in detached leaves (Arimura *et al.,* 2000; Vranova *et al.,* 2002) and responses to the application of signal molecules and in various signaling-enhanced or -deficient mutants (Schenk *et al.,* 2000; Maleck *et al.,* 2000). While the potential of array studies to characterize signal transduction pathways and identify transcription factors of co-regulated genes is being realized, arrays are rarely used to investigate transcriptional responses to biotic challenges or to a combination of ecologically relevant elicitation events.

It is abundantly clear that a plant's response to a biotic challenge is mediated not by simple linear signal transduction cascades but by a network of cross-talking pathways (Dong, 1998; Reymond and Farmer, 1998; Walling, 2000; de Bruxelles and Roberts, 2001), which presumably enables a fine-tuned, specific response. Given the complexity of the responses elicited during biotic interactions, substantial efforts have been made to simplify the elicitation process. Thus plant-pathogen interactions are frequently studied with purified pathogen-specific elicitors, often in combination with synchronized plant cell cultures to help clarify the rapidly induced dynamics of the responses (Mandujano-Chavez *et al.,* 2000; Nuhse *et al.,* 2000; Namdeo *et al.,* 2002; Chico *et al.*, 2002). Similar approaches are being adopted in the study of herbivore-specific responses. Herbivores, unlike most pathogens, are physiologically independent of their host plant. They force their way through a plant's outer protective barriers with mandibles and mouthparts that cause wounds into which herbivore-specific elicitors are likely to be introduced during the interaction. Hence some herbivore-specific plant responses can be mimicked by adding herbivore-specific elicitors, frequently isolated from larval oral secretions or oviposition fluids, to mechanical wounds. Many factors have been identified as herbivore-specific elicitors in the oral secretions of *Pieris rapae*, *Spodoptera exigua*, *Manduca* spp, and *Helicoverpa zea* larvae, including, β-glucosidase, volicitin, other fatty acid-amino acid conjugates (FACs), and glucose oxidase,

respectively (Mattiacci et al., 1995; Alborn *et al.,* 1997; Pohnert *et al.*, 1999; Halitschke *et al.,* 2001; Musser *et al.,* 2002).

While the kinetics of the transcriptional response to herbivore attack can be simplified by simulating the interaction - adding herbivore-specific elicitors to mechanical wounds - this simplification has drawbacks. For most herbivore species, the potential cocktail of elicitors introduced into wounds during feeding, remains to be characterized. Moreover, the temporal and spatial dynamics of wounding as well as the quantities of elicitor introduction into the wounds are lost in the simulations, but may provide the plant with valuable information with which to tailor its responses. Signaling pathways and the resistance responses they mediate, although proximately activated by chemical elicitors, are ultimately selected by biotic agents. Thus characterizing a plant-herbivore interaction in all its complexity is more illuminating than with exogenously applied elicitors (de Bruxelles and Roberts, 2001).

Why study plant-insect interaction of a non-agricultural plant? For the most part, induced defenses involve changes in metabolism *sensu lato* and are mediated by complex polygenic traits (Simms and Rausher, 1992). Since agricultural plants have been under intense selection for particular yield-enhancing traits, genetic associations mediating their defense traits are likely to have been altered during agricultural selection and hence are difficult to interpret. Therefore, we have chosen to produce microarrays enriched in herbivore-elicited genes for *Nicotiana attenuata*, a desert annual native to southwestern USA (Baldwin, 2001). Among the herbivores colonizing *N. attenuata* are leaf tissue feeders (larvae of the sympatric sibling species *Manduca quinquemaculata* and *Manduca sexta, Spodoptera exigua, Trichoplusia ni,* and adults of *Epitrix hirtipennis*), cell content feeders (*Tupiocoris notatus*), seed feeders (corimelaenid or 'negro' bugs), root feeders (larvae of *E. hirtipennis*) and others. Herbivores typically arrive sequentially, and even in adjacent areas plant populations frequently differ in their herbivore communities. For example, plant populations with established mirid populations (*T. notatus*) are rarely found supporting large hornworm populations. In the companion paper (Kessler and Baldwin, 2004) the mechanism responsible for this lack of co-occurrence of *N. attenuata's* two most abundant herbivores is described. Kessler and Baldwin (2004) found that initial mirid feeding 'vaccinates' the plants by repelling *Manduca* oviposition and reducing hornworm survival, which, in turn, results from reduced hornworm performance and increased predator attraction: responses, which are all mediated by the host plant. Since the fitness consequences of mirid attack for *N. attenuata* are dwarfed by those of *Manduca* attack,

mirids, the less harmful herbivores, protect the plant in environments in which both herbivores are present (Kessler and Baldwin, 2004).

To understand the transcriptional processes elicited by these two herbivores, which have such profoundly different fitness consequences for their host, we infested *N. attenuata* plants in two different experiments (I and II) with one or the other species and with both species sequentially or simultaneously in order to simulate the order of attack observed in different plant populations (Fig. 2, right panel). We use a combination of array technologies (a cDNA microarray in experiment I and an oligonucleotide microarray in experiment II) and a multivariate statistical procedure (Principal Components Analysis, PCA) to answer the following questions: (i) Does each herbivore species reproducibly engrave a distinct transcriptional imprint? (ii) If so, can sequential or parallel attack by another species 'erase' or alter this transcriptional imprint? (iii) Do herbivore-induced changes in transcript levels correlate with herbivore-induced changes in direct and indirect defense metabolites as measured by Kessler and Baldwin (2004)? While the relevant elicitors of herbivore-specific interactions from *Manduca'*s oral secretions are known (Halitschke *et al.,* 2001; Halitschke *et al.,* 2003), nothing is known about the elicitors of *T. notatus.* The saliva of related mirid species, such as *Lygus rugulipennis*, *L. lineolaris*, and *Creontiades dilutus*, contains polygalacturonases, amylases, and proteases (Laurema *et al.,* 1985, Colebatch *et al.,* 2002; Zeng *et al.,* 2002), which in addition to their digestive roles may function to generate elicitors.

Results

Comparison of M. sexta- and T. notatus-Induced Transcriptional Changes in N. attenuata

Repeatability. Due to differences in conception and design, criteria for differential expression differed between the cDNA and the oligonucleotide array (see Experimental Procedures). For the cDNA array, a combination of statistical significance (t-test, expression ratio (ER) $\neq 1$) and arbitrary thresholds (ER>1.3 and ER<0.76) was sufficient for determining the significance of ERs (Halitschke *et al.*, 2003). However, due to the overall lower signal strengths of the oligonucleotide array, an average sum of signal strengths >1000 was added as a criterion, in addition to the statistical test (t-test, ER$\neq$1) and higher arbitrary threshold (ER>1.5. and ER<0.67) criteria (Heidel and Baldwin, in review). Shifts in the transcriptome of *N. attenuata* in response to a 24 hour attack from *M. sexta* or *T. notatus* (Fig. 2, treatment 1) were analyzed with both arrays with RNA originating from

independent biological replicates. An evaluation of the regulation scores for genes represented on both arrays yielded an overall reproducibility of ca. 60%; e.g. in the 182 cases when a gene on the cDNA array was regulated (up or down) by either of the species, 59.3% showed the same response on the oligonucleotide array, 24.2% showed no response, 8.2% showed the opposite response, and in 8.2% of the cases no interpretable results could be obtained with the oligonucleotide array (Table 1). For the reverse suite of comparisons (comparing results obtained with the oligonucleotide array with those from the cDNA array) see Table 1, and for details (species-specific changes) see Supplementary Material (ExpII-SupplMat5). To compare transcriptional changes induced by *M. sexta* with those induced by *T. notatus*, we depict results obtained with the oligonucleotide array (Fig. 1, Table 2), simply because this array contained 388 additional genes (see ExpII-SupplMat1 and ExpI-SupplMat1) and therefore reveals a more complete picture.

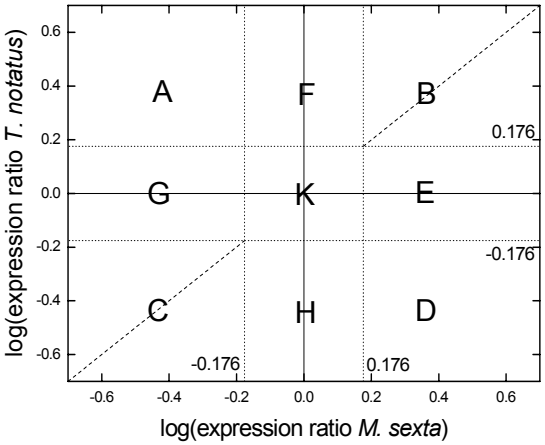
Table 1. Data from treatment 1 (see Fig. 2) were analyzed with both the cDNA and the oligonucleotide array. For genes present on both arrays and responsive to herbivory in experiments I or II, this table summarizes how the results obtained with the cDNA array compared with those obtained with the oligonucleotide array and vice versa; e.g. of the 182 cases in which a gene was regulated (up or down) by attack from either *M. sexta* or *T. notatus* or both species on the cDNA array, in 108 (61+47) cases, the gene showed the same regulation/trend on the oligonucleotide array, resulting in a repeatability of 59.3%. There were circa 20-25% inconsistent cases (a gene was regulated on one array but not the other) and circa 10% contrary cases (a gene was up-regulated on one array but down-regulated on the other). For details on regulation (down-, up-, *M. sexta*-, *T. notatus*-regulation and combinations thereof) see Supplementary Material (ExpII-SupplMat5).

results from/confirmed by	cases	total	equal regulation/trend	not regulated	contrary trend/regulation	not analyzable
cDNA array/oligo array	#	182	61/47	44	9/6	15
	%	**100**	**59.3**	**24.2**	**8.2**	**8.2**
oligo array/cDNA array	#	131	61/24	29	6/8	3
	%	**100**	**64.9**	**22.1**	**10.7**	**2.3**

Commonly regulated genes. Most plant genes up-regulated by both herbivore species, irrespective of feeding guild, play a role in signaling and secondary metabolism, while most genes down-regulated by both herbivores are involved in photosynthesis and primary metabolism (Table 2). For a detailed discussion of individual gene changes and their role in various signaling pathways, direct and indirect defense mechanism against herbivores and pathogens, and primary metabolism see Supplementary Material (ExpII-SupplMat8).

Fig. 1

Zone overview



Zone	*M. sexta*	*T. notatus*
A	down	up
B	up	up
C	down	down
D	up	down
E	up	-
F	-	up
G	down	-
H	-	down
K	-	-
diagonal line	equal regulation by both species	
dotted lines	arbitrary thresholds (log0.67, log1.5)	

Individual zones

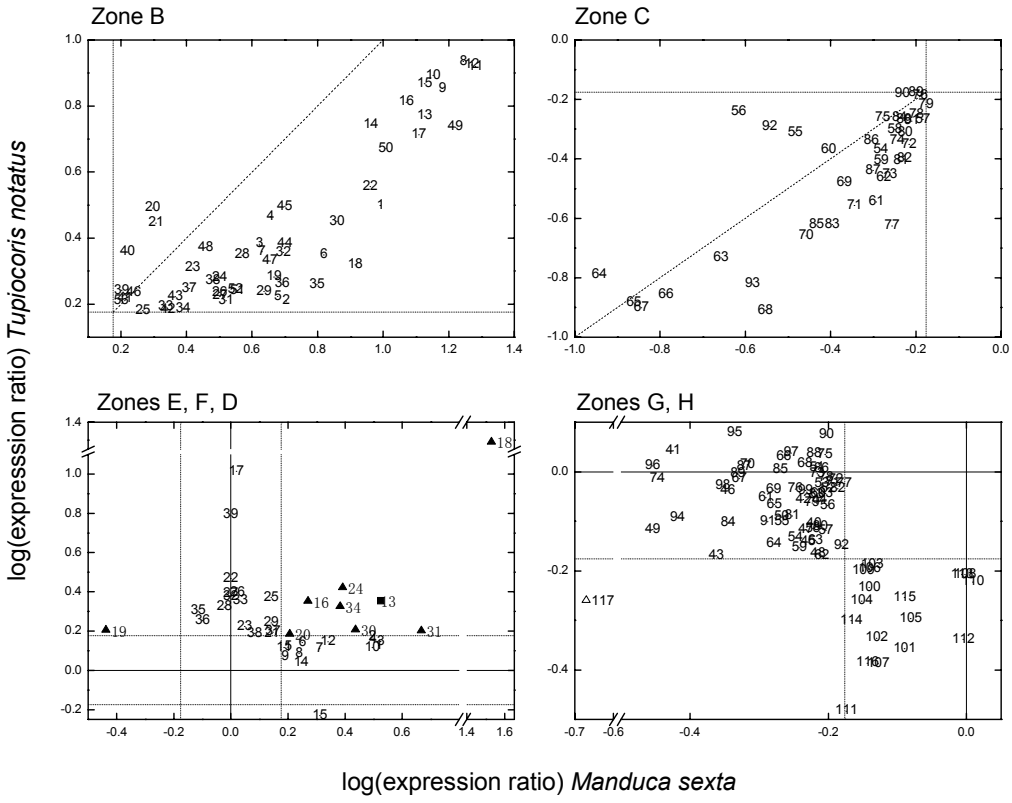


Fig. 1. Logarithmic mean expression ratios of insect-induced genes for *Manduca sexta* (x-axis) and *Tupiocoris notatus* (y-axis) obtained from hybridizations with the oligonucleotide array. Arabic numerals refer to gene probes listed in Table 2. Three criteria were used to define differential expression: 1. Mean expression ratio (ER) exceeds arbitrary thresholds (<0.67 (log0.67 = -0.176) for down-regulated genes, >1.5 (log1.5 = 0.176) for up-regulated genes, thresholds depicted by dotted lines); 2. ER is significantly different from 1 as determined by t-tests; 3. Average sum of signal strengths for Cy3 and Cy5 values exceeds 1000. The upper panel depicts zones representing different types of regulation; the lower panel depicts transcripts following the respective regulation patterns. **Zones B, C:** Genes induced by both insect species (all 3 criteria are fulfilled for

ERs of both species) **Zones F, E, H, G:** Genes induced by either *M. sexta* or *T. notatus* (all 3 criteria are fulfilled for ERs of only one species). Genes depicted in zone A, B, and C (*M. sexta*, squares; *T. notatus*, triangles): 1st criterium is fulfilled by both species, but 2nd and/or 3rd criteria are fulfilled only by one species. **Zone D**: Genes oppositely regulated by both species; note that only one gene shows this reverse response.

Specifically regulated genes. According to our criteria, 117 oligonucleotide probes revealed differential expression of the respective genes between *M. sexta* and *T. notatus* attack. However, for reasons listed in supplementary results, we do not consider all of these to represent truly different responses elicited by the two herbivores. In other words, to strictly interpret the data according to the criteria would mean to reject a true null hypothesis (Type I error), i.e. to propose differential expression although it cannot be reliably inferred from this array analysis alone (ExpII-SupplMat8).

Conspicuous herbivore-specific changes include the *M. sexta*-specific down-regulation of genes involved in photosynthesis (47, 48), carbohydrate metabolism (56, 58-60), nitrogen assimilation and refixation of photorespiratory ammonium (49-52), and genes from diverse signal transduction pathways, such as GTP binding proteins, SNF-1-interacting proteins, receptor proteins, and a triacyl glycerol lipase. For more hornworm-specific changes, see Supplementary Material (ExpII-SupplMat8).

Genes specifically up-regulated by *T. notatus* include asparagine synthetase (provides a source of mobile nitrogen), anthranilate synthase (tryptophan biosynthesis), α-amlyase (hydrolytic starch degradation), flavanone-3-hydroxylase (quercetin synthesis), lignin-forming peroxidase, a WRKY type transcription factor, which is thought to bind to the W-box motif of defense-related genes (Eulgem *et al.,* 2000), NPR1, an essential regulator of plant systemic acquired resistance (Mou *et al.,* 2003), and others (Table 2). Genes specifically down-regulated by *T. notatus* include the large subunit of Rubisco; a lipid transfer protein, which may play a role in systemic signaling (Maldonado *et al.,* 2002); a MAP kinase (MEK2), which acts upstream of WIPK in disease resistance signaling pathways (Yang *et al.,* 2001); metallothionins, which may function as metal chelators or be involved in senescence or hypersensitive response pathways; ubiquitin, which is involved in the degradation of short-lived and abnormal proteins via proteasomes; an adenine nucleotide carrier, which catalyzes mitochondrial ATP export; and others (Table 2).

Five of the genes depicted in Table 2 (small subunit of rubisco, xyloglucan-endotransglycosylase (XTH), sucrose phosphate synthase, luminal binding protein, 5-epi-aristolochene synthase) exhibit contradictory regulation patterns, i.e. some probes indicate

Table 2. Homologies/identities and graph labels of insect-responsive gene probes, grouped by zones as defined in Fig. 1. Genes were assigned to the following categories: PM, primary metabolism; SM, secondary metabolism; PS, photosynthesis; SI, signaling; PD, pathogen defense; PT, protein translation; TR, transcription; SR, stress response; CS, cytoskeleton; UN, unknown; with a few not categorized (NC) exceptions. For original data, gene numbers, and heterologous/homologous probe identification see extended version of this table in Supplementary Material (ExpII-SupplMat4).

Zone	Gene name	Category	Graph label
Commonly regulated genes (1-93)			
B	beta-tubulin	CS	1
	SNF1	SI	2
	pto-responsive gene 1	PD	3
	rubisco small subunit	PS	4
	sucrose-phosphate synthase	PM	5
	phenylalanine ammonia-lyase (PAL)	SM	6, 7
	inhibitor 2 of Ser proteases; pin 2	SM	8, 9
	proteinase inhibitor	SM	10-13
	proteinase inhibitor IIb	SM	14, 15
	proteinase inhibitor IIa	SM	16, 17
	SAM decarboxylase (SAMDC)	SM	18, 19
	xyloglucan endo-transglycosylase (XTH)	SM	20, 21
	5-epi-aristolochene synthase	SM	22
	SAM:JA carboxyl methyltransferase (JMT)	SI	23
	cinnamic acid 4-hydroxylase C4H	SM	24
	12-oxophytodienoate reductase 3 (OPR3)	SM	25
	hydroperoxide lyase (HPL)	SM	26
	rhamnosyltransferase (flavanoids)	SM	27
	polyphenol oxidase (PPO)	SM	28
	putrescine N-methyltransferase 1 (PMT1)	SM	29
	4-coumarate-CoA ligase (St4C1-1)	SM	30
	1-aminocyclopropane-1-carboxylate oxidase (ACO)	SI	31, 32
	pathogen induced a-dioxygenase	SI	33-38
	13-lipoxygenase (13-LOX)	SI	39, 40
	SAM:SA carboxyl methyltransferase (SMT)	SI	41
	luminal binding protein blp4 + blp5 + blp8	SR	42
	glutathione peroxidase	SR	43
	wound-induced protein kinase (WIPK)	SI	44
	9 unknowns	UN	45-53
C	histone H3 gene	TR	54
	pathogenesis-related protein P2	PD	55, 56
	pathogenesis-related protein P4	PD	57
	pathogenesis-related protein P5	PD	58
	rubisco activase	PS	59-62
	rubisco small subunit	PS	63-68
	lhbc1 gene for LHCII type III	PS	69
	photosystem II	PS	70
	photosystem I, subunit PSI-E	PS	71
	photosystem II, O2-evolving complex	PS	72
	plastidic aldolase	PM	73, 74
	glyceraldehyde-3-phosphate dehydrogenase	PM	75, 76
	thiazole biosynthetic enzyme precursor	PM	77
	phosphoribulokinase	PM	78
	triose phosphate translocator	PM	79
	ferredoxin-NADP reductase	PM	80
	phosphoglycerate kinase	PM	81
	glutamine synthetase	PM	82
	RNA-binding glycine-rich protein (RGP-1a)	TR	83
	glycine hydroxymethyltransferase	SM	84
	phospholipase C2	SI	85
	germin	SR	86
	7 unknowns	UN	87-93
Specifically regulated genes (1-117)			
E	SNF1-related protein kinase	SI	1
	sucrose-phosphate synthase	PM	2
	thionins	SM	3, 4
	1-aminocyclopropane-1-carboxylate oxidase (ACO)	SI	5
	allene oxide synthase (AOS)	SI	6
	calcium-dependent protein kinase 3	SI	7
	wound stimulated protein Sn-1	SR	8, 9
	5 unknowns	UN	10-14
D	pathogenesis-related protein 3	PD	15
F	NPR1	SI	16
	asparagine synthase	PM	17
	threonine deaminase	PM	18
	anthranilate synthase alpha-2 chain	PM	19
	molybdopterin synthase sulphurylase gene	PM	20
	nitrate reductase	PM	21
	a-amylase	PM	22
	WRKY3	SI	23
	xyloglucan endo-transglycosylase (XTH)	SM	24-26
	putative flavanone 3-beta-hydroxylase	SM	27
	peroxidase C (POC1)	SM	28
	polyphenol oxidase (PPO)	SM	29
	allene oxide synthase 2 (AOS2)	SI	30
	pathogen induced a-dioxygenase	SI	31
	13-lipoxygenase 3 (13-LOX)	SI	32
	wound-induced protein kinase (WIPK)	SR	33
	6 unknowns	UN	34-39
G	GTP-binding proteins	SI	40, 41
	virus coat proteins	NC	42-44
	pore protein	NC	45
	pathogenesis-related protein P4	PD	46
	Mg protoporphyrin IX chelatase	PS	47
	6.1 kDa polypeptide of photosystem II	PS	48
	ferredoxin-dependent glutamate synthase	PM	49-51
	NADH-dependent glutamate synthase	PM	52
	glutamine synthetase	PM	53, 54
	NADPH thioredoxin reductase	PM	55
	fructokinase (Frk1)	PM	56
	phosphoglycerate kinase (cytosolic isoform)	PM	57
	triosephosphate isomerase	PM	58
	cytosolic fructose-1,6-bisphosphatase	PM	59
	beta-amylase	PM	60
	sucrose-phosphate-synthase	PM	61
	putative 60S ribosomal protein	PT	62
	ribosomal protein L7	PT	63
	(E)-beta-farnesene synthase	SM	64
	xyloglucan endotransglycosylase (XTH)	SM	65
	ethylene receptor	SI	66, 67
	SNF-1 anchoring protein SIP1	SI	68
	systemin receptor SR160	SI	69
	EDS1 similar to triacyl glycerol lipase	SI	70
	GAL 83	SI	71
	chaperonin 60	SR	72
	luminal binding protein 5	SR	73
	transformer-SR ribonucleoprotein	TR	74
	25 unknowns	UN	75-99
H	mitogen-activated protein kinase 2	SI	100
	ubiquitin	NC	101
	induced stolon tip protein	NC	102
	pathogenesis-related protein P6	PD	104
	rubisco large subunit	PS	105
	glyceraldehyde-3-phosphate dehydrogenase	PM	106
	phosphoglycerate kinase	PM	107
	ADP/ATP translocator	PM	108
	transketolase	PM	109
	5-epi-aristolochene synthase	SM	110
	metallothionein-like protein	SR	111, 103
	lipid transfer protein 1	SR	112
	5 unknowns	UN	113-117

an increase but others a decrease in gene expression after herbivore attack. For a possible explanation of this inconsistent behavior see Supplementary Material (ExpII-SupplMat8).

Comparison of Responses to the Different Attack Treatments

A principal components analysis (PCA) is an unconstrained ordination technique we used to configure samples (= different treatments represented by individual microarrays, Fig. 2) in ordination space so that the distances between the samples best reflect the dissimilarities of the elements composing a sample. These elements are the oligonucleotides with their treatment-dependent expression ratios. A PCA reduces the dimensionality of the dataset by defining variables (= ordination axes) that explain the largest proportion of the total variance in the smallest number of dimensions. From the PCA analysis of experiment I, three patterns emerged. First, ordination axes 1 and 2 accounted for 80% of variance in the data and the two single species treatments (1M, 1T) were clearly distinguished by these axes. Hence, attack from each herbivore species leaves a distinct imprint in *N. attenuata*'s transcriptome. Second, the transcriptional response to an attack from both herbivore species depended on the order of attack. If mirids attacked first and were followed by attack from *Manduca* (2T), the response was more similar to that observed after a mirid-only attack (1T) than to a simultaneous mirid/*Manduca* attack (3M+T). If *Manduca* attacked first followed by an attack from mirids (2M), the response was different from a *Manduca*-only attack (1M) but very similar to a simultaneous mirid/*Manduca* attack (3M+T). In other words, a mirid's imprint is more resistant to erasure by *Manduca* attack than a *Manduca*'s imprint is to erasure by mirid attack. Third, the imprint resulting from the simultaneous attack from both herbivores (3M+T) is different from either imprint left by attack from single herbivore species (1M, 1T; Fig. 2, experiment I).

From the PCA plot of experiment II the following patterns can be inferred (Fig. 2, experiment II). First, ordination axes 1 and 2 accounted for 50% of the variance in the data, and although the single species 24h treatments (1M, 1T) clearly differed, their correlation with the two axes was weaker than in experiment I. Second, insect-specific changes in the transcriptome occurred after 24h (1M, 1T; Fig. 4), but after 5d of continuous attack the *M. sexta*- and *T. notatus*-elicited transcriptional imprints were similar not only to each other (6M, 6T; Fig. 4) but also to those obtained after a 24h attack followed by a 4d 'relaxation' period (5M, 5T). Thus, the transcriptome responds specifically within 24h but this difference disappears after 5d. Third, similarly to experiment I in which the imprint from 24h of simultaneous attack (3M+T) was very different from that of 24h attack by either species alone (1T+1M), in experiment II the imprint from 5d simultaneous attack (7M+T) was very different from 5d individual attack (6M, 6T, Fig. 4), except that after 5d the individual attack treatments did not differ from each other. Fourth, the 4d individual attack

Fig. 2

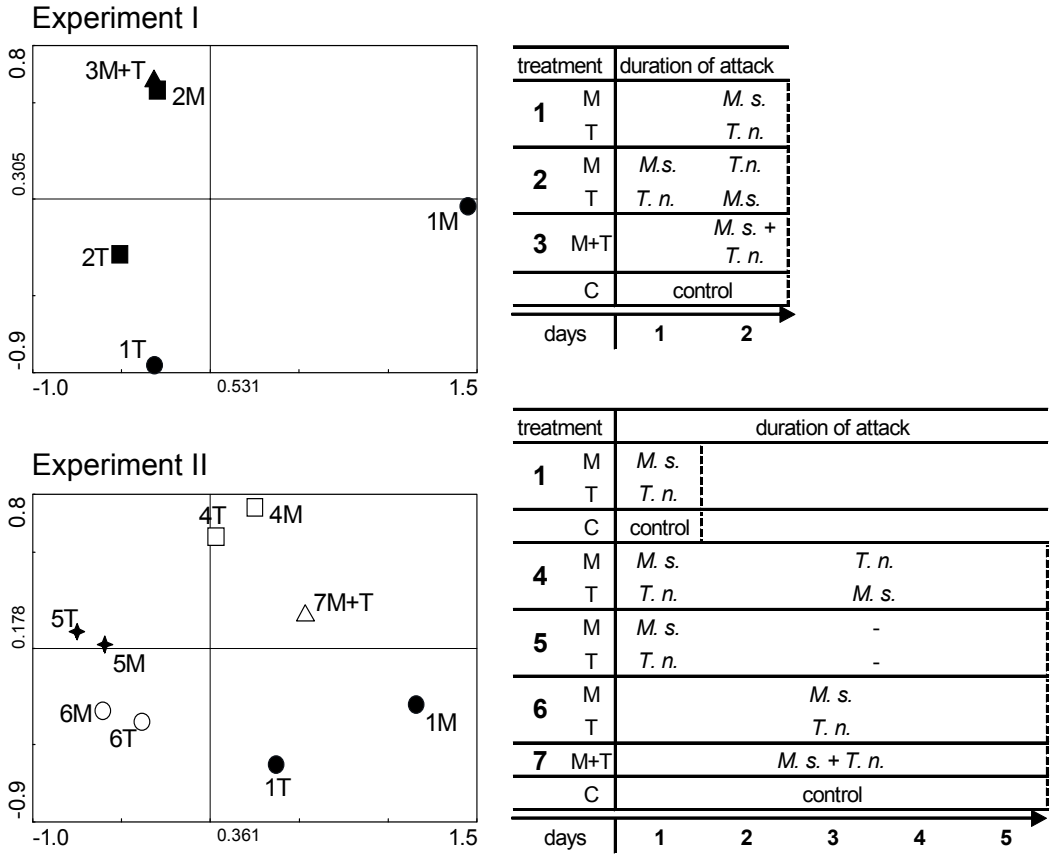


Fig. 2. Left panel: Principal Components Analysis of logarithmic expression ratios obtained from 5 hybridized cDNA arrays (experiment I) and 9 hybridized oligonucleotide arrays (experiment II). Numbering and description of treatments of both experiments are given in the **right panel** (1 – single species attack for 24h; 2 – symmetrical switch: 24h attack by one species followed by 24h attack by the other species; 3 – simultaneous attack by both species for 24h; 4 –asymmetrical switch: 24h attack by one species followed by 96h of attack by the other species; 5 – 24h attack by one species followed by 96h of no attack; 6 – 120h attack by a single species; 7 - simultaneous attack by both species for 120h). Dashed lines indicate when plants were harvested for RNA extraction. Cy3-labeled cDNA from treated plants was mixed with Cy5-labeled cDNA from untreated plants (harvested after 1d or 5d, respectively) for each of the 14 hybridizations.

treatments with a preceding 1d attack of the other species (4M, 4T) were as similar to each other as the 5d individual attack treatments were (6M, 6T), and the latter pair of treatments was clearly distinguishable from the former pair. Hence, the transcriptome reacts differently to the same treatment (4d individual attack) depending on the previous events (1d attack of the same or 1 d attack of the other species) (Fig. 4). In other words, it is important whether after 24h a species continues to attack or is replaced by a second species. When the second species begins its attack, the process of herbivore recognition followed by transcriptional

relaxation is interrupted and redirected to a different transcriptional state. Fifth, while in experiment I with the symmetrical switch treatment (2T, 2M), a mirid imprint persisted longer than a *Manduca* imprint; in experiment II with the asymmetrical switch treatment (4M, 4T), the initial imprints (1T, 1M) were erased to the same extent after 4d. Hence, over a longer time scale, the initial imprints are similarly erasable.

Fig. 3

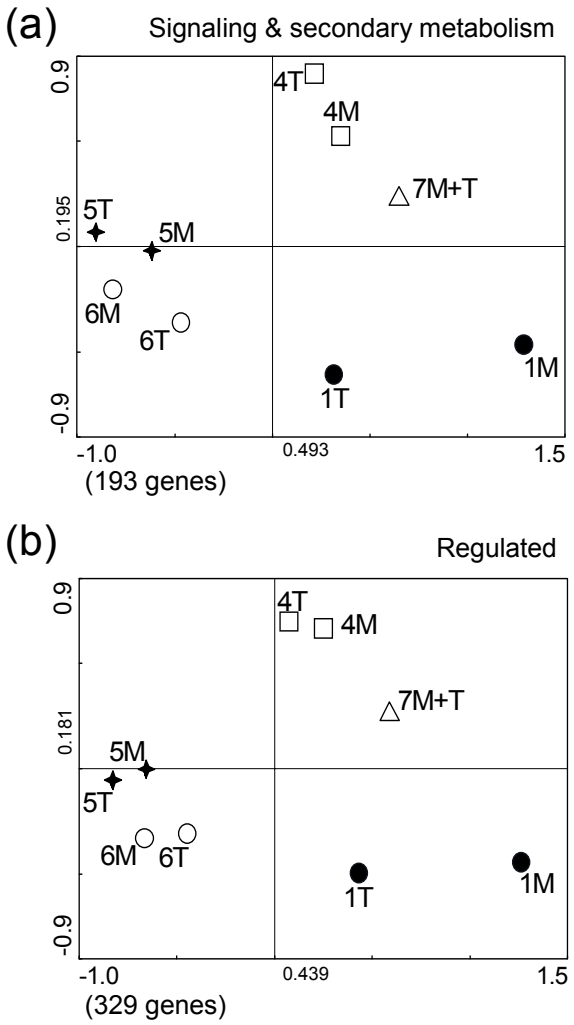


Fig. 3. Principal Components Analysis (PCA) of two sets of selected genes (A, B) from experiment II. Genes involved in secondary metabolism and signaling (193 of 628) reflect the pattern depicted in Fig. 2, experiment II (conducted with the complete set of 628 genes). A similar pattern is obtained when genes that do not fulfill the criteria for differential expression in any of the 9 arrays (299) are omitted and the analysis is performed only with the regulated genes (329). See Supplemental Material (ExpII-SupplMat7) for additional PCAs with other subsets of genes.

Fig. 4

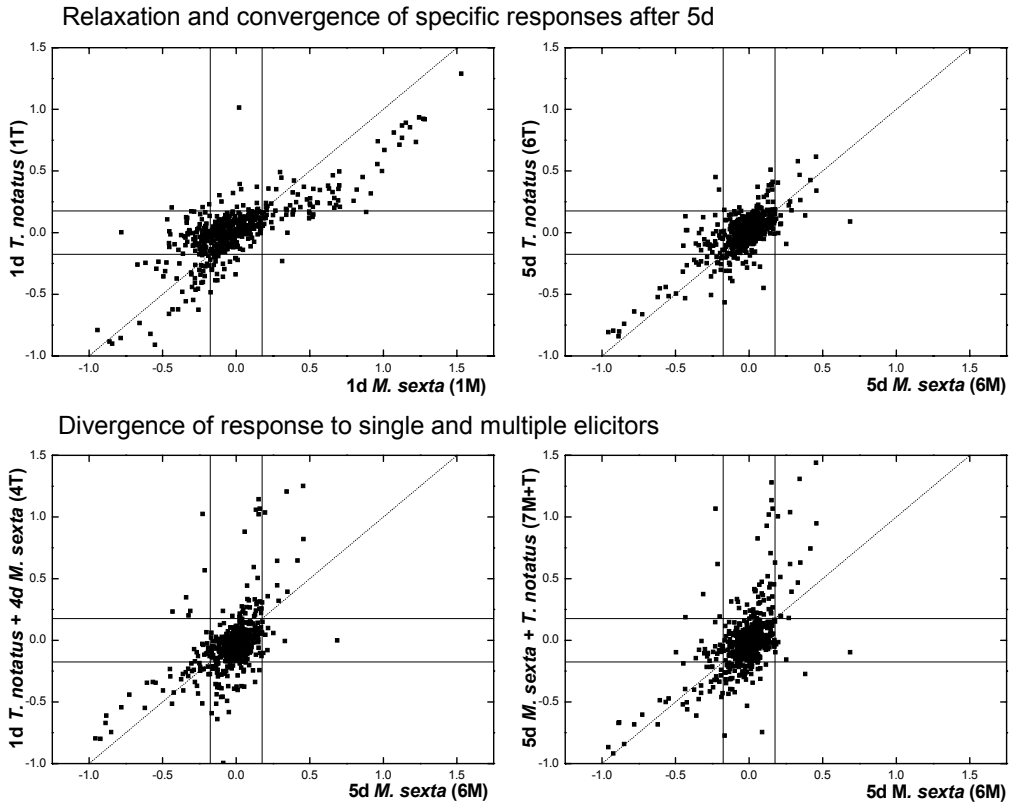


Fig. 4. Upper panel: Scatter plots from a comparison of *M. sexta* versus *T. notatus* induced genes after 1d as opposed to after 5d. Note the overall 'relaxation' of the response and the decrease in specific changes after 5d of continuous attack. **Lower panel:** Scatter plots from a comparison of sequential versus single attack and parallel versus single attack. Note the shift in expression ratios caused by prior or parallel presence of the second insect species.

By analyzing different gene subsets, we examined the robustness of the PCA's characterization of the transcriptional imprint. The pattern observed in the entire dataset (Fig. 2, experiment II, Fig. 3B) was retained when only those genes were included in the analysis that showed differential regulation in any of the treatments according to our criteria (329 genes). In other words, the PCA's synopsis was unchanged when the individual genes providing statistically significant 'signals' were separated from those providing 'noise' (for a PCA plot with only the non-regulated genes see ExpII-SupplMat7). Moreover, the PCA pattern from the entire dataset was retained with an analysis using only genes involved in signaling pathways and secondary metabolism (Fig. 2, experiment II, Fig. 3A) as opposed to the PCA patterns observed when only genes involved in photosynthesis and primary metabolism (ExpII-SupplMat7) were used. In summary, the transcriptional activity of the

genes involved in signaling pathways and secondary metabolism dominated the transcriptional imprints revealed by PCA.

Discussion

In contrast to our expectations, the plant's transcriptional response to attack by herbivores of different feeding guilds comprises considerably more common than specific elements. This common response predominantly includes an up-regulation of transcripts involved in jasmonic acid (JA), salicylic acid (SA), ethylene, sugar, and wound signaling; phenylpropanoid metabolism; production of digestibility reducers; green leaf volatile, polyamine, and nicotine biosyntheses; cell wall modification; and pathogen resistance as opposed to a down-regulation of transcripts involved in photosynthesis and primary metabolism and PR protein production. These alterations indicate an insect-induced switch from a growth-oriented transcriptional phenotype to a defense-oriented one and correlate with the insect-induced changes in secondary metabolism measured by Kessler and Baldwin (2004). In particular, increases in phenylpropanoid metabolism and polyamine synthesis genes (phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-coumarate-CoA-ligase (4CL), S-adenosylmethionine decarboxylase (SAMDC)) correlate with changes in chlorogenic acid, cryptochlorgenic acid, and hydroxycinnamic acid amids such as caffeoyl putrescine; increases in trypsin proteinase inhibitor (TPI) transcripts correspond to increases in TPI activity; increases in lipoxygenase (LOX) and hydroperoxide lyase (HPL) correspond to increases in green leaf volatiles, such as cis-3-hexen-1-ol, cis-3-hexenylacetate, and cis-3-hexenyl butyrate; increases in SAM:SA carboxyl methyl-transferase (SMT) correspond to increases in methyl salicylate emission (Kessler and Baldwin, 2004).

While the oxylipin-cascade and the wound response it mediates (e.g. induction of TPIs) is known to be activated by and elicit resistance to chewing lepidopteran larvae or beetles (Howe *et al.,* 1996; Halitschke *et al*., 2003), its role in the plant's response to cell content-feeding herbivores has only recently been established (Li *et al.*, 2002). JA and proteinase inhibitor (PI) activity as well as transcript levels of three PI genes, LOX, and allene oxide synthase (AOS) increased after attack from two-spotted spider mites (*Tetranychus urticae*) in wild type tomato plants as opposed to JA-deficient *def-1* plants. Moreover, plants with a constitutively activated JA pathway were more resistant to *T. urticae* and to another cell content feeder, the western flower thrips (*Frankliniella occidentalis*). In contrast, phloem-feeding aphids (*Macrosiphum euphorbiae*, *Myzus persicae*, Fidantsef *et al.,* 1999; *M.*

persicae, Moran and Thompson, 2001) induce LOX, but neither aphids nor whiteflies (*Trialeurodes vaporariorum, Bemisa argentifolii*) induce PIs (Fidantsef *et al.*, 1999; Walling, 2000). The difference in wound response between cell content and phloem feeders likely reflects the different amounts of damage inflicted by the two types of stylet feeders.

Surprisingly, except for signaling genes, differential gene regulation after attack from the two different herbivore species occurs largely in primary rather than secondary metabolism. While in response to *M. sexta* attack, all genes catalyzing glutamate synthesis are down-regulated, attack by *T. notatus* greatly amplifies asparagine synthetase transcription. Glutamate and asparagine are nitrogen transport amino acids, but the former is readily metabolized in the biosynthesis of amino and nucleic acids and the latter serves primarily as a nitrogen transport and storage compound. Moreover, among genes involved in photosynthesis and carbohydrate metabolism, not only more but different ones are down-regulated after *M. sexta* as compared to *T. notatus* attack (Table 2). Additionally, α-amylase and anthranilate synthase were up-regulated by mirids. From the specific changes in signaling genes, it is clear that the interplay of the signal cascades, which mediate plant responses, is different between the two herbivore species. The up-regulation of a flavonol- and a lignin synthesis gene by mirids and the down-regulation of a farnesene synthase by *M. sexta* are the few specific changes occurring in secondary metabolism. Kessler and Baldwin (2004) observed a great reduction in fitness in *N. attenuata* plants after *M. sexta* attack but almost no reduction in fitness after *T. notatus* attack. In part, this may be explainable by the different herbivore feeding modes; in other words, the tissues lost during *M. sexta* feeding may be harder to compensate for than those lost during *T. notatus* feeding. The assumption that the reprogramming of primary metabolism, probably resulting in differential use of carbohydrates and nitrogen storage compounds, contributes to the plant's ability to more easily tolerate mirid versus *Manduca* attack, emerges as a testable hypothesis from the results of this study.

This large transcriptional reorganization argues forcefully for the existence of *trans*-activating factors that coordinate this polygenic response. While these *trans*-factors remain unknown, plant biologists are ill prepared to describe the transcriptional responses elicited by these factors, if they are discovered. The PCA defines ephemeral transcriptional imprints elicited by environmental stimuli in the plant's transcriptome as they mature into phenotypic responses – a procedure analogous to the use of whole-brain imaging techniques to understand the effects of environmental stimuli on the function of animal brains. If we

are to find *trans*-activating factors that shape these imprints, their precise characterization is a prerequisite that a PCA can provide.

Moreover, for sedentary organisms such as plants, it will be essential to have tools that can characterize the transcriptional 'memory' of environmental responses. Such a memory allows a plant to alter how it responds to subsequent environmental stresses. Little data exist on the flexibility and erasability of a plant's transcriptome by consecutive stimulations, but plants do increase the rate of metabolite accumulation in response to subsequent attacks after an initial attack (Baldwin and Schmelz, 1996). This form of immunological memory is presumably responsible for the phenomenon of induced resistance (Karban and Baldwin, 1997). Certain treatments and chemicals, such as β-aminobutyric acid (BABA), while not directly eliciting responses themselves are known to 'potentiate' or 'prime' a plant for subsequent elicitors, resulting in larger or longer lasting elicitations (Conrath *et al.,* 2002; Toquin *et al.,* 2002). The phenomenon of priming underscores the importance of context in understanding environmental stress responses and the need to experimentally characterize context-dependent patterns of expression. Our analysis of the *Manduca*- and *Tupiocoris*-induced transcriptional imprints provides an ecologically motivated analysis of context-dependent expression, which reveals important insights into these plant-herbivore interactions.

First, herbivore-specific induced shifts in the transcriptome of *N. attenuata* occur after 24h (1M, 1T, experiments I and II) but disappear (6M, 6T) as the transcriptome approaches the unchallenged (relaxed) state (5M, 5T). The transcriptome changes reveal a rapid recognition response that may peak even before 24h but rapidly declines while the actual defense is mounted. Second, sequential or parallel attacks by both herbivore species lead to transcriptional imprints that are different from those elicited by the attack of a single herbivore species. At least in symmetrical switch treatments, the order of attack clearly has a major role in shaping the transcriptional imprint. On the individual gene level this means that when a plant experiences different biological stressors sequentially or simultaneously, a different suite of genes is induced (Fig. 4). A recent analysis of the effects of a combination of heat and drought stress using cDNA filter arrays revealed a comparable pattern. For example, transcripts induced during drought (e.g. dehydrin, catalase, and glycolate oxidase) and transcripts induced during heat shock (e.g. thioredoxin peroxidase and an ascorbate peroxidase) were suppressed when both stressors were combined. Other transcripts, including alternative oxidase, glutathione peroxidase, PAL, PR proteins, WRKY, and an

ethylene response transcriptional co-activator, were elicited only when both heat and drought stress were combined (Rizhsky *et al.*, 2002).

From our analysis we infer that there are only a few genes whose expression is dominated by *trans*-activating factors that mediate only species-specific responses; in other words, the existence of a mirid-specific transcription factor is unlikely. Most genes are regulated in a complex, context-dependent pattern; hence, experimental protocols that explicitly examine context-specific expression (i.e. simultaneous and sequential elicitation experiments in a Boolean framework: Genoud and Metraux, 1999; Genoud *et al.,* 2001) should be encouraged. Similarly, Rizhsky *et al.* (2002) predicted a unique genetic program in response to stress combination as well as the existence of key regulators of gene clusters activated during such a combination of stresses.

Array studies, by providing analyses of hundreds of transcripts simultaneously, allow plant biologists to describe the early ontogenetic stages of a plant's adaptive phenotypic response to complex environmental stresses. Since transcriptional changes do not necessarily translate into changes in protein activity or metabolite levels, such studies offer the possibility of analyzing environmental signals that a plant perceives but ignores and thereby increases the depth with which the plant's behavioral repertoire can be analyzed. Moreover, the ability to characterize the mechanisms of gene expression underlying ecological phenomena such as an herbivore-mediated plant vaccination will likely be the key to understanding adaptive behavior in complex environments.

Experimental Procedures

Plant and insect growth. An inbred line of *Nicotiana attenuata* Torr. ex Wats., originally collected from southwestern Utah in 1988, was used in both experiments. For experiment I seed germination and hydroponic plant growth were conducted as described by Hermsmeier *et al.* (2001). A day before placing herbivores on plants, 1 mL of 1 M KNO_3 was added to each 1-L hydroponic chamber, and 24 randomly chosen, similar rosette-stage plants were placed in wood insect cages (30x30x60cm, each cage accommodating four plants). For experiment II seeds were sterilized and smoke-germinated on phytagel as described by Krügel *et al.* (2002). Twelve days later seedlings were planted in soil in Teku pots (Waalwijk, The Netherlands) and after 12 more days, transferred to 0.5 L pots. Plants were grown at 26–28°C and 65% humidity under 16h of light supplemented by Philips Sun-T Agro 400- or 600-W sodium lights in a peat-perlite substrate. A day before placing

herbivores on plants, 64 randomly chosen, similar rosette- stage plants were placed in glass insect cages (30x30x60cm, each cage accommodating four plants).

Eggs of *Manduca sexta* (Lepidoptera, Sphingidae) came from Carolina Biological Supply (Burlington, NC, USA; experiment I) and North Carolina State University-Entomology Insectary (experiment II) and were hatched at 28 °C. Nymphs and adults of *Tupiocoris notatus* (Hemiptera, Miridae) were taken from a colony started in summer 2000 with individuals from our Utah Apex mine field site (Kessler and Baldwin, 2001).

Experimental design. From four to eight rosette-stage plants were used in each of the following treatments. *Experiment I*: 24h *M. sexta* feeding (1M); 24h *T. notatus* feeding (1T); 24h *T. notatus* feeding with previous 24h *M. sexta* feeding (2M); 24h *M. sexta* feeding with previous 24h *T. notatus* feeding (2T); 24h *M. sexta* and *T. notatus* feeding (3M+T); control, i.e. unattacked plants. *Experiment II*: 24h *M. sexta* feeding (1M); 24h *T. notatus* feeding (1T); 4d *T. notatus* feeding with previous 24h *M. sexta* feeding (4M); 4d *M. sexta* feeding with previous 24h *T. notatus* feeding (4T); 24h *M. sexta* feeding followed by 4d of no attack (5M); 24h *T. notatus* feeding followed by 4d of no attack (5T); 5d *M. sexta* feeding (6M); 5d *T. notatus* feeding (6T); 5d *M. sexta* and *T. notatus* feeding (7M+T); control, i.e. unattacked plants (Fig. 2, left panel). Either 20 first instar *M. sexta* larvae, with from one to three larvae per leaf, depending on the leaf size, or 20 *T. notatus* individuals of all developmental stages, were placed exclusively (treatments 1, 5, 6), sequentially (treatments 2, 4), or simultaneously (treatments 3, 7) on each plant. All plants, including controls, were kept in cages for the duration of the experiments to avoid cross-infestation. After feeding periods as depicted in Fig. 2 (left panel, dashed lines indicate harvesting point), herbivores and their frass were removed, and shoots of plants were harvested, flash-frozen in liquid nitrogen, and stored at –80 °C until used for microarray analysis.

cDNA-array fabrication: 234 genes, which were cloned by Differential Display Reverse Transcription-PCR (DDRT-PCR) and magnetic bead-assisted subtractive hybridization (SHMB) of *M. sexta* larvae-attacked *N. attenuata* plants (Hermsmeier *et al,.* 2001, Hui *et al.,* 2003), or by cDNA-amplified fragment length polymorphism (AFLP) display of *N. attenuata* plants under simulated *M. sexta* attack by applying oral secretions and regurgitate to leaf wounds (Halitschke *et al.*, 2003), and 6 well-characterized *Manduca*-induced genes (putrescine-N-methyltransferase (PMT), XTH, AOS, HPL, TPI, WRKY) were PCR-amplified and spotted on epoxy coated slides as described in Halitschke *et al.* (2003). For each cDNA, two PCR fragments, with 5'-aminolink on either strand, were synthesized, and each PCR fragment was spotted four times. Hence, each gene was represented by two

independent PCR fragments that, in turn, were spotted in quadruplicate. A complete list of positions and identities of spotted PCR products on the cDNA array can be found in Supplementary Material (ExpI-SupplMat1, 2).

Oligonucleotide array fabrication. Seven hundred ninety 50-mer oligonucleotides were printed onto epoxy-coated glass slides four times each (Quantifoil Microtools, Jena, Germany). Template genes for the design of oligonucleotides include all genes from the cDNA array (except ribosomal RNA genes), genes cloned from *M. sexta*- and *T. notatus*-elicited plants by DDRT-PCR and SHMB (Voelckel and Baldwin, 2003), and genes from trichome and flower cDNA libraries of *N. attenuata*. Additional 50-mers were designed from heterologous sequences from genes suspected to play a role in plant defense. A complete list of positions and identities of spotted oligonucleotides on the oligonucleotide array can be found in Supplementary Material (ExpII-SupplMat1, 2).

Microarray hybridization and quantification: Samples were ground under liquid nitrogen and total RNA was isolated according to the methods of Pawlowski *et al.* (1994) for experiment I or extracted with TRI REAGENT^{TM} (SIGMA, St. Louis, MO, USA) according to the manufacturer's instructions for experiment II. CDNA derived from herbivore-infested plants (Cy3) was competitively hybridized with cDNA originating from untreated plants (Cy5). For cDNA synthesis, Cy3/Cy5 labeling, hybridization procedures, array scanning, evaluation of images and signal strengths (AIDA Image Analyzer and AIDA software package, Raytest Isotopenmessgräte GmbH, Straubenhardt, Germany), and calculation of array-specific normalization factors and normalized Cy3/Cy5 ratios see Halitschke *et al.* (2003) and Schmidt *et al.* (2004).

Criteria for differential expression. Normalized Cy3/Cy5 ratios were calculated for each individual spot (expression ratio: ER) as well as the mean of the four replicate spots for each cDNA (two for each gene: mean ER1, mean ER2) or oligonucleotide (mean ER). ERs based on negative quantum level data as well as obvious outliers were excluded from further analysis. With the cDNA array a transcript was defined as being differentially regulated if both of the following criteria were fulfilled: (1) the final ER ((mean ER1+mean ER2)/2) was equal to or exceeded the arbitrary thresholds ($\leq$0.77 (log0.77 = -0.11) for down-regulated genes or $\geq$1.3 (log1.3 = 0.11) for up-regulated genes); (2) mean ER1 and mean ER2 were significantly different from 1 as evaluated by t-tests to control for ER variance and ER sample size. For justification and evaluation of these criteria see Halitschke *et al.* (2003). With the oligonucleotide array a transcript was defined as being differentially regulated if the following three criteria were fulfilled: (1) the mean ER

exceeded the arbitrary thresholds (<0.67 (log2/3 = -0.176) for down-regulated genes or >1.5 (log1.5 = 0.176) for up-regulated genes); (2) the mean ER was significantly different from 1 as evaluated by t-tests; (3) the combined signal fluorescent intensity from both Cy3 and Cy5 averaged over the four spots was greater than 1,000 QL. For justification and evaluation of these criteria see Heidel and Baldwin (in review). Original data from both experiments can be found in Supplementary Material (ExpI-SupplMat3, ExpII-SupplMat3).

Indirect gradient analysis. A Principal Components Analysis (PCA) was conducted with log-transformed mean expression ratios of all transcripts from the five cDNA arrays of experiment I (ExpI-SupplMat4) and the nine oligonucleotide arrays of experiment 2 (ExpII-SupplMat6) to compare the full transcriptional response of *N. attenuata* to the different treatments (Canoco for Windows 4.5, Microcomputer Power, Ithaca, NY, USA). Since an oligonucleotide array harbors single-strand sequences, for genes whose orientation was not known, two oligonucleotides (sense, antisense) were spotted. Antisense signals were diagnosed by their lower signal strength as compared to sense signals and were not included in any PCA. In the case of some genes, diagnosis failed or genes were spotted only once, which is why initially two PCAs were performed, one with and one without the ambiguous signals (628 versus 557 genes). Since their PCA plots differed only marginally (ExpII-SupplMat7), the ambiguous signals were included in all subsequent PCAs with different subsets of genes (Fig. 3, ExpII-SupplMat7).

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Manuscript IV

An Analysis of Plant-Aphid Interactions by Different Microarray Hybridization Strategies

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Abstract

Aphids have long been considered "stealthy" herbivores that subvert a plant's induced defenses and manipulate its source-sink signaling, but these hypotheses are largely untested at a transcriptional level. We analyzed gene expression in native tobacco plants (*Nicotiana attenuata*) infested with *Myzus nicotianae* aphids, without resorting to the use of clip-cages, with a cDNA microarray containing 240 defense-related *N. attenuata* genes. Using a hybridization scheme ('ratio analysis' and 'state analysis') broadly applicable in two-factor analyses, we examined how the aphids influenced source-sink relationships and determined if their feeding preference, apart from benefiting from the sink strength of young leaves, was associated with the expression of known plant defense genes. In contrast to the responses elicited by attack from tissue-feeding lepidopteran larvae and mesophyll-sucking bugs, attack from phloem-feeding aphids elicited only weak responses. Similar to other herbivores, *M. nicotianae* feeding increased the expression of trypsin protease inhibitors (TPI), lipoxygenase, and xyloglucan-endotransglycosylase genes, and decreased small RUBISCO subunit and ubiquitin carrier protein transcripts. Aphid-specific changes included the up-regulation of glutamate synthase and the down-regulation of a germin-like protein. Aphids preferentially settled on younger leaves, which expressed more hydroperoxide lyase and TPI than did older leaves, suggesting that these genes, which mediate the synthesis of compounds reported to be toxic for aphids in other plant systems, are either not under transcriptional control or not important in this system. By identifying aphid-responsive genes, we have made a first step in identifying the 'genes that matter' in plant-aphid interactions.

Keywords: feeding guild, feeding preferences, *Myzus persicae*, source-sink manipulation

Introduction

From a plant's perspective, phloem feeders, such as aphids, or phloem-parasitizing plants (e.g. *Cuscuta* spp., *Orobanche* spp.) are sinks for sugars and nutrients similar to newly expanding leaves, developing buds or maturing fruits. However, phloem sap is the 'junk food' of plant diets, rich in carbohydrates but very low in protein and amino acids (aa) (Sandstrom & Moran 1999). To cope with this unbalanced diet, aphids consume large amounts of phloem sap, excrete the excess carbohydrates as 'honeydew', scavenge the nitrogen-containing constituents, and house bacterial endosymbionts that provide the aphids with essential aa. Some aphid species manipulate aa composition in the phloem (Sandstrom *et al.* 2000); others, such as gall-forming *Pemphigus betae* aphids, manipulate plant allocation patterns while competing with plant sinks for resources (Larson & Whitham 1997 and references therein). The mechanisms responsible for these manipulations are largely unknown. Microarrays provide the opportunity to monitor transcriptional responses of a large number of genes and are being increasingly used to study plant-insect interactions (Reymond *et al.* 2000, Arimura *et al.* 2000, Hui *et al.* 2003). Using a cDNA array containing genes from a native tobacco that are differentially regulated by attack from leaf-chewing and single cell-feeding herbivores (Voelckel & Baldwin 2004), we determine whether or not aphids manipulate transcriptional processes in sink and source tissues of their host plant.

Studies that have examined aphid-induced alterations in plant gene expression include Fidantsef *et al.* (1999), who compared the effects of phloem feeders to chewing insects on tomato plants. They found that after *Macrosiphium euphorbiae/Myzus persicae* attack, lipoxygenase (LOX) and pathogenesis-related protein P4 (PR1) were strongly elicited but proteinase inhibitor (PI) II was not expressed, whereas after *Helicoverpa zea* attack, the opposite occurred. Similarly, the transcriptional signatures of salicylic acid signaling (apoplastic β-1,3-glucanase, PR-1) and to a lesser degree the signatures of jasmonic acid /ethylene-signaling (antimicrobial defensin PDF1.2) and wound signaling (LOX2, but not LOX1) were found in *M. persicae*-attacked *Arabidopsis thaliana* plants (Moran & Thompson 2001). *M. persicae*-mediated increases in phenylalanine ammonia lyase (PAL1) and monosaccharide symporter mRNAs suggest imbalances of phenolics and sugars at the wound site due to sequestration of phenolics in stylet sheaths and the generation of metabolic sinks, respectively (Moran & Thompson 2001).

Examining the *M. persicae-Arabidopsis* interaction further using arrays, Moran *et al.* (2002) discovered many diverse responses. For example, oxidative stress genes

(glutathione-S-transferases, superoxid dismutases), Ca^{2+}/calmodulin-related signaling genes, PR genes (BGL2, PR-1, hevein-like protein), ethylene biosynthesis genes (ACC oxidase1), aromatic biosynthesis genes (PAL2, chalcone synthase, tyrosine decarboxylase), and tryptophan biosynthetic pathway genes (anthranilate synthase β-subunit, tryptophan synthase) were found to be up-regulated or down-regulated after 72-96h of *M. persicae*-attack. A similar comprehensive array-analysis was used by Zhu-Salzman *et al.* (2004) to compare the transcriptional responses in *Sorghum bicolor* plants elicited either by greenbugs (*Schizaphis graminae*), salicylic acid (SA), or jasmonic acid (JA). Greenbug attack caused changes in the expression of defense genes (PRs, PIs, phenolics biosynthesis genes), anti-oxidant genes (glutathione-S-transferases, lactoylglutathione lyase, catalase), abiotic stress-related genes (drought-, salt- and low-temperature-responsive genes, aldehyde oxidase), nitrogen-assimilation genes (nitrite reductase), photosynthesis genes, and genes of unknown function (two of which were greenbug-specific). While some PR genes (thaumatin like proteins) responded stronger and more rapidly to greenbug attack than to salicylic acid, jasmonic acid-regulated genes (LOX, a cytochrome P450, dhurrinase, PI) were only marginally and transiently induced by the aphid, as revealed by Northern blot analysis. The latter also identified two additional greenbug-specific genes; a leucine-rich repeat-containing protein and a defense-related protein (DRP) known to be induced by sugar depletion. Zhu-Salzman *et al.* (2004) interpret the induction of DRP in the light of a fourfold decrease in soluble carbohydrate concentration in greenbug-infested barley (Cabrera *et al.* 1994). Finally, a faster and stronger accumulation of PR-1 transcripts was found in incompatible as compared to compatible *M. euphorbiae* - *L. esculentum* interactions (de Ilarduya *et al.* 2003).

In summary, phloem feeders often elicit the transcriptional signature of SA- and pathogen signaling. This elicitation may reflect responses to virus vectoring by aphids and whiteflies, aphid-associated bacterial endosymbionts, or it may suggest the similarities of intercellular fungal hyphae growth and aphid stylet penetration (Fidantsef *et al.* 1999, Walling 2000, McKenzie *et al.* 2002). The limited elicitation of JA-mediated defense responses may be due to antagonistic crosstalk with SA and ethylene signaling or stealthy feeding behavior which minimizes the amount of tissue damaged (Zhu-Salzmann *et al.* 2004).

Plant-aphid interaction studies frequently used Tanglefoot or clip cages to confine insects to leaves. Clip cages are known to decrease CO_2-exchange rates and soluble leaf protein in cotton (Crafts-Brandner & Chu 1999), indicating their adverse effects on plant

metabolism. Here we take advantage of the strong feeding preference of *Myzus nicotianae* for young leaves in order to examine local and systemic plant responses without the potential confounding influence of clip-cages. We use a native tobacco (*Nicotiana attenuata*) and analyze its responses to a naturally occurring tobacco aphid (*M. nicotianae*) with a cDNA microarray enriched in defense-related genes. We ask the following questions: (1) Does *M. nicotianae*, a phloem feeder, elicit transcriptional changes different from those elicited by representatives of other feeding guilds, such as *Manduca sexta*, a leaf chewer, and *Tupiocoris notatus*, a cell-content feeder (Voelckel & Baldwin 2004), and if so, what is the nature of these specific changes? (2) Can we detect differences in defense gene expression between sources and sinks that explain feeding preferences for sink leaves? (3) Can we detect transcriptional evidence that aphids manipulate source-sink relationships?

Array-analyses based on competitive hybridizations of two differentially labeled cDNAs allow for different sets of binary comparisons. How a factor, e.g. aphid herbivory, modulates gene expression in the absence of this factor is a question readily answered using one array (referred to as 'state analysis'). How a relative gene expression ratio, e. g. gene expression in sink relative to source leaves, is modulated by insect herbivory is a second a question, which requires the use of two arrays (referred to as 'ratio analysis'). We use both approaches and consider their respective advantages and disadvantages.

Methods

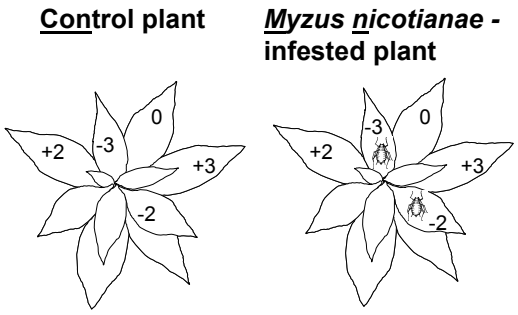
Plant and insect cultivation. Seeds of an inbred line of *Nicotiana attenuata* Torr. Ex Wats. (synonymous with *N. torreyana* Nelson and Macbr.), which was originally collected in southwestern Utah in 1992, were germinated and grown hydroponically as described by Hermsmeier *et al.* (2001). Throughout the experiment plants were grown at 26–28°C under 16h of light. A day before placing aphids on plants, 1 mL of 1 M KNO_3 was added to each 1-L hydroponic chamber, and 36 randomly chosen rosette-stage plants were paired by rosette size.

A red strain of *M. nicotianae* aphids, initially obtained from a *N. tabacum* field near Heidelberg (Germany), was bred for several generations on greenhouse-grown *N. attenuata* plants before being used in this experiment. Formerly considered a tobacco-adapted form of generalist *M. persicae*, tobacco-associated aphids were described as *M. nicotianae* in 1987 (Blackman 1987). Meanwhile, recent genetic, biochemical, and behavioral evidence suggests that *M. nicotianae* and *M. persicae* are conspecifics (Clements *et al.* 2000a, b). In

the greenhouse, *M. nicotianae* has been observed to preferentially aggregate on bolting tissue and younger leaves, as reported in the literature (*M. persicae*, Moran & Thompson 2001). In a preliminary experiment, we monitored aphid movements within rosette-stage plants over 2d. Apterous adult females placed on each of two sink leaves (the 2nd and the 3rd younger than the source-sink transition leaf, designated as leaves at nodes -2, -3) were observed to remain and reproduce on these leaves or move toward the center of the rosette to newly expanding leaves (data not shown). We used this feeding preference for young leaves to avoid the use of clip cages. One plant in each pair of experimental plants was infested with two apterous females on each of the leaves at nodes -2 and -3 (infested plant), while the other plant received no aphids (control plant). The location of each of the four

Fig. 1

A



-2, -3	local leaves (sinks)
+2, +3	systemic leaves (sources)
0	source-sink-transition leaf

B

slide	Cy5		Cy3	
1	con	loc	*M. n.*	loc
2	con	sys	*M. n.*	sys
3	con	sys	con	loc
4	*M. n.*	sys	*M. n.*	loc

Fig. 1. A Experimental design: Two sink leaves (nodes -2, -3; local) of 18 plants were infested each with two female, viviparous adult *Myzus nicotianae* and harvested after 48 h as well as two non-infested, source leaves (nodes +2, +3; systemic) from the same plants. Corresponding sink and source leaves from 18 control plants were harvested in parallel. **B** Scheme of hybridizations depicting the sources of Cy5- and Cy3-labeled cDNA of the four binary comparisons. With slides 1 and 2 local (loc) and systemic (sys) leaves of infested plants are directly compared to their counterparts on non-infested (con) plants ('state analysis'); on the remaining slides the relative gene expression between sink and source leaves is evaluated ('ratio analysis'): without aphids (slide 3) and with aphids (slide 4).

aphids per infested plant was monitored twice daily; if an aphid had moved to younger leaves or had died, it was replaced with a new one to ensure a constant aphid density on the local leaves throughout the 48h experiment. Plants typically supported a population of 4-8 females plus nymphs on their sink leaves. After 2d of feeding, all aphids were removed and the two attacked leaves (-2, -3) and two unattacked, source leaves (+2, +3) were harvested from all plants and pooled separately from infested and non-infested plants. Hence four samples (local infested, systemic infested, local control, systemic control) from 18 replicate plants were obtained (Fig. 1). Leaves were flash-frozen in liquid nitrogen and stored at -80°C until used in microarray analysis.

cDNA-array fabrication, hybridization, and quantification: 234 genes that were cloned by differential display reverse transcription-polymerase chain reaction (DDRT-PCR), subtractive hybridization with magnetic beads (SHMB), and cDNA-amplified fragment length polymorphism (AFLP) from *N. attenuata* plants subjected to real and simulated herbivory from *Manduca sexta* larvae (Hermsmeier *et al.* 2001, Hui *et al.* 2003, Halitschke *et al.* 2003) and six well-characterized *Manduca*-induced genes (*PMT, XTH, AOS, HPL, TPI, WRKY*) were PCR-amplified and spotted on epoxy coated slides as described in Halitschke *et al.* (2003). For each cDNA, two PCR fragments, with 5'-aminolink on either strand, were synthesized, and each PCR fragment was spotted four times. Hence each gene was represented by two independent PCR fragments, which, in turn, were spotted in quadruplicate. A complete list of positions and identities of spotted PCR products on the cDNA-array can be found in supplementary materials (SupplMat1, 2).

Samples were ground under liquid nitrogen and total RNA was isolated according to the methods of Pawlowski *et al.* (1994). Altogether, four hybridizations were performed and cDNAs were labeled with either Cy3 or Cy5 fluorescent dyes as specified in Fig. 1B. For cDNA synthesis, Cy3/Cy5 labeling, hybridization procedures, array scanning, evaluation of images and signal strengths (AIDA Image Analyzer and AIDA software package, Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany), and calculation of array-specific normalization factors and normalized Cy3/Cy5 ratios, see Halitschke *et al.* (2003).

Criteria for differential expression. Normalized Cy3/Cy5 ratios for each individual spot (expression ratio: ER) and the mean of the four replicate spots for each cDNA (2 for each gene: mean ER1, mean ER2) were calculated. Mean ERs that included negative values as well as obvious outliers were excluded from further analysis. A transcript was defined as being differentially regulated if both of the following criteria were fulfilled: 1) the final ER ((mean ER1+mean ER2)/2) was equal to or exceeded the arbitrary thresholds (≤0.77 for

down-regulated genes (log0.77 = -0.11) or ≥1.3 for up-regulated genes (log1.3 = 0.11); 2) mean ER1 and mean ER2 were significantly different from 1 as evaluated by t-tests to control for ER-variance and ER-sample size. For justification and evaluation of these criteria see Halitschke *et al.* (2003). Original data are organized according to their order in Figures 2 and 3 (see SupplMat3).

Results

In a previous study, the cDNA array was competitively hybridized with RNA from uninfested plants and RNA stemming from whole-plant infestations with either *M. sexta* (leaf chewer) or *T. notatus* (cell content feeder) (Voelckel & Baldwin 2004). *M. nicotianae* aphids elicited substantially fewer genes than did attack from these two species and the plants' responses to these two herbivores were more similar than either response was to the response to aphids (Fig. 2). Aphid attack did not elicit (mentioning only the prominent differences): a burst in threonine deaminase (TD) mRNA levels, an increase in allene oxide synthase, α-dioxygenase (α-DOX), hydroperoxide lyase (HPL), a pto-responsive gene, or a light-harvesting complex gene. Neither did it decrease the expression of glycine hydroxymethyltransferase, histone 3, heatshock protein 70, a GTP-binding protein, a metallothionein, a Gap dehydrogenase, or a protein translation factor (SupplMat4). Considering the differences in sampling time (24 h in the *M. sexta/T. notatus*- and 48h in

Fig. 2

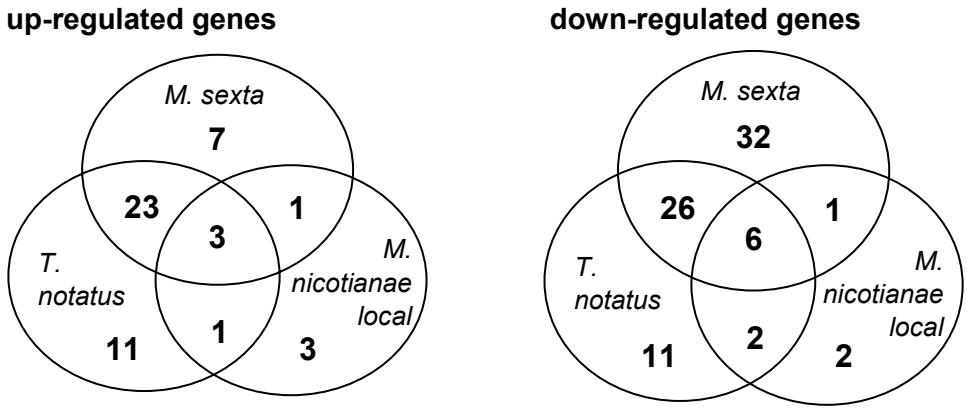


Fig. 2. Venn diagram depicting the number of cDNAs showing common or differential expression in response to attack by a leaf chewer (*Manduca sexta*), a cell content feeder (*Tupiocoris notatus*), and a phloem feeder (*Myzus nicotianae*). For details see Supplementary Material (SupplMat4).

the *M. nicotianae* experiment), sampled tissue (whole rosette versus two specific leaf positions), and herbivore infestation densities (15-20 versus 4-8 individuals), this comparison may over- or underestimate differences in gene expression.

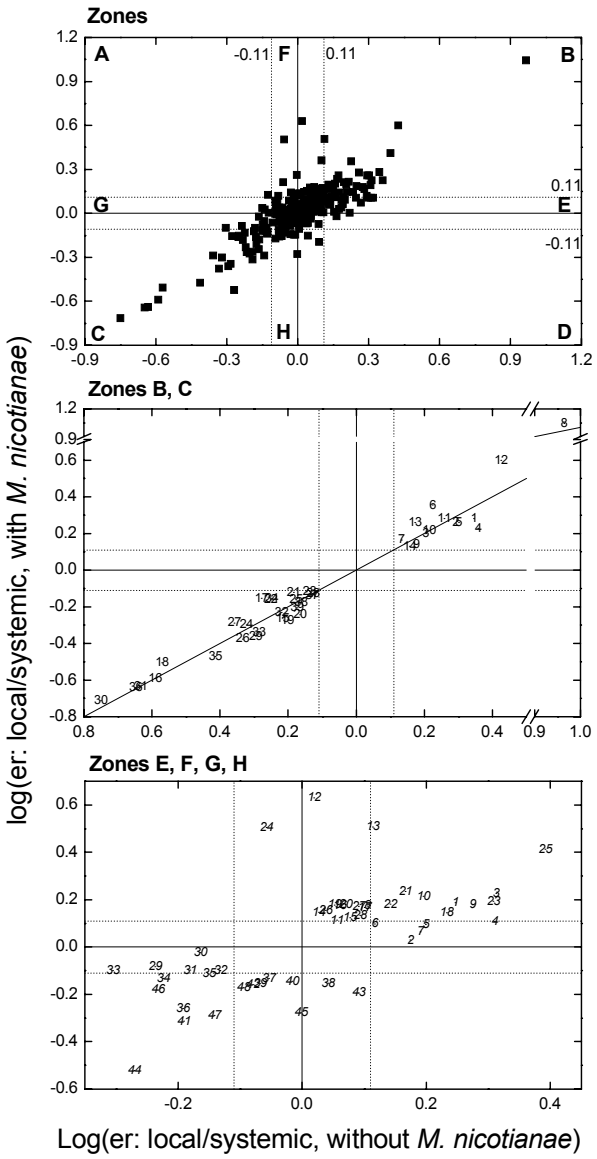
Since *M. nicotianae* preferably attacks young leaves, constitutive defense gene expression in younger relative to older leaves may correlate with this feeding preference. Moreover, constitutive expression patterns may be actively manipulated by aphids. To test these hypotheses, two hybridizations were performed as described in Fig. 1 (slides 3 and 4), and the results of this 'ratio analysis' are summarized in Fig. 3.

Interestingly, in control plants, the expression of defense genes such as *TPI* and *HPL*, photosynthesis genes such as chelatase, RUBISCO ssu, O_2-evolving and light-harvesting complex proteins, and 12 unknown genes was higher in sink leaves than it was in source leaves. On the other hand, source leaves expressed defense-related genes, such as α-DOX, 13-lipoxygenase (LOX), TD, epi-aristolochene synthase, luminal binding protein (BiP), and other genes such as a senescence-upregulated protein, a protein translation factor, an ubiquitin carrier protein, histone 3, a MAR-binding protein, and 14 unknown genes to a greater extent than did sink leaves.

While these sink/source expression differences remained in most cases (Fig. 3, upper half of table), in some cases (Fig. 3, lower half of table), the differences vanished in response to aphid attack (e.g. light-harvesting- and O_2-evolving complex proteins, LOX, TD). Moreover, some genes that had displayed no differences in expression between sink and source leaves before aphid attack (ratio=1) showed higher expression in sink (ratio>1, e.g. xyloglucan-endotransglycosylase (XTH) and glutamate synthase (Fd-GOGAT)) or source (ratio<1, e.g. germin and HMGR) leaves after aphid attack (Fig. 3, lower half of table). No matter if aphid attack erases the normal sink/source difference in expression of a gene (ratio≠1→ratio=1; e.g. TD) or elicits a difference in sink/source expression when none existed (ratio=1→ ratio≠1; e.g. germin), this analysis can not determine whether the difference results from a change in sink or in source leaves. For example, in case of LOX (<1→1), we cannot discern whether aphid attack caused an increase in sink expression or a decrease in source expression. Other ratio changes (1→<1, 1→>1, and >1→1) remain similarly irresolvable. Because of these limitations inherent to 'ratio' analysis, we performed another set of comparisons, referred to as 'state' analysis (Fig. 4).

LOX, Fd-GOGAT, and XTH were locally up-regulated whereas germin and light harvesting complex protein were locally down-regulated (Fig. 4, lower half of table). These results not only explain which type of regulation led to the change in the sink/source

Fig. 3

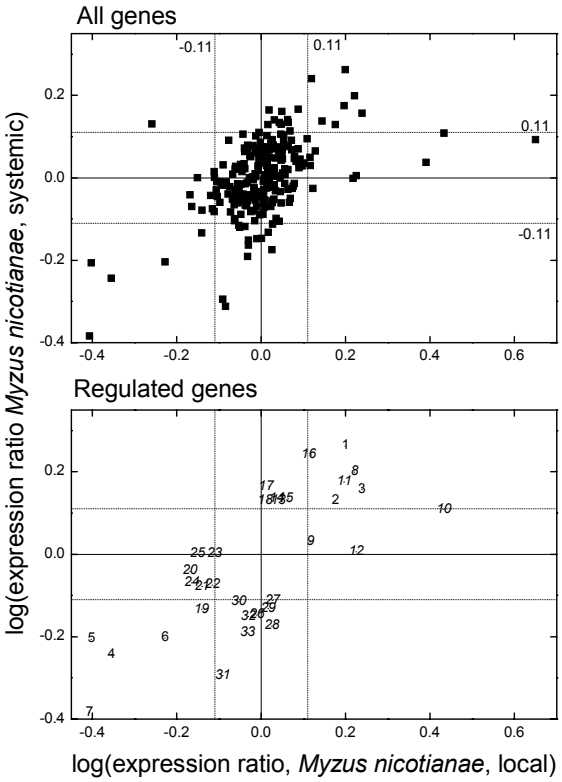


mRNA levels in sink- compared to source leaves			
Description	Label	- *M.n.*	+ *M.n.*
Genes not influenced by *M. nicotianae* attack (Zones B+C)			
18S rRNA	2	high	high
hydroperoxide lyase (HPL)	9	high	high
proteinase inhibitor (PI)	8	high	high
Mg protoporphyrin IX chelatase	4	high	high
pore protein	14	high	high
ssu pseudogene	11	high	high
9 unknowns	1, 3, 5-7, 10, 12, 13	high	high
ubiquitin carrier protein	24, 25	low	low
alpha-dioxygenase	16, 36	low	low
metallothionein-like protein	30, 31	low	low
5-epi-aristolochene synthase	28	low	low
C-7	32, 34	low	low
PII10	26	low	low
luminal binding protein (BiP)	17	low	low
putative protein translation factor SUI	38	low	low
senescence upregulated protein	18	low	low
12 unknowns	15, 19, 20-23, 27, 29, 33, 35, 37, 39	low	low
Genes influenced by *M. nicotianae* attack (Zones E, F, G, H)			
beta-tubulin	1	high	equal
lhcb1	2	high	equal
PSII O2 evolving complex peptide	4	high	equal
chloroplast genome DNA	6	high	equal
3 unknowns	3, 5, 7	high	equal
60S ribosomal protein	23	equal	high
Sn-1 gene	8	equal	high
xyloglucan endo-transglycosylase B1	12, 13, 24	equal	high
ripening regulated protein	26	equal	high
26S rRNA	11	equal	high
Fd-GOGAT precursor	9	equal	high
actin gene Sfa 15B	19	equal	high
12 unknowns	10, 14-18, 20-22, 25, 27, 28	equal	high
13-lipoxygenase	33	low	equal
threonine deaminase	29	low	equal
MAR-binding protein	32	low	equal
histone H3	31	low	equal
2 unknowns	30, 34	low	equal
germin homolog	37	equal	low
ITS 26S + 18S	43	equal	low
WRKY2	46	equal	low
HMGR	44	equal	low
major intrinsic protein 2	45	equal	low
9 unknowns	35, 36, 38-42, 47, 48	equal	low

Fig. 3. Left panel: Analysis of the relative expression of defense-related transcripts in sink as compared to source leaves (x-axis) and the influence of *Myzus nicotianae*, a phloem feeder, on this relative expression (y-axis). The upper graph depicts the mean ratio of all 240 genes and distinguishes individual zones of which the relevant ones are depicted separately in the graphs below. The lower graphs show only differentially expressed genes (i.e. genes fulfilling the criteria specified in Materials and Methods). **Right panel**: Identification of genes from the graphs. Independent of *M. nicotianae* attack, a number of genes are constitutively more highly expressed in sink leaves than in source leaves (Zone B) and vice versa (Zone C). For some genes this differential expression between sinks and sources is removed when *M. nicotianae* aphids attack plants (Zones E, G), for others with no initial difference in constitutive sink-source expression, *M. nicotianae* attack elicits differential expression (Zones F, H).

expression ratio as revealed by the previous 'ratio analysis', but also serve as an indirect replication of the analysis. Not all patterns of expression, however, were as neatly replicated (Fig. 4, lower half of table): for example, the systemic up-regulation of triose phosphate isomerase ('state analysis') did not lead to a change in its sink/source ratio ('ratio analysis'). The behavior of two other genes (local down-regulation of a ribosomal protein, systemic down-regulation of HMGR) even contradicts their behavior in the 'ratio analysis'. Moreover, some genes exhibiting regulatory behavior in the 'ratio analysis' (Fig. 3, lower table half, e.g. Sn-1) do not exhibit local or systemic regulation in the 'state analysis'. All results in which both analyses fail to confirm each other should be interpreted with care.

Fig. 4



Description	Label	local	systemic
locally and systemically regulated			
18S rRNA	2	up	up
trypsin inhibitor (TPI)	3	up	up
unknown	1	up	up
ITS 26S-18S	6	down	down
small subunit of rubisco	4	down	down
ssu pseudogene	5, 7	down	down
locally or systemically regulated			
13-lipoxygenase	12	up	non
Fd-GOGAT	8	up	non
Mg protoporphyrin IX chelatase	9	up	non
xyloglucan endo-transglycosylase B1	10	up	non
unknown	11	up	non
triosephosphate isomerase	18	non	up
5 unknowns	13-17	non	up
germin	19	down	non
lhbC1	20	down	non
putative 60S ribosomal protein	23	down	non
ssu pseudogene	25	down	non
ubiquitin carrier protein	22	down	non
unknown	21, 24	down	non
alpha-dioxygenase	28	non	down
HMGR	32	non	down
metallothionein-like protein	33	non	down
PII10	31	non	down
senescence upregulated protein	29	non	down
3 unknowns	26, 27, 30	non	down

Fig. 4. Left panel: Logarithms of mean gene expression ratios derived from the array analysis of locally *Myzus nicotianae*-infested (x-axis) versus systemically non-infested leaves (y-axis) of the same plant. The upper graph depicts the mean ratios of all 240 genes. In the lower graph only those genes are depicted whose logarithmic mean expression ratios exceed the arbitrary thresholds (log 1.3=0.11, log0.76=-0.11) and differ significantly from 1 as evaluated by t-tests. Upright numbers indicate the fulfillment of these criteria for local and systemic expression; italicized numbers indicate the fulfillment of these criteria for either local or systemic expression. **Right panel**: Identification of genes from the graphs and their regulation pattern (local, systemic, up, down, non).

'State analysis' reveals changes in local *and* systemic expression (e.g. for PIs) which are otherwise not detectable in the 'ratio analysis' since they do not change the sink/source ratio but are only superimposed on it. Only considerably larger changes in expression in either sink or source tissue are detectable as a change in the ratio.

Discussion

A major challenge to the use of microarrays in ecological analysis is the need to have adequate replication within the financial constraints of the study. By using an alternative hybridization scheme (slides 3 and 4) instead of simply replicating the hybridization scheme represented by slides 1 and 2 we showed that (1) some of the results obtained with both approaches are consistent with each other (which justifies considering the alternative hybridizations as replicate hybridizations) and (2) two different hybridization schemes can be used to provide additional information. For example, a hybridization scheme in which RNA from several treatments is hybridized with the same reference RNA allows these treatments to be compared directly. In our case, hybridizations of RNA from aphid-elicited plants with RNA from insect-free plants (slides 1 and 2) enabled comparisons with hybridizations of RNA from *M. sexta*- or *T. notatus*-induced plants with RNA from insect-free plants. On the other hand, the analysis of sink/source gene expression ratios in the absence and presence of aphids (slides 3 and 4) allowed specific hypotheses to be tested, such as whether sink tissues express defense genes differently from source tissues and whether aphid-induced shifts in source-sink expression occur.

In the following, we discuss (1) how *M. nicotianae* modulates plant gene expression differently from insects of other feeding guilds, (2) whether some of the changes indicate the aphids are manipulating plant metabolism for their benefit, and (3) if the feeding preference of the aphids for sink leaves correlates with differences in the transcription of defense genes between source and sink tissues.

Our microarray analysis revealed that aphid attack elicited the transcriptional signatures characteristic of *M. sexta* and *T. notatus* attack, namely an up-regulation of defense- and a down-regulation of photosynthesis-related genes, exemplified by local and systemic up-regulation of TPIs and down-regulation of the small subunit of Rubisco, 48h after aphid attack. While increases in PI transcripts were not seen in tomato after one week of potato aphid feeding (Fidantsef *et al.* 1999), and no information on PI responses is available in the *M. persicae-Arabidopsis* interaction (not tested with the expressed sequence tag (EST) array of Moran *et al.* 2002), greenbug aphid attack increased transcripts of several PI genes in

Sorghum after 1d (Zhu-Salzman *et al.* 2004) and *M. euphorbiae/M. persicae* feeding increased PI-I and II transcripts after 6-12h in two tomato varieties (de Ilarduya *et al.* 2003). Collectively, these results underscore the importance of selecting the 'right' time point in elicitation studies and demonstrate that genes that are commonly associated with the wound response – PIs – are elicited by supposedly 'stealthy' feeders such as aphids. Furthermore, attack from members of all three feeding guilds elicited a common set of genes: increases in LOX- and XTH-expression and a decrease in ubiquitin carrier protein transcripts (genes that were regulated by aphids in the tissues they directly attack). Apart from these similarities, aphids elicited a comparably small transcriptional response, both qualitatively (fewer genes were regulated) and quantitatively (the -fold regulations were smaller). This weak transcriptional response may be a consequence of either the aphid's stealthy feeding behavior or the selection of genes on the array. If this collection of genes had been enriched with aphid-responsive genes (compare the Zhu-Salzman *et al.* 2004 study), a stronger response may have emerged**.** On the other hand, although this array was not enriched in mirid-induced genes, a strong mirid-induced response was nevertheless found (Fig. 2, Voelckel & Baldwin 2004). While this array does not comprehensively characterize a plant's response to attack from any one of the insects, it compares the response of a subset of *M. sexta*-responsive genes to attack from three different herbivore species. Regardless of the array's limitations, an interesting pattern was observed: the up-regulation of glutamate synthase and the down-regulation of germin, a H_2O_2-generating enzyme, in leaves attacked by aphids. This pattern was detected with both hybridization approaches and appears to be unique to the *M. nicotianae-N. attenuata* interaction, since these genes were not similarly induced or repressed by *M. sexta* or *T. notatus* attack. On the contrary, glutamate synthase, a gene pivotal in nitrogen assimilation, was down-regulated by *M. sexta* attack.

The up-regulation of glutamate synthase suggests an aphid-induced increase in glutamate production. Glutamate is one of the nitrogen transport molecules in plants and supplies reduced nitrogen for aa synthesis. Interestingly, an increase in tryptophan biosynthesis genes was induced by *M. persicae* in *Arabidopsis* (Moran *et al.* 2002). The up-regulation of aa synthesizing genes by aphids could explain the mechanism for a phenomenon observed earlier by Sandstrom *et al.* (2000): the greenbug aphid not only elevated the aa concentration in the phloem sap of wheat and barley, but it also enhanced the proportion of essential aa therein as validated by stylet exudate- and cut leaf exudate analyses. This manipulation of phloem-sap composition was interpreted as a means of becoming more independent from bacterial endosymbionts (Sandstrom *et al.* 2000). Phloem

aa composition seemingly influences the nutritional quality of plants for aphids, as supported in another correlative study in which the potato- and the green peach aphid performed better on pre-tuber-filling potato plants with high glutamine levels than on tuber-filling plants with low glutamine levels (Karley *et al.* 2002). An array containing additional aa metabolism genes may reveal the extent to which aphids manipulate primary metabolism. However, in order to feed efficiently on phloem, aphids not only have to increase the nutritional value of their diet, but they must also cope with a plant's constitutive defenses. Therefore we examined differential transcription of defense genes between potential aphid feeding sites, namely source and sink leaves.

Young leaves had higher Trypsin PI- and HPL-transcript levels than did old leaves, confirming previous results from different (Vancanneyt *et al.* 2001, Howe *et al.* 2000) and the same plant systems (Schittko & Baldwin 2003). These results are in accord with the 'Optimal Defense Theory', which predicts tissues with high fitness values to be best defended (Feeny 1976, Rhoades 1979). C-6 aldehydes derived from HPL-mediated catabolism of 13-hydroperoxides were shown to adversely affect the fecundity of *M. nicotinae/M. persicae* feeding on tobacco leaves exposed to these compounds (Hildebrand *et al.* 1993) or wild type as opposed to antisense HPL potato plants (Vancanneyt *et al.* 2001). Although PIs are assumed to have little effects on phloem-feeders whose diet contains mainly free aa, PIs from potato increased the mortality of three cereal aphid species (*Diuraphis noxia, Schizaphis graminum, Rhoalosiphum padi)* in feeding trials (Tran *et al.* 1997). Similarly, Rhabé *et al.* (2003a) found weight and fecundity of *M. persicae* aphids to be reduced on oilseed rape plants that constitutively expressed the cysteine proteinase inhibitor oryzacystatin (OC-1). The deleterious effects of OC-1 correlated with a decrease in cathepsin L/H-type cysteine protease activity in extracts of whole insects. OC-1 itself was not only found in the digestive tract but it was associated with bacteriocytes, suggesting that OC-1 interacts with the bacterial symbioses which are essential for aphid reproduction (Rahbé *et al.* 2003a). In another study, Bowman-Birk bi-functional trypsin/chymotrypsin inhibitors purified from pea were toxic to pea aphids despite the lack of chymotrypsin activity in aphid guts (Rahbé *et al.* 2003b).

Why do aphids preferentially feed on young leaves that exhibit higher mRNA expression of genes (*HPL, PI*) with proven adverse effects on aphid performance? Vancanneyt *et al.* (2001), who found higher HPL transcripts in younger leaves, did not find differences in HPL activity of young compared with old leaves in potato, which suggests that this gene is not under simple transcriptional regulation. PI activity (which is measured

as PI /mg total protein) is reported to be lower in younger than in older *N. attenuata* leaves (J. Zavala & I. T. Baldwin, unpublished results), but these differences likely reflect the greater protein contents of young leaf extracts, rather than true differences in the amounts of PI proteins. However, total leaf protein contents should not be critical for aphid nutrition, and ascertaining whether an increase in PI mRNA levels translates into elevated PI activity in phloem elements, which in turn requires phloem-specific detection of PI proteins, would be more appropriate. Such an analysis has recently been accomplished by Haebel & Kehr (2001), who have found PIs in phloem exudates of cucumber. On the other hand, PIs and HPL are not the only relevant resistance traits for aphids. For example, Goundoudaki *et al.* (2003) found that aphid performance was positively correlated with leaf sugar levels but negatively correlated with trichome density. Taken together, these findings underscore the need to analyze mRNA, protein, and secondary metabolites levels in the exact tissue types on which aphids feed and contact: phloem and epidermal cells, and trichomes.

Aphids reproduce quickly and produce large populations on *N. attenuata* plants. Their success likely results from their ability to simultaneously suppress plant defense responses and manipulate phloem flow and composition. This study represents a first step in elucidating the transcriptional mechanisms behind these suppressions and manipulations. Functional studies using knock-out plants silenced in lipoxygenase, proteinase inhibitor, xyloglucan-endotransglycosylase, glutamate synthase or germin expression are needed to test the predictions of this study: namely that the induction of these genes alters the susceptibility of *N. attenuata* to *M. nicotianae* attack and that these genes ‘matter’ for the interaction.

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Manuscript V

Generalist and Specialist Lepidopteran Larvae Elicit Different Transcriptional Responses in *Nicotiana attenuata,* Which Correlate with Larval FAC Profiles

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Abstract

Unlike generalist herbivores, specialists are believed to share a history of evolutionary interactions with their host plants. We determine whether a specialist lepidopteran species alters plant metabolism differently from two generalist species despite similarities in feeding mode and larval elicitors. With a cDNA microarray enriched in defense-related genes, we compared the transcriptional responses elicited in the native tobacco *Nicotiana attenuata* by the oligophagous larvae of *Manduca sexta* and the polyphagous larvae *Heliothis virescens* and *Spodoptera exigua,* which are all members of *N. attenuata*'s natural herbivore community. We found the differences in plant responses to be correlated with the profile of larval elicitors [fatty acid amino acid conjugates: (FAC)] and discuss how variation in FAC composition may shape the interaction between generalist or specialist lepidopteran larvae and plants.

Keywords: Elicitors, fatty acid–amino acid conjugates, induced plant defense, Noctuidae, Sphingidae.

Introduction

Generalist herbivores feed on a wide range of plant taxa while specialists are associated with a few closely related or even a single plant species. Frequently, the latter are less affected by plant defense responses than the former (Agrawal 2000), probably resulting from the more intimate evolutionary interactions between specialists and their hosts (Farrell 1998). During this period of host-herbivore association, some specialists have succeeded in suppressing plant defense responses and detoxifying plant metabolites (Karban & Agrawal 2002, Wink & Theile 2002), sometimes sequestering them for their own defense (Brower 1984, Malcolm 1995, Dyer & Bowers 1996). The circumvention of the plant's defense responses often starts with a transcriptional response: for example, the suppression of wound-inducible nicotine production in *Nicotiana attenuata* by the specialist herbivore *Manduca sexta* is accomplished by an ethylene-mediated inhibition of the expression of putrescine-N-methyltransferase (Winz & Baldwin 2001), the enzyme, which catalyzes the committed step in nicotine biosynthesis. Consequently, we hypothesize that attack from specialist herbivores elicits transcriptional responses in their hosts that differ from those elicited by attack from generalists.

When plants are attacked by herbivores, their normal wound responses are frequently altered by the introduction of elicitors from the herbivore's saliva or regurgitate to the wounds at the feeding site. These modifications of the wound response are readily seen at the transcriptional as well as the metabolite level. For example, volicitin, a fatty acid-amino acid conjugate (FAC) first isolated from *Spodoptera exigua* regurgitate, not only elicits the emission of terpenoids and indole (Alborn *et al.* 1997), but also increases the expression of maize genes that catalyze terpenoid and indole formation (Frey *et al.* 2000, Shen et al. 2001, Schnee et al. 2002). Moreover, only two of the eight FACs found in *Manduca* regurgitate (N-linolenoyl-L-glutamine and N-linolenoyl-L-glutamate), account for a majority of *N. attenuata*'s transcriptional responses to real and simulated *M. sexta* feeding (Halitschke *et al.* 2003, Roda *et al.* 2004). FACs have been found in all lepidopteran larvae (generalist and specialist feeders alike) studied to date (Pohnert *et al.* 1999; Mori *et al.* 2003; Halitschke *et al.* 2001; Alborn *et al.* 2003) and all lepidopteran larvae are chewing herbivores that cause extensive damage during feeding. Given the similarity in feeding modes and elicitors, plants may have similar transcriptional responses to attack by lepidopteran larvae irrespective of their host range.

Our goal was to compare the changes in gene expression in a species of native tobacco when it is attacked by the specialist larvae of *M. sexta* (Sphingidae) with those elicited by

the generalist larvae of *Heliothis virescens* and *Spodoptera exigua* (Noctuidae). Larvae of *Manduca* spp. almost exclusively feed on solanceaous plants (except for two *Proboscidea* host species reported by Mechaber & Hildebrandt 2000) and are major herbivores of *N. attenuata.* On the other hand, *H. virescens* and *S. exigua* feed on a wide range of plant families and are only occasionally observed feeding on *N. attenuata* populations. With a cDNA array enriched in *M. sexta*-responsive genes, we tested whether plants respond differently to lepidopteran larvae that belong to the same feeding guild, possess similar elicitors, but differ in host range.

Methods

Plant and insect cultivation. *Nicotiana attenuata* Torr. Ex Wats. (synonymous with *N. torreyana* Nelson and Macbr.) plants were grown hydroponically at a 16h 32°C : 8h 28°C hour day:night cycle at 65% humidity as described by Hermsmeier *et al.* (2001). A day before placing caterpillars on plants, 16 rosette stage plants of equal size were randomly distributed in four wood cages (30x30x60cm). Eggs of *M. sexta* (Lepidoptera, Sphingidae) and *H. virescens*/*S. exigua* (Lepidoptera, Noctuidae) were obtained from Carolina Biological Supply (Burlington, VT, USA) and Bayer (Leverkusen, Germany), respectively, and were hatched at 25-28 °C.

Experimental design. Each plant was treated either with 20 first-instar *M. sexta* (first cage) or 40-50 first-instar *H. virescens* (second cage) and *S. exigua* (third cage) larvae or left untreated (control cage). Had each treatment been present in each cage, caterpillars would have moved between plants and contaminated control plants. However, cage effects were likely negligible since cages were identical, contained the same number of plants, and were all situated on the same bench of the glasshouse. During hybridizations, RNA from each treatment cage was hybridized with RNA from the same control cage, which should minimize potential cage effects. Plant replication, on the other hand, was required to ensure comparable damage levels: because not every larvae feeds on the same leaf position or to the same degree, the damage after 24h might have been different had only one plant been used in each treatment. With four plants per treatment, these differences were minimized. Different larvae numbers (20 *M. sexta* as opposed to 40-50 *S. exigua* and *H. virescens*) served to standardize the amount of damage per plant, because first-instar larvae of the two noctuids are only half the size of the first-instar larvae of *M. sexta.* The large number of larvae served to amplify the transcriptional responses to improve the resolution of the

analysis and to eliminate transcriptional responses resulting from differences in feeding mode (solitary versus gregarious feeding). In short, the experimental design examines an amplified local response to herbivore attack at a whole-rosette scale to focus the analysis on differences due to herbivore traits other than feeding kinetics, amounts, or locations on leaves (e.g. elicitor profiles).

Larvae were evenly placed across the canopy of each plant. After 24h of feeding, herbivores and their frass were removed, and shoots of plants were harvested, flash-frozen in liquid nitrogen, and stored at –80 °C.

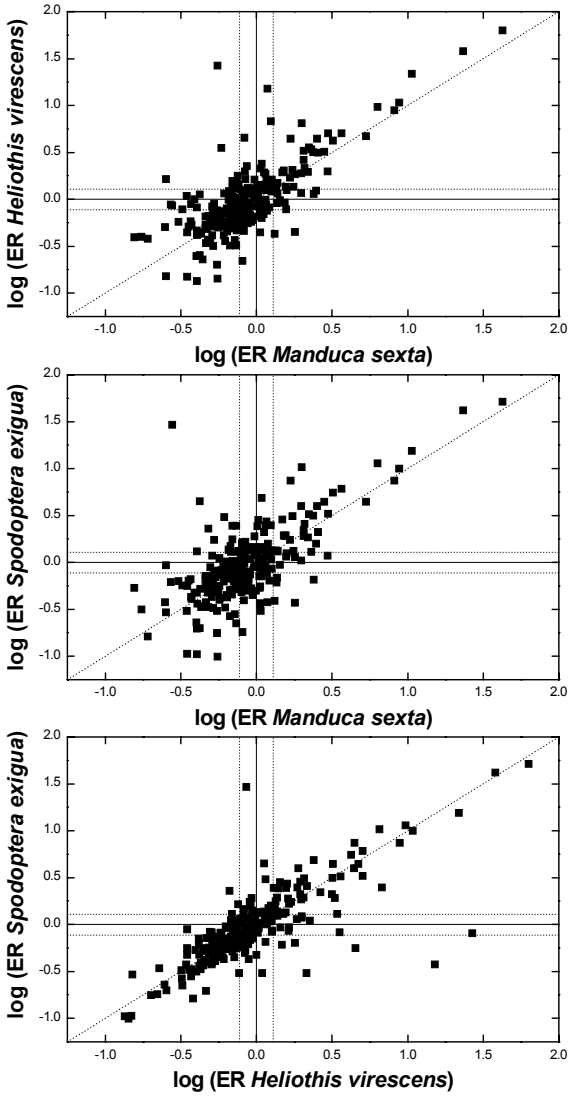
cDNA-array analysis: A total of 240 *M. sexta*-responsive genes (Hermsmeier *et al.* 2001; Hui *et al*. 2003; Halitschke *et al.* 2003) were PCR-amplified and spotted on epoxy coated slides (Halitschke *et al.* 2003). Each gene was represented by two independent PCR fragments, which were spotted in quadruplicate. A gene list is provided in Appendix S1 in the Supplementary material. Total RNA was isolated according to Pawlowski *et al.* (1994). Altogether, three hybridizations were performed and cDNAs derived from caterpillar-infested and non-infested plants were labeled with Cy3 or Cy5 fluorescent dyes, respectively. For cDNA synthesis and labeling, hybridization procedures, and data acquisition and normalization see Halitschke *et al.* (2003).

Criteria for differential expression. Normalized Cy3/Cy5 expression ratios (ER) were calculated for each spot as well as the mean of the four replicate spots for each cDNA (2 for each gene: ER1, ER2). A transcript was defined as being differentially regulated if both of the following criteria were fulfilled: 1) the final ER [(ER1+ ER2)/2)] was equal to or exceeded the arbitrary thresholds ($\leq$0.77 for down-regulated genes (log0.77 = -0.11) or $\geq$1.3 for up-regulated genes (log1.3 = 0.11); 2) ER1 and ER2 were significantly different from 1 as evaluated by t-tests to control for ER-variance and ER-sample size. For justification and evaluation of these criteria see Halitschke *et al.* (2003). Original data can be found in the Supplementary material where they are listed by the regulation patterns depicted in Figs 1 and 2.

Results

Attack from all three lepidopteran larvae elicited similar responses in a large number of genes: 61.9% and 60% of the genes up- and down-regulated by *M. sexta* were similarly regulated by *H. virescens* and *S. exigua* (Fig. 1). Among the commonly up-regulated genes were: 13-lipoxygenase and α-dioxygenase, which produce 13-hydroperoxides and 2-hydro-

Fig. 1



Description	Array ID	*Manduca sexta*	*Heliothis virescens*	*Spodoptera exigua*
commonly up-regulated				
13-lipoxygenase	438	2.80	3.21	4.44
13-lipoxygenase	672	6.34	9.66	11.43
hydroperoxide lyase	668	8.19	8.89	7.46
pathogen-inducible α-dioxygenase	382	1.99	6.50	10.40
pathogen-inducible α-dioxygenase	788	1.68	4.45	7.49
proteinase inhibitor	666	42.49	63.15	51.40
Pto-responsive gene 1	800	2.52	4.41	3.97
threonine deaminase	342	23.32	37.92	41.75
WRKY	678	1.48	1.95	2.88
xyloglucan endo-transglycosylase B1	446	3.65	5.03	6.12
unknown	496	2.19	1.97	1.86
unknown	520	2.36	3.19	3.15
unknown	532	8.80	10.77	9.97
commonly down-regulated				
6.1 kDa polypeptide photosystem II	562	0.67	0.32	0.27
axi 1	586	0.67	0.57	0.46
beta-alanine synthase	560	0.57	0.52	0.42
GTP-binding protein	656	0.57	0.51	0.40
histone H3	632	0.56	0.50	0.48
ITS 26S-18S	610	0.72	0.69	0.52
metallothionein-like protein	664	0.52	0.54	0.53
metallothionein-like protein	718	0.67	0.60	0.68
Mg protoporphyrin IX chelatase	420	0.62	0.36	0.33
PII10	608	0.42	0.25	0.20
pore protein	774	0.68	0.50	0.51
putative 60S ribosomal protein	620	0.71	0.78	0.74
ripening regulated protein	714	0.66	0.60	0.67
rubisco ssu	414	0.55	0.14	0.10
rubisco ssu pseudogene	584	0.35	0.15	0.11
rubisco ssu pseudogene	612	0.40	0.13	0.10
rubisco ssu pseudogene	726	0.55	0.20	0.18
senescence upregulated protein	390	0.70	0.65	0.68
ubiquitin carrier protein	574	0.53	0.51	0.49
ubiquitin carrier protein	576	0.70	0.66	0.67
unknown	336	0.35	0.44	0.56
unknown	338	0.54	0.73	0.56
unknown	344	0.25	0.15	0.29
unknown	386	0.19	0.38	0.16
unknown	392	0.47	0.48	0.57
unknown	394	0.46	0.65	0.67
unknown	418	0.64	0.45	0.42
unknown	434	0.40	0.42	0.48
unknown	484	0.64	0.71	0.45
unknown	524	0.57	0.60	0.56
unknown	530	0.75	0.58	0.54
unknown	538	0.52	0.32	0.33
unknown	540	0.67	0.49	0.50
unknown	564	0.44	0.23	0.34
unknown	636	0.48	0.37	0.33
unknown	658	0.46	0.34	0.37
unknown	702	0.17	0.40	0.32
unknown	742	0.77	0.63	0.67
unknown	810	0.51	0.52	0.55
not regulated by any of the species: 92 gene probes				

Fig. 1 Left panels: Pairwise comparisons of mean logarithmic expression ratios (ERs) from plants attacked by one lepdidoperan species (x-axis) against another lepdiopteran species (y-axis). Microarrays were hybridized with fluorescently labeled cDNA from plants attacked for 24h by *Manduca sexta, Heliothis virescens or Spodotera exigua* lavae against cDNA from unattacked plants. Diagonal line indicates equal expression by attack from both species. Dotted lines depict arbitrary expression thresholds (≤log0.76 = -0.11 for down-regulated genes, ≥log1.3 = 0.11 for up-regulated genes). Not all data points located outside the threshold lines reflect differential expression as ERs may not be significantly different from 1 when subjected to a one tailed t-test. Transcriptional responses elicited by *S. exigua* and *H. virescens* attack are more similar to each other than either response is to the response elicited by *M. sexta* attack. **Right panel**: ERs from genes with similarly up- or down-regulated expression patterns across all herbivore treatments.

peroxides from linolenic acid; hydroperoxide lyase, which converts fatty acid hydroperoxides to aldehydes and oxoacids; proteinase inhibitors, a potent anti-herbivore defense; threonine deaminase, which may provide isoleucine for the formation of jasmonic acid-isoloeucine conjugates (J. Kang & I. T. Baldwin, unpublished results); xyloglucan-endotransglycosylase, which modifies cell wall structure and may also generate elicitors; a WRKY type transcription factor, and a cDNA with homology to a *pto*-responsive gene. Various photosynthesis genes were commonly down-regulated (Mg protoporphyrin IX chelatase: committed step in chlorophyll biosynthesis, polypeptides of photosystem II, small subunit of Rubisco), as well as regulatory genes (ubiquitin carrier protein, GTP-binding protein), a metallothionein, which may be involved in senescence- or hypersensitive response-pathways, a histone, ripening- and senescence regulated proteins and others (Fig. 1). Three genes of unknown function were particularly strongly regulated (532 up, 344, 386 down). Of the cDNAs present on the array, 38.3% did not respond to attack from any of the larvae.

Interestingly, *N. attenuata's* transcriptional responses to attack from *H. virescens* and *S. exigua* larvae were more similar to each other than either response was to that elicited by *M. sexta* attack (Fig. 1). The similarity in the *H. viresecens*- and *S. exigua-elicited* responses resulted from the large proportion of commonly down-regulated genes (23 genes, Fig. 2).

Fig. 2

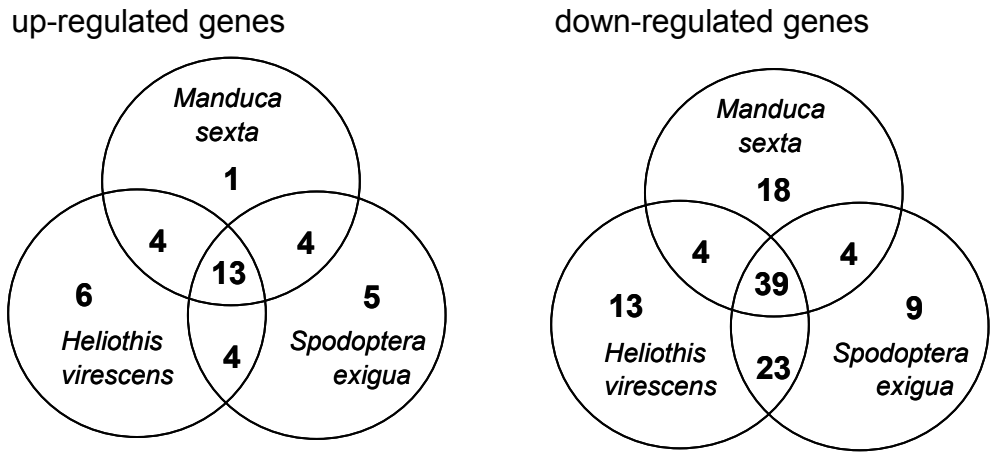


Fig. 2 Venn Diagram depicting the number of cDNAs with common or different expression in response to attack by the larvae of a sphingid (*M. sexta*) or two noctuid (*H. virescens, S. exigua*) moth species. Note the large overlap of down-regulated cDNAs between *H. virescens*- and *S. exigua-attacked plants* and the largest number of species-specific responses in *M. sexta*-down-regulated cDNAs. For gene identities and expression ratios see Supplementary material.

The 18 cDNAs down-regulated by *M. sexta*-attack (Fig. 2) represent the largest proportion of species-specific responses observed in the study. For up-regulated genes, no pair of treatments elicited responses more similar to one another than to the third response, but the two noctuid larvae elicited a larger number of specific responses than did attack by the sphingid larva (Fig. 2, SupplMat).

Discussion

Despite the large overlap in transcriptional responses to all three lepidopteran larvae, plants responded more similarly to attack from the two generalists *(S. exigua* and *H. virescens*) than to attack from the specialist (*M. sexta)*. Interestingly, this pattern of transcriptional elicitation coincides with the FAC composition described from the three species. While glutamine-based FACs are major components of regurgitates from all three larvae there are also qualitative differences in FAC composition. The FAC profiles of *S. exigua* and *H. virescens* regurgitates are almost identical (Pohnert *et al.* 1999), but *M. sexta* regurgitate differs in that it contains neither volicitin nor its hydroxylated analogs and is dominated by fatty acid-glutamic acid conjugates (N-linolenoyl-L-glutamate and N-linoleoyl-L-glutamate, Halitschke *et al.* 2001), which, in turn, are lacking from the regurgitate of *S. exigua* and *H. virescens* (Pohnert *et al.* 1999; Alborn *et al.* 2003).

Successful pathogens are known to counter a plant's ability to recognize attack by maintaining a diversity of avirulence proteins (Holt *et al*. 2003) and a mechanism comparable to the gene-for-gene co-evolution model between plants and parasites (Thompson & Burdon 1992) may determine the success of specialist herbivores. Specialists may manipulate a plant's metabolism by changing a plant's perception of biotic agents; modifying elicitors (glutamine-based FACs→glutamate-based FACs) may alter down-stream processes or silence cascades by blocking putative FAC receptors. Consistent with this hypothesis, a volicitin binding protein was found in plasma membrane fractions of *Z. mays* leaves, whose binding activity was influenced by the L-glutamine and hydroxylated moieties of volicitin (Truitt *et al.* 2004). Hence, a specialist larva may be able to alter plant responses for its own fitness benefit by manipulating its elicitor profile.

A recent analysis of *S. litura* regurgitate suggests that caterpillars can influence its FAC composition through the selective incorporation of glutamine independently of its dietary concentration (Yoshinaga *et al.* 2003). In addition to differential FAC synthesis, a larger FAC degrading activity has been found in *H. virescens* as opposed to *H. zea* larvae, which

accounted for the lower proportion of N-linolenoyl-L-glutamine in *H. virescens* frass (Mori *et al.* 2001). The particular FAC composition of *M. sexta* (no volicitin, glutamine- and glutamate-based FACs) did not depend on a diet of solanceous hosts (Alborn *et al.* 2003). Taken together, these results underscore that many processes will influence FAC composition and understanding whether they are of microbial (Spiteller *et al.* 2000) or larval origin (Lait *et al.* 2003) will help to clarify the observed patterns.

The herbivore-induced transcriptome provide a high resolution analysis of the plant's ability to differentiate between elicitors. Analyses of down-stream responses are necessarily more coarse-grained and have provided ambiguous results. *Tupiocoris notatus*- attacked *N. attenuata* plants release a blend of volatiles that is qualitatively similar but quantitatively different from that released by *M. sexta*-attacked plants (Kessler & Baldwin 2001). Since *T. notatus* does not contain FACs (A. Roda & I.T. Baldwin, unpublished results), other elicitors are clearly involved. The volatile profiles of *H. zea*-attacked cotton, tobacco, and maize plants were different from those elicited by *H. virescens* (DeMoraes *et al.* 1998), which may be due to different FAC degrading activities in larval guts (Mori *et al.* 2001). In contrast, similar volatiles were released from maize in response to *M. sexta* and *S. exigua* attack despite the herbivores' different FAC profiles (Alborn *et al.* 2003).

N. attenuata plants elicit clearly different transcriptional signatures in response to attack from native generalist and specialist lepidopteran herbivores and these differences correlate with differences in larval FAC composition. To precisely determine whether the observed transcriptional patterns are due to dissimilarities in FAC profiles, it is necessary to (1) examine the efficiency of different FACs in modulating the expression of target genes; (2) compare the transcriptional response to *M. sexta* regurgitate with that of *M. sexta* regurgitate lacking the Glu-based FACs; and (3) compare the transcriptional response to noctuid regurgitate to that of noctuid regurgitate containing the Glu-based FACs. Similarly, experiments in which volicitin is added to or removed from *M. sexta* or noctuid regurgitate, would establish the importance of hydroxylated FACs in eliciting noctuid-specific responses. Such experiments will identify the particular components of regurgitate that are responsible for the herbivore-specific responses and also likely targets of selection in the evolutionary interplay between plants and lepidopteran herbivores. While additional work with other native host-herbivore systems is required, these results demonstrate that plants are able to distinguish attack between generalist and specialist herbivores of the same feeding guild.

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4. Discussion

The following discussion integrates the findings from manuscripts II and III resulting in the suggestion of candidate genes for further studies (4.1. and 4.2), describes how the results from this thesis help answer an array of functional questions in plant-insect interactions (4.3.), gives an overview of the first manipulative studies performed in the *Nicotiana* system (4.3.), anticipates the use of Rubisco activase knock-out plants as an example for studying the adaptive value of herbivore-induced down-regulation (4.4.), and highlights the potentials of transcription profiling from an 'herbivore-centric' view (4.5.).

4.1. Putative Differentials – What Was Real?

During Northern blot analysis (NA) some of the hypothetical 'differentials' cloned by DDRT-PCR and SHMB techniques exhibited regulation patterns different from those predicted by these cloning procedures (manuscript II). After most of the clones had been spotted onto an oligonucleotide array (manuscript III), even more contradictory regulation patterns were seen. Table 1 summarizes the regulation behavior of all clones obtained by DDRT-PCR (upper part) and SHMB (lower part) when measured in NA and microarray analyses (MA). Using all these procedures, gene regulation after 24 hours of continuous herbivory from *M. sexta* larvae or *T. notatus* bugs was examined. For a more comprehensive view on gene expression, treatments with other herbivore infestation regimes (4M, 4T etc., manuscript III) were included in Table 1. Some genes cloned by DDRT-PCR had been previously 'fished' under the same experimental conditions (Hermsmeier et al. 2001) and for half of them two series of array signals are available (e.g. cv57.4 and pDH41.6 are both ESTs of α-dioxygenase and were both spotted onto the oligonucleotide array). The following paragraph identifies candidate genes (*) based on the data assembled in Table 1.

Alpha-dioxygenase*, a thionin*, a rhamnosyl transferase*, and an unknown gene (cv86.1*) were consistently up-regulated after attack from both *M. sexta* and *T. notatus*. Despite the prediction of *M. sexta*-specific up-regulation by DDRT-PCR, cv47.4* (an unknown gene) is likely up-regulated by both herbivores according to MA. Two genes with inconsistent expression patterns tend to be up-regulated after herbivory; they code for a wound stimulated protein* and another unknown gene (cvs90*). Except for NA, where SAM decarboxylase* was up-regulated only by *T. notatus* attack, DDRT-PCR and MA indicated up-regulation of this gene by attack from both herbivores. Surprisingly, the primary metabolism genes cloned as being up-regulated by *T. notatus* with SHMB (small subunit of Rubisco*, Rubisco activase*, photosystem I subunit E*, plastidic aldolase*,

Table 1. Comparison of gene regulation patterns predicted by differential procedures (DDRT-PCR, SHMB) with those obtained by Northern blot and microarray analyses.

Gene description	Clone	Chip ID	OO	DD, SHMB M	DD, SHMB T	NA M	NA T	Oligonucleotide array analysis 1M	1T	4M	4T	5M	5T	6M	6T	7M+T	Candidate gene?
cloned by DDRT-PCR (DD)																	
N. tabacum NeIF-4A15	cv11.3	5	s	down	down			1.12	1.00	1.07	1.02	1.92	1.18	1.07	1.12	1.13	NO
unknown	cv11.4	6	s	down	down			0.72	1.07	0.66	0.98	0.54	-	1.12	0.90	1.24	NO
G. hirsutum dehydration induced RD22	cv14.2	9	s					0.53	-	0.57	1.24	0.80	0.83	1.07	1.45	1.03	
	pDH19.3	291	u	mp					0.86		0.74	1.09	0.76	0.68	0.95	1.00	NO
	pDH19.3	292	u					0.61	0.83	0.43	0.51	0.94	0.20	0.87	0.79	0.69	
A.thaliana putative mutT protein	cv15.3	10	s	down	down			1.24	1.06	0.68	0.94	-	-	1.12	1.02	1.34	NO
unknown	cv16.3	11	s	down	non			0.90	1.33	1.00	1.03	1.35	1.35	1.26	1.43	1.34	NO
unknown	cv18.1	-	-	down	down			-	-	-	-	-	-	-	-	-	NO
	pDH76.2	357	s					0.78	1.20	0.94	1.01	0.86	1.15	1.19	0.93	1.35	
A.thaliana oxysterol-binding protein	cv2.4	13	s	up	non			1.29	1.30	1.26	1.01	1.53	1.20	1.16	1.22	1.11	NO
	pDH75.7	356	s					0.48	1.02	1.20	0.75	0.79	0.84	0.85	0.79	0.91	
unknown	cv28.1	14	s	mp				0.81	1.22	0.96	1.04	1.46	0.87	0.93	0.82	1.45	NO
unknown	cv29.4	16	s	mp				0.86	1.30	1.03	1.24	1.02	1.28	1.07	1.27	1.08	NO
unknown	cv30.4	-	-	mp				-	-	-	-	-	-	-	-	-	NO
	pDH39.5	320	s					0.78	1.28	0.89	0.83	0.98	1.09	1.00	0.91	0.82	
unknown	cv42.1	18	s	mp				1.03	1.07	0.95	0.96	0.99	1.01	1.07	1.01	1.06	NO
unknown	cv45.2	-	-	up	non			-	-	-	-	-	-	-	-	-	NO
unknown	cv45.4	-	-	down	non			-	-	-	-	-	-	-	-	-	NO
	pDH32.1	314	s					0.64	1.01	1.09	0.90	0.71	1.28	0.92	0.96	0.83	
N. tabacum blp5 luminal binding protein	cv46.1	20	s	up	non	up	up	1.20	1.14	0.55	0.53	0.60	0.48	0.66	0.90	0.90	NO
	pDH46.4	328	s					0.61	0.99	0.53	0.56	0.48	0.43	0.50	0.81	0.85	
unknown	cv47.4	-	-	up	non			-	-	-	-	-	-	-	-	-	YES
	pDH23.5	297	s					3.55	1.76	1.64	1.54	1.20	1.33	1.07	1.34	1.78	
unknown	cv50.1	21	s	down	down			1.11	1.15	0.93	1.16	1.27	1.00	1.20	1.40	1.05	NO
C.annum wound stimulated protein Sn-1	cv52.1	23	s	mp				1.73	1.22	0.69	1.16	1.83	1.43	1.35	2.19	1.31	YES
	pDH6.1	343	s					1.55	1.18	1.12	1.02	1.51	1.58	1.05	1.42	1.11	
A. thaliana HMGR 2	cv53.3	24	s	down	down			0.78	1.04	0.53	0.98	0.86	0.48	0.93	1.13	1.05	NO
	pDH77.2	360	s					0.87	0.85	0.96	0.96	1.05	1.14	0.97	0.80	0.85	
unknown	cv53.4	-	-	down	non			-	-	-	-	-	-	-	-	-	NO
	pDH73.2	353	s					0.49	1.49	0.96	0.79	0.91	1.31	1.24	1.19	1.37	
N. attenuata α-dioxygenase	cv57.4	25	s	up	up	up	up	4.92	1.82	1.43	1.67	0.87	1.36	1.47	1.19	2.85	YES
	pDH41.6	323	s					2.17	1.56	2.02	1.21	1.11	0.73	1.16	1.16	1.99	
unknown	cv62.3	26	s	down	non			0.62	1.08	1.08	1.09	0.86	1.17	1.01	1.08	1.06	NO
N. tabacum cytochrome f	cv63.1	28	s	down	down			0.86	0.92	0.92	1.07	0.71	1.05	0.80	0.82	0.56	NO
unknown	cv67.3	-	-	down	non			-	-	-	-	-	-	-	-	-	NO
	pDH68.1	349	s					0.69	0.70	0.90	0.97	0.93	1.00	1.03	0.85	0.94	
unknown	cv74.2	29	s	up	up			1.16	1.30	1.30	0.95	1.18	1.14	1.08	1.25	1.25	NO
unknown	cv78.4	-	-	up	up			-	-	-	-	-	-	-	-	-	NO
unknown	cv8.2	31	s	down	non			1.13	1.20	1.09	1.23	1.08	1.31	1.11	1.01	1.31	NO
N.tabacum FtsH-like protein Pftf precursor	cv8.4	33	s	down	non			0.82	0.81	0.99	1.07	1.18	1.33	0.84	0.82	0.88	NO
unknown	cv80.2	34	s	mp				0.85	0.88	0.93	1.08	1.10	1.47	0.98	0.94	0.99	NO
Solanaceae flower-specific thionin	cv84.4	37	s	up	up	up	up	3.15	1.43	4.55	3.69	1.68	1.28	0.61	1.16	4.17	YES
unknown	cv85.1	38	s	up	up			0.83	0.99	1.13	0.99	1.02	1.58	1.10	0.88	0.97	NO
unknown	cv86.1	42	s	up	up			2.42	2.12	1.76	1.70	0.87	1.33	1.10	1.13	2.52	YES
unknown	cv86.6	44	s	up	up			0.70	0.98	0.84	0.77	0.78	1.13	0.89	0.82	0.91	NO
unknown	cv88.3	47	s	mp				1.32	1.07	0.97	1.06	1.05	1.40	0.95	0.86	0.75	NO
unknown	cv90.5	51	s	down	down			1.01	1.62	0.29	-	1.35	1.25	1.07	-	0.89	NO
unknown	cv91.3	53	s	non	up			0.98	0.62	1.36	1.34	1.00	1.35	0.78	0.79	1.10	NO
unknown	cv94.1	55	s	up	non			0.74	1.06	0.46	1.14	0.86	0.78	0.93	1.31	1.16	NO
P. hybrida rhamnosyltransferase	cv95.1	56	s	up	up			3.18	1.68	1.10	1.38	0.77	1.13	0.82	0.94	2.16	YES
unknown	cv95.4	57	s	up	up			0.79	1.10	1.10	1.12	1.17	1.37	1.09	1.01	1.18	NO
A. thaliana elongation factor Tu family	cv96.2	59	s	non	up			0.97	0.89	1.09	1.09	1.22	1.21	0.95	0.82	0.96	NO
unknown	cv1.3	4	pa	up	non			1.02	1.26	0.91	1.57	2.13	1.20	1.32	1.53	1.39	NO
	pDH27.4	303	s					0.78	1.21	0.84	1.09	0.93	1.51	1.15	1.41	1.19	
unknown	cv12.2	8	pa	mp				0.56	1.09	0.88	0.92	1.17	1.20	1.19	1.36	1.26	NO
N.tabacum 16S-23S ITS + 23S	cv81.4	36	r	up	non			0.17	0.19	0.19	0.25	0.28	0.26	0.18	0.15	0.19	?
unknown	cv85.2	40	u	mp				1.29	1.22	0.78	1.15	1.09	1.66	1.08	0.82	1.53	NO
unknown	cv85.2	41	u	mp				0.91	1.07	0.78	1.10	0.59	1.53	1.36	1.15	1.37	NO
unknown	cv88.2	46	pa	mp				0.44	0.93	0.49	0.79	0.57	0.63	0.63	0.68	0.83	NO
unknown	cv90.2	49	pa	mp				0.94	1.18	1.08	1.23	1.28	1.34	1.13	1.13	1.07	NO
cloned by SHMB																	
unknown	cvs11	60	s		up			0.68	0.50	0.72	0.77	0.97	1.17	0.61	0.57	0.78	NO
N. tabacum elongation factor 2	cvs13	62	s		up	up	up	0.70	0.68	0.85	0.84	1.14	0.93	0.95	0.86	0.84	NO
unknown	cvs14	63	s		up			0.71	0.95	1.00	1.15	1.13	1.20	0.95	1.01	1.17	NO
unknown	cvs17	65	s		up			0.74	0.75	0.92	0.99	0.94	1.15	0.88	0.86	0.88	NO
unknown	cvs20	67	s		up			0.98	1.02	0.75	1.05	0.83	1.13	1.03	0.94	1.22	NO
unknown	cvs22	69	s		up			0.89	1.24	0.88	1.36	0.90	1.47	1.10	1.20	1.16	NO
A. thaliana probable RNA helicase F21E10.	cvs25	71	s		up			0.70	0.95	1.13	1.10	1.20	1.14	1.22	1.00	0.96	NO
N. tabacum α tubulin	cvs30	72	s		up			0.75	0.78	0.63	0.72	0.92	0.90	0.74	0.55	0.61	NO
N. tabacum phospholipase C2	cvs43	75	s		up	-	-	0.37	0.24	0.28	0.45	0.59	0.34	0.25	0.35	0.33	YES
N. tabacum TMV response-related gene	cvs44	76	s		up			1.22	1.32	0.74	0.62	1.13	0.99	0.92	0.81	1.10	NO
N. sylvestris glutamine synthetase	cvs45	-	-		up	down	down	-	-	-	-	-	-	-	-	-	NO
unknown	cvs46	77	s		up			0.68	0.87	0.78	0.87	0.78	1.00	0.87	0.89	0.99	NO
N. sylvestris small subunit of rubisco	cvs47	79	s		up			0.22	0.19	0.15	0.29	0.15	0.21	0.17	0.23	0.21	YES
O. sativa ribosomal protein L7	cvs49	80	s		up			0.60	0.73	0.82	0.84	1.02	0.92	0.93	0.92	0.88	NO
N. tabacum phosphoglycerate kinase	cvs50	81	s		up	-	-	0.58	0.39	0.60	0.81	0.92	0.75	0.68	0.73	0.55	YES
N. sylvestris SAM decarboxylase	cvs52	82	s		up	non	up	4.65	1.91	2.45	2.11	1.17	1.16	1.22	1.22	2.98	YES
N. paniculata plastidic aldolase	cvs53	-	-		up	-	-	-	-	-	-	-	-	-	-	-	NO
unknown	cvs70	86	s		up			0.50	0.36	0.47	0.58	0.91	0.75	0.60	0.64	0.48	YES
unknown	cvs75	88	s		up			1.06	0.96	1.05	1.05	0.83	0.99	0.85	0.77	0.84	NO
N. paniculata plastidic aldolase	cvs78	89	s		up			0.57	0.46	0.49	0.65	0.84	0.70	0.56	0.69	0.61	YES
N. sylvestris photosystem I subunit PSI-E	cvs80	90	s		up	down	down	0.45	0.28	0.41	0.59	0.75	0.62	0.47	0.58	0.37	YES
unknown	cvs81	91	s		up			1.45	1.01	0.93	1.13	1.02	1.00	0.86	1.01	1.37	NO
unknown	cvs82	93	s		up			1.01	0.83	1.22	0.90	0.98	0.98	0.91	0.93	0.72	NO
unknown	cvs89	97	s		up			0.98	0.90	0.83	1.18	1.11	1.60	0.88	1.15	1.12	NO
unknown	cvs90	99	s		up			3.13	1.31	1.32	1.58	1.12	1.49	0.94	1.07	1.68	YES
unknown	cvs91	101	s		up			0.59	0.54	0.62	0.85	0.77	0.81	0.52	0.63	0.74	YES
unknown	cvs92	103	s		up			0.88	1.23	0.84	1.31	0.77	1.48	1.00	1.01	1.02	NO
N. tabacum rubisco activase	rca inas	461	s		up			0.51	0.29	0.19	0.39	0.49	0.47	0.44	0.58	0.25	YES
	rca out	462	s					0.53	0.35	0.31	0.38	0.53	0.53	0.38	0.54	0.31	
unknown	cvs38	73	u		up			0.57	0.92	0.73	1.40	1.25	2.10	1.17	1.25	0.75	NO
unknown	cvs38	74	u		up			0.76	1.01	0.72	0.94	1.09	1.16	0.98	0.81	1.16	NO
unknown	cvs59	83	u		up			0.81	0.56	0.50	0.58	1.21	0.96	0.60	0.80	0.51	?
unknown	cvs59	84	u		up			0.72	0.41	0.29	0.43	1.20	1.05	0.54	0.75	0.38	
unknown	cvs87	95	u		up			1.32	1.12	0.75	0.97	0.60	1.17	1.45	1.77	0.95	NO
unknown	cvs87	96	u		up			0.32	0.44	0.42	-	-	0.70	0.72	-	0.84	NO

Table 1 continued

Color code		Oligonucleotide orientation (OO) on oligonucleotide microarray		Abbreviations
up-regulated		s	sense	M *Manduca sexta* T *Tupiocoris notatus*
down-regulated		pa	potentially antisense (unknown gene, one strand spotted)	NA Northern blot analysis
not regulated, no data		u	unclear (unknown gene, both strands spotted, strands with similar strong signals)	DDRT-PCR, SHMB, NA (manuscript II)
multiple patterns		r	ribosomal RNA (array regulation measurements unreliable)	1M,1T,4M,4T,5M,5T,6M,6T,7M+T (manuscript III)
Genes represented by two clones (cvX and pDHX) were cloned by two independent DDRT-PCRs, which were performed under the same experimental conditions (Hermsmeier et al. 2001, manuscript II); hence, for some of them two series of array signals are available.				

phosphoglycerate kinase*) are down-regulated in NA/MA. This is also the case with phospholipase C* and two other unknown genes (cvs70*, cvs91*). Taken together, both DDRT-PCR and SHMB delivered very few 'differentials' with consistent regulation patterns and two types of 'false positives': genes showing opposite than predicted regulation (type 1) and genes with no herbivore-induced regulation (type II). True 'differentials' and type I 'false positives' are indicated in the table as being target genes for further research.

In face of the small number of true 'differentials' delivered by them, the efficiency of the 'ask the plant procedures' might be questioned. For comparison:

1. In a DDRT-PCR analysis comparing apical leaves from whitefly-infested and non-infested *Cucurbita pepo* plants, 28 primer-pair combinations identified 11 differentially expressed cDNAs. Eight of these failed to detect an mRNA in total RNA blots or were false positives and the other three were differentially expressed with two of them encoding the same gene (van de Ven et al. 2000). In the DDRT-PCR analysis from this thesis 20 primer-pair combinations identified six candidate genes out of 45 contigs (13.3%), placing the analysis within the same efficiency range of that performed by van de Ven et al. (2000) (18.2%).

2. After forward and reverse subtractions of cDNAs from *Sorghum* seedlings infested and uninfested by greenbugs, 82 cDNA contigs of 672 cDNAs were found to be regulated by either greenbug attack, JA, or SA treatments; only greenbug-responsive were 33 (23 up, 10 down) genes (4.9%; Zhu-Salzmann et al. 2004). The subtractive analysis from this thesis yielded 10 candidate genes out of 32 contigs; a quite high efficiency (31.3%), if it is ignored that only two genes responded as predicted (6.3%). From Zhu-Salzmann's analysis it is not discernable whether the 23 up-regulated and the 10 down-regulated genes stem from the libraries enriched in greenbug-induced and greenbug-suppressed genes, respectively.

Despite the fact that different plant-insect interactions were studied and each will likely be characterized by a different number of plant gene elicitations, the 'hit-rate' of enrichment procedures such as DDRT-PCR and subtractive libraries seems to be small. On the other hand, spotting a non-subtracted, normal cDNA library (generated from herbivore-infested leaves) on an array will greatly enlarge the number of spots and increase redundant spotting.

Array analyses would become more expensive and the removal of redundancy within large datasets would be very time consuming (Gibson 2002). In summary, although being multi-step processes (including enrichment and verification procedures) the unbiased 'ask the plant' approaches have proven worthwhile since they identified candidate genes which would not have been found using biased procedures unless by accident.

4.2. Microarray Analysis Identifies More Candidate Genes

Manuscript III mainly contrasts *M. sexta*- and *T. notatus*-induced elicitations after 24 hours of continuous attack (treatments 1M and 1T of the oligonucleotide array analysis, OA) and uses the results to distinguish common and specific plant responses to these two herbivores. There were more common responses than expected, but also a few distinct specific responses. An example is the mirid-specific tenfold up-regulation of asparagine synthetase (AS). A first step in interpreting such an elicitation is to get familiar with the gene's regulation behavior established by prior investigations. AS (EC 6.3.5.4.) catalyzes the reaction of glutamine and aspartate to glutamate and asparagine, but also accepts NH_4^+ as a nitrogen donor. By synthesizing asparagine, which has a higher nitrogen/carbon (N/C) ratio than glutamine, AS plays a role in re-assimilating ammonia that is released by carbohydrate deprivation-induced proteolysis. Due to its relative inertness compared to other N-transporting amino acids, asparagine is used in long range N transport and storage. AS expression is repressed by light and metabolizable sugars but induced by carbohydrate deprivation, nitrogen compounds in the absence of glucose, Cu stress, nitrogen deficiency, and salt stress (Chevalier et al. 1996 and references therein). Interestingly, AS mRNA levels increased several-fold in the roots of the hemiparasitic *Triphysaria versicolor* in response to root exudates of *Trifolium repens* and *Arabidopsis,* establishing the first case of a parasitic plant gene being differentially regulated by host root signals (Delavault et al. 1998). The up-regulation of the AS is an early response to mirid attack (1T) because it is not seen after five days (6M) of elicitation (Table 2). AS is an example of a candidate gene (a mirid marker gene?) not found by the 'ask the plant' approaches but by arbitrarily selecting primary metabolism genes suspected to show a response.

When it comes to the identification of genes that could be the focus of future research, a dissection of the seven remaining transcriptional imprints of the OA (so far analyzed only by PCA, manuscript III) reveals more insights in herbivore-induced gene regulation (Table 2). Table 2 depicts additional regulation scores for the genes regulated in 1M and 1T and includes genes not showing any regulation in 1M or 1T but being regulated under at least

one of the other seven herbivore infestation regimes (4M, 4T, etc.). The table is organized by predominant gene regulation patterns some of which are listed below:

→ Some genes were only regulated by sequential herbivory (4M, 4T) but not by single species herbivory (6M, 6T) or parallel herbivory (7M+T).

→ Other genes were exclusively regulated by parallel attack (7M+T).

→ Some genes were regulated when attack had already ceased for four days (5M, 5T), indicating consequences for plant metabolism when the stress itself is already history ('echo' responses).

→ There are late response genes requiring five days of continuous attack (6M, 6T) for their elicitation (e.g. the up-regulation of the sesquiterpenoid cyclase germacrene C synthase by *M. sexta* and the up-regulation of the regulatory GAL83 protein by *T. notatus*). Thus specific responses may occur also in the late phase of plant induction, although, as clearly shown by PCA (manuscript III, Fig. 2) and Table 2, specific responses are more numerous within the early phase.

→ Some gene regulations being declared common responses after 24 hours of attack (e.g. Gene No. 660, 786) change into specific responses five days after herbivore attack.

→ Some of the initial common responses sustain (such as the down-regulation of photosynthesis genes and the up-regulation of almost all proteinase inhibitor probes, α-dioxygenase, epi-aristolochene-synthase, β-tubulin), but the majority declines before five days.

→ Similarly, some of the *M. sexta*- and *T. notatus*-specific responses sustain through a period of five days (e.g. mirid-up-regulated nitrate reductase and α-amylase), while most initial responses are not detectable anymore after five days (e.g. mirid-up-regulated AS).

→ Interestingly, while there was one oppositely regulated gene after 24 hours of attack (pathogenesis-related protein P3, 1M:up, 1T:down), which showed no regulation at five days, there was also one oppositely regulated gene after five days of attack (unknown gene, Gene No. 197, 1M:down, 1T:up), which showed common up-regulation after 24 hours. Except for this gene, both thionins (Gene No. 37, 400) showed a similar response: *M. sexta*-specific up-regulation after 24 hours, but *M. sexta*-specific down-regulation after five days.

All these patterns clarify that (1) specificity may not only be visible by presence/absence of gene regulation at one data point but in the kinetics of an induced response, (2) candidate genes might be chosen based on either the specificity, the longevity, or the 'echo' of the response, (3) a PCA is helpful in extracting general patterns from complex data sets (manuscript III), but can not replace a reductionist analysis of gene expression (Table 2),

Table 2. Genes regulated under at least one of the nine herbivore infestation regimes described in manuscript III sorted by regulation pattern.

Gene description	Oligo origin	Cate-gory	Gene Nr.	Strand	1M	1T	4M	4T	5M	5T	6M	6T	7M+T
Commonly down-regulated genes													
16S-23S ITS + 23S	N. a.	PS	36	r	0.17	0.19	0.19	0.25	0.28	0.26	0.18	0.15	0.19
phospholipase C2	N. a.	SI	75	s	0.37	0.24	0.28	0.45	0.59	0.34	0.25	0.35	0.33
rubisco small subunit	N. a.	PS	79	s	0.22	0.19	0.15	0.29	0.15	0.21	0.17	0.23	0.21
Photosystem II 10	N. a.	PS	134	s	0.35	0.22	0.20	0.28	0.63	0.34	0.24	0.30	0.21
unknown	N. a.	UN	154	s	0.26	0.15	0.28	0.36	0.28	0.63	0.19	0.22	0.25
RNA-binding glycine-rich protein (RGP-1a)	N. a.	TR	159	s	0.40	0.24	0.47	0.46	0.25	0.49	0.27	0.36	0.32
rubisco small subunit pseudogene	N. a.	PS	177	s	0.14	0.13	0.17	0.21	0.18	0.16	0.13	0.14	0.21
rubisco small subunit pseudogene	N. a.	PS	193	s	0.14	0.13	0.15	0.25	0.15	0.27	0.13	0.16	0.22
light harvesting complex II type III	N. a.	PS	344	s	0.43	0.33	0.37	0.48	0.55	0.44	0.42	0.60	0.40
rubisco small subunit	N. a.	PS	346	s	0.11	0.16	0.10	0.16	0.08	0.12	0.11	0.16	0.14
rubisco activase (inas)	N. a.	PS	461	s	0.51	0.29	0.19	0.39	0.49	0.47	0.44	0.58	0.25
rubisco activase (out)	N. a.	PS	462	s	0.53	0.35	0.31	0.38	0.53	0.53	0.38	0.54	0.31
rubisco small subunit pseudogene	N. a.	PS	504	s	0.16	0.14	0.11	0.18	0.10	0.17	0.14	0.18	0.14
rubisco small subunit (inas)	N. a.	PS	519	s	0.28	0.12	0.10	0.16	0.10	0.12	0.12	0.16	0.12
rubisco activase	L. pl.	PS	765	s	0.52	0.39	0.23	0.37	0.43	0.53	0.55	0.31	0.46
rubisco activase	L. pl.	PS	766	s	0.39	0.43	0.48	0.45	0.37	0.31	0.28	0.31	0.34
pathogenesis-related protein P2	L. e.	PD	749	s	0.33	0.49	0.35	0.51	0.37	0.33	0.36	0.79	0.65
pathogenesis-related protein P2	L. e.	PD	750	s	0.24	0.57	0.41	0.65	0.44	0.34	0.49	0.88	0.78
triose phosphate translocator	L. e.	PM	660	s	0.67	0.61	0.48	0.63	0.62	0.50	0.91	0.56	0.62
unknown	N. a.	UN	500	s	0.29	0.51	0.25	0.59	0.47	0.63	0.69	0.79	0.69
Photosystem I subunit PSI-E	N. a.	PS	90	s	0.45	0.28	0.41	0.59	0.75	0.62	0.47	0.58	0.37
thiazole biosynthetic enzyme precursor	N. a.	PM	133	s	0.55	0.24	0.46	0.59	0.72	0.46	0.35	0.48	0.30
phosphoribulokinase	L. e.	PM	630	s	0.63	0.56	0.52	0.61	0.68	0.48	0.64	0.50	0.52
Photosystem II O2-evolving complex 23	N. a.	PS	498	s	0.61	0.45	0.57	0.57	0.73	0.66	0.57	0.78	0.42
GAP-dehydrogenase	N. t.	PM	615	s	0.53	0.55	0.52	0.60	0.67	0.65	0.73	0.57	0.66
germin	L. e.	SR	579	s	0.50	0.46	0.59	0.55	0.73	0.65	0.74	0.80	0.58
unknown	N. a.	UN	86	s	0.50	0.36	0.47	0.58	0.91	0.75	0.60	0.64	0.48
plastidic aldolase	N. a.	PM	89	s	0.57	0.46	0.49	0.65	0.84	0.70	0.56	0.69	0.61
plastidic aldolase-like protein	N. a.	PM	495	s	0.55	0.35	0.43	0.51	0.71	0.73	0.47	0.72	0.43
ferredoxin-NADP reductase	N. t.	PM	565	s	0.60	0.49	0.34	0.51	0.86	0.72	0.75	0.68	0.49
pathogenesis-related protein P4	L. e.	PD	751	s	0.65	0.54	0.55	0.83	0.72	0.66	0.65	0.55	0.81
unknown	N. a.	UN	101	s	0.59	0.54	0.62	0.85	0.77	0.81	0.52	0.63	0.74
glutamine synthetase	N. a.	PM	266	s	0.59	0.40	0.42	0.75	0.68	0.85	0.66	0.73	0.37
histone H3 (PcH3-20)	N. a.	NC	174	s	0.52	0.43	0.62	0.89	0.79	0.88	0.71	0.64	0.63
phosphoglycerate kinase	N. a.	PM	81	s	0.58	0.39	0.60	0.81	0.92	0.75	0.68	0.73	0.55
glycine hydroxymethyltransferase	N. a.	SM	224	s	0.58	0.54	0.56	0.76	0.78	1.03	0.70	0.77	0.45
cytosolic GAP-dehydrogenase	N. a.	PM	390	s	0.65	0.65	0.66	0.68	0.74	0.71	0.75	0.80	0.48
unknown	N. a.	UN	488	s	0.62	0.53	0.63	0.69	0.82	0.97	0.72	0.79	0.61
pathogenesis-related protein P5	L. e.	PD	754	s	0.56	0.50	0.67	0.64	0.49	0.62	0.74	0.49	0.78
unknown	N. a.	UN	207	s	0.63	0.67	1.03	0.95	0.92	0.75	0.80	0.86	0.62
unknown	N. a.	UN	294	s	0.59	0.66	0.85	0.99	0.80	0.87	0.94	0.88	0.72
Oppositely regulated genes													
pathogenesis related protein 3	N. s.	PD	628	s	2.05	0.59	1.31	1.81	1.54	0.78	1.48	0.98	1.46
Commonly up-regulated genes													
xyloglucan endo-transglycosylase B1	N. a.	SM	412	s	1.98	3.09	0.83	0.31	0.68	0.46	0.73	1.39	1.41
xyloglucan endotransglycosylase (out)	N. a.	SM	530	s	2.03	2.79	0.80	0.32	1.14	1.03	0.87	1.29	1.03
wound-induced protein kinase	N. t.	SR	786	s	5.02	2.42	1.36	0.57	1.27	0.91	1.43	2.23	2.26
phenylalanine ammonia-lyase	N. a.	SM	219	s	4.27	2.29	1.21	1.05	0.63	0.50	0.85	1.06	1.82
unknown	N. a.	UN	137	s	1.73	1.72	1.13	0.90	1.16	1.52	1.18	1.32	1.15
unknown	N. a.	UN	168	s	2.88	2.36	1.10	0.58	0.97	0.76	1.13	1.65	1.29
12-oxophytodienoate reductase 3	L. e.	SM	596	s	1.85	1.51	1.44	1.21	0.79	1.11	0.98	1.20	1.23
putrescine N-methyltransferase 1 (out)	N. a.	SM	620	s	4.33	1.73	0.76	0.95	1.04	0.70	1.21	1.58	1.34
luminal binding protein blp4 + blp5 + blp8	N. t.	SR	686	s	2.20	1.52	1.10	0.95	0.82	0.80	0.87	0.83	1.14
α-dioxygenase	N. a. + N. t.	SI	741	s	2.56	1.77	1.32	1.40	1.14	0.95	1.06	1.22	1.27
rhamnosyltransferase	N. a.	SM	56	s	3.18	1.68	1.10	1.38	0.77	1.13	0.82	0.94	2.16
unknown	N. a.	UN	158	s	1.59	1.63	1.02	0.71	1.09	1.18	1.19	1.60	1.66
Pto-responsive gene 1 protein	N. a.	PD	240	s	4.19	2.41	1.39	1.49	1.12	1.51	1.10	0.98	2.49
unknown	N. a.	UN	250	s	3.59	1.74		1.01	1.08	0.63	1.15	1.69	2.26
cinnamic acid 4-hydroxylase	C. a.	SM	549	s	3.17	1.90	1.04	1.07	1.18	1.10	1.03	1.11	1.56
phenylalanine ammonia-lyase	L. e.	SM	731	s	6.59	2.23	1.30	1.31	0.90	0.85	1.01	1.36	1.75
SNF1	L. e.	NC	771	s	5.06	1.62	0.94	1.26	1.36	1.04	1.64	1.19	1.57
sucrose-phosphate synthase	L. e.	PM	776	s	4.76	1.67	1.00	1.20	1.17	1.07	1.41	1.32	1.71
unknown	N. a.	UN	199	s	5.01	3.14	0.75	0.99	2.91	2.02	2.15	3.80	2.95
α-dioxygenase	N. a.	SI	25	s	4.92	1.82	1.43	1.67	0.87	1.36	1.47	1.19	2.85
unknown	N. a.	UN	146	s	4.52	2.14	1.16	1.87		0.94	1.17	1.03	2.82
rubisco small subunit	L. e.	PS	764	s	4.53	2.92	0.98	2.04	0.97	0.92	1.39	1.39	2.87
polyphenol oxidase	S. t.	SM	746	s	3.71	2.23	1.16	1.66	0.79	0.62	1.15	1.94	1.47
α-dioxygenase	N. a.	SI	232	s	2.46	1.53	2.02	1.20	1.08		1.36	1.18	2.17
1-aminocyclopropane-1-carboxylate oxidase (inas)	N. a.	SI	268	s	3.32	1.62	1.75	1.36	1.24	1.22	1.22	1.13	2.40
lipoxygenase 3 (out)	N. a.	SI	281	s	1.66	2.28	1.82	0.99	0.78	0.81	0.84	1.17	2.06
α-dioxygenase	N. a.	SI	323	s	2.17	1.56	2.02	1.21	1.11	0.73	1.16	1.16	1.99
glutathione peroxidase	N. a.	SR	395	s	2.32	1.67	2.38	1.12	1.39	1.75	1.13	1.57	1.43
13-lipoxygenase	N. a.	SI	408	s	1.59	1.75	2.04	0.99	1.23	1.12	1.21	1.17	1.19
4-coumarate-CoA ligase	S. t.	SM	551	s	7.23	2.82	1.66	1.45	1.20	1.07	1.05	1.19	1.75
S-adenosylmethionine decarboxylase	N. a.	SM	82	s	4.65	1.91	2.45	2.11	1.17	1.16	1.22	1.22	2.98
unknown	N. a.	UN	195	u	16.61	5.44	3.51	3.92	1.46	0.85	1.31	2.01	8.50
1-aminocyclopropane-1-carboxylate oxidase (out)	N. a.	SI	269	s	4.96	2.27	2.08	2.02	1.18	1.53	1.50	1.55	4.27
unknown	N. a.	UN	297	s	3.55	1.76	1.64	1.54	1.20	1.33	1.07	1.34	1.78
beta-tubulin	N. a.	CS	361	s	9.80	3.15	3.06	2.79	1.39	1.28	1.90	1.79	4.16
S-adenosylmethionine decarboxylase	N. a.	SM	369	s	8.21	2.08	2.41	1.96	1.31	0.89	1.37	1.44	2.68
SAM:JA carboxyl methyltransferase	N. a.	SM	399	s	2.63	2.04	2.71	1.85	1.28	1.75	1.24	1.15	2.33
hydroperoxide lyase (inas)	N. a.	SM	603	s	3.17	1.72	1.63	1.80	1.19	1.07	1.44	1.10	1.42
α-dioxygenase (inas)	N. a.	SI	618	s	6.28	1.82	1.95	2.15	1.22	1.08	1.34	1.26	3.31
SAM:SA carboxyl methyltransferase	A. b.	SI	673	s	1.63	1.65	2.29	2.07	1.15	1.27	1.45	1.54	1.85
5-epi-aristolochene synthase	N. t.	SM	779	s	9.13	3.59	1.95	2.48	1.61	0.99	2.23	1.84	4.26
trypsin proteinase inhibitor (inas)	N. a.	SM	374	s	13.36	5.88	5.63	7.59	2.77	1.37	1.14	2.36	6.70
α-dioxygenase	N. a. + N. t.	SI	742	s	3.02	1.86	1.56	2.10	1.56	1.11	1.94	1.52	2.49
proteinase inhibitor IIa	S. a.	SM	770	s	12.85	5.17	2.79	4.44	2.66	1.46	2.61	2.67	5.55
unknown	N. a.	UN	197	s	10.19	4.68	7.68	10.60	4.14	1.72	0.59	2.84	11.67
trypsin proteinase inhibitor first repeat region	N. a.	SM	376	s	14.19	7.81	13.63	11.70	2.65	1.58	1.45	2.57	11.69
trypsin proteinase inhibitor for signal peptide	N. a.	SM	377	s	19.10	8.31	9.50	11.49	2.80	1.74	1.36	2.42	10.50
proteinase inhibitor IIb	S. a.	SM	767	s	9.17	5.53	10.19	10.54	1.78	1.51	1.42	2.44	13.72
proteinase inhibitor IIb	S. a.	SM	768	s	13.36	7.38	14.33	13.93	2.43	1.73	1.42	2.47	19.12
trypsin proteinase inhibitor (full length)	N. a.	SM	373	s	18.63	8.39	8.57	10.87	2.93	1.81	1.57	2.55	10.12
inhibitor 2 of Ser proteases	L. e.	SM	739	s	17.54	8.61	17.63	17.84	3.96	3.26	2.84	4.14	27.60
inhibitor 2 of Ser proteases	L. e.	SM	740	s	15.16	7.14	17.95	16.16	3.32	1.96	2.21	2.94	20.39
proteinase inhibitor IIa	S. a.	SM	769	s	11.76	6.48	5.46	6.61	2.52	1.61	2.86	2.20	8.88

Table 2 continued

***M. sexta*-specifically down-regulated genes**													
luminal binding protein	N. a.	SR	328	s	0.61	0.99	0.53	0.56	0.48	0.43	0.50	0.81	0.85
heterotrimeric GTP binding protein	N. a.	NC	420	u	0.38	1.10	0.53	0.58	0.57	0.71	0.52	0.57	0.61
Fd-GOGAT	A. t.	PM	691	s	0.35	0.76	0.34	0.61	0.53	0.39	0.57	0.98	0.80
xyloglucan endotransglycosylase (XET)	L. e.	SM	789	s	0.53	0.86	0.64	0.54	0.95	0.57	0.84	0.62	0.47
unknown	N. a.	UN	46	pa	0.44	0.93	0.49	0.79	0.57	0.63	0.63	0.68	0.83
unknown	N. a.	UN	459	s	0.52	0.79	0.40	0.94	0.54	0.44	0.63	0.91	0.92
transformer-SR ribonucleoprotein	N. a.	TR	514	s	0.36	0.97	0.53	0.85	0.51	0.47	0.66	0.90	0.84
unknown	N. a.	UN	183	s	0.65	0.92	0.42	0.89	0.65	0.63	0.84	1.07	0.98
unknown	N. a.	UN	481	s	0.63	0.90	0.58	0.98	0.62	0.47	0.74	1.07	1.02
unknown	N. a.	UN	490	s	0.38	0.81	0.30	0.70	0.61	1.07	1.06	0.81	0.91
ethylene receptor homolog	N. t.	SI	612	s	0.47	0.97	0.52	0.74	0.77	0.50	1.05	1.04	0.82
pathogenesis-related protein P4	L. e.	PD	752	s	0.45	0.91	0.43	0.84	0.75	0.44	0.83	0.96	0.73
Photosystem II 6.1	N. a.	PS	509	s	0.61	0.68	0.62	0.82	0.68	0.73	0.77	0.66	0.81
unknown	N. a.	UN	178	u	0.56	0.81	0.37	0.79	0.69	0.80	0.81	1.08	0.91
unknown	N. a.	UN	194	u	0.61	0.90	0.65	1.09	0.84	1.07	1.05	1.06	1.17
triosephosphate isomerase	N. a.	PM	245	s	0.60	0.77	0.65	0.80	0.83	0.88	0.77	0.71	0.77
unknown	N. a.	UN	510	u	0.35	1.03	0.46	0.86	0.67	0.70	0.86	1.04	0.77
tobacco rattle virus coat protein	TRV	NC	663	s	0.58	0.88	0.64	0.77	1.07	0.92	1.36	1.11	0.98
Beet Curly Top Virus coat protein	BCTV	NC	541	s	0.43	0.68	0.36	2.24	0.51	0.41	0.46	0.99	0.84
unknown	N. a.	UN	234	s	0.45	0.79	0.89	0.61	0.70	0.58	0.72	0.82	0.97
β-amylase	S. t.	PM	540	s	0.61	0.90	1.23	0.60	0.82	1.02	1.08	1.33	1.00
unknown	N. a.	UN	439	s	0.63	1.19	0.74	1.00	0.61	0.87	0.87	0.97	1.12
(E)-β-farnesene synthase	M. p.	SM	781	s	0.53	0.71	0.81	0.73	0.64	0.64	0.69	0.63	0.55
NADPH thioredoxin reductase	N. a.	PM	218	s	0.54	0.79	0.74	0.77	0.70	0.49	0.82	0.86	0.95
unknown	N. a.	UN	284	s	0.54	1.01	0.72	0.74	1.08	0.65	1.04	1.09	0.92
Mg protoporphyrin IX chelatase	N. a.	PS	350	s	0.59	0.76	0.88	0.86	0.76	0.78	0.72	0.92	0.64
sucrose-phosphate-synthase	S. t.	PM	646	s	0.51	0.89	0.84	0.87	0.98	0.87	0.83	0.88	0.65
unknown	N. a.	UN	506	u	0.46	1.20	0.79	1.18	1.17	1.04	1.07	1.46	1.63
unknown	N. a.	UN	73	u	0.57	0.92	0.73	1.40	1.25	2.10	1.17	1.25	0.75
unknown	N. a.	UN	8	pa	0.56	1.09	0.88	0.92	1.17	1.20	1.19	1.36	1.26
unknown	N. a.	UN	26	s	0.62	1.08	1.08	1.09	0.86	1.17	1.01	1.08	1.06
ribosomal protein L7	N. a.	PT	80	s	0.60	0.73	0.82	0.84	1.02	0.92	0.93	0.92	0.88
unknown	N. a.	UN	132	s	0.67	0.95	0.85	0.88	0.67	0.91	0.88	0.86	1.01
GTP-binding protein (Ran-A1)	N. a.	NC	142	u	0.60	0.78	0.81	0.77	1.03	0.86	0.93	0.89	0.88
unknown	N. a.	UN	144	s	0.63	0.97	0.79	1.07	0.88	1.19	0.73	0.87	0.75
putative 60S ribosomal protein	N. a.	PT	151	s	0.62	0.68	0.76	0.86	1.06	1.10	0.92	0.96	1.16
unknown	N. a.	UN	153	s	0.60	0.86	0.97	1.13	0.90	0.94	1.15	1.10	1.27
unknown	N. a.	UN	170	u	0.61	0.78	0.82	1.14	1.03	1.03	1.05	0.98	0.91
chaperonin 60	N. a.	SR	192	s	0.65	0.96	0.92	0.83	0.98	1.15	1.07	0.97	0.97
pore protein	N. a.	NC	221	s	0.59	0.72	0.70	0.85	0.93	0.83	0.84	0.88	0.89
SNF1-related protein kinase β-subunit (inas)	N. a.	SI	263	s	0.64	0.95	0.83	0.98	1.08	0.95	1.06	1.05	0.68
unknown	N. a.	UN	325	s	0.62	1.01	0.78	0.91	0.75	0.95	0.86	1.14	1.10
Fd-GOGAT precursor	N. a.	PM	340	s	0.61	1.02	1.09	1.09	1.10	1.07	0.96	1.10	0.69
unknown	N. a.	UN	342	pa	0.58	0.92	0.94	0.82	0.65	0.97	0.94	1.00	1.08
unknown	N. a.	UN	356	s	0.48	1.02	1.20	0.75	0.79	0.84	0.85	0.79	0.91
unknown	N. a.	UN	411	s	0.60	1.09	1.10	0.74	0.73	1.05	0.89	1.03	1.03
unknown	N. a.	UN	418	s	0.47	0.99	0.86	0.82	0.75	0.91	0.77	0.87	0.79
unknown	N. a.	UN	469	s	0.66	0.71	0.77	0.76	0.82	0.80	1.05	0.69	0.95
Tobacco rattle virus coat protein	TRV	NC	523	s	0.61	0.87	1.02	0.85	0.78	0.81	0.92	0.96	0.79
cytosolic fructose-1,6-bisphosphatase	S. t.	PM	546	s	0.57	0.70	0.72	0.95	0.99	1.07	0.87	1.02	0.71
cytosolic phosphoglycerate kinase	N. t.	PM	552	s	0.63	0.76	0.79	0.91	0.90	0.90	0.85	1.00	0.77
triacyl glycerol lipase	N. t.	SI	554	s	0.48	1.03	1.16	0.85	0.71	0.72	0.95	1.11	0.94
Fd-GOGAT	L. e.	PM	563	s	0.54	0.81	0.80	0.86	0.91	1.00	0.95	0.98	0.72
fructokinase	L. e.	PM	566	s	0.63	0.85	0.94	0.75	1.04	0.81	1.16	0.92	0.95
glutamine synthetase	N. t.	PM	580	s	0.57	0.73	0.94	0.92	0.92	1.25	1.00	0.89	0.92
chloroplast glutamine synthetase	N. p.	PM	581	s	0.62	0.94	0.72	0.78	0.82	0.79	1.01	1.01	1.01
ethylene receptor	L. e.	SI	592	s	0.54	1.07	1.06	0.91	0.76	0.97	0.90	1.07	0.90
NADH-GOGAT	L. e.	PM	602	s	0.63	0.92	0.95	0.97	0.90	0.79	0.97	1.05	0.93
SNF-1 kinase complex anchoring protein SIP1	L. e.	SI	644	s	0.58	1.04	1.13	0.98	1.07	1.01	1.03	1.01	0.72
systemin receptor SR160	L. pv.	SI	648	s	0.53	0.92	0.88	0.90	0.93	0.80	0.88	1.08	0.74
***M. sexta*-specifically up-regulated genes**													
unknown	N. a.	UN	106	s	1.53	1.31	1.00	0.73	1.05	1.09	0.79	1.18	1.13
unknown	N. a.	UN	135	u	2.19	1.40	1.26	1.01	1.05	1.27	1.21	1.11	1.35
unknown	N. a.	UN	202	s	3.34	2.26	1.28	1.27	1.08	0.64	0.94	1.35	1.19
unknown	N. a.	UN	309	s	1.76	1.09	0.94	1.10	1.13	0.98	1.15	1.04	1.30
allene oxide synthase (inas)	N. a.	SI	599	s	1.78	1.39	1.18	0.78	0.72	1.07	0.96	1.02	1.41
calcium-dependent protein kinase 3	N. t.	SI	610	s	2.04	1.29	1.09	1.20	0.79	1.14	1.28	1.40	1.29
1-aminocyclopropane-1-carboxylate oxidase	L. e.	SI	675	s	1.59	1.31	1.34	1.10	0.99	1.07	1.21	1.06	1.12
SNF1-related protein kinase	S. t.	NC	774	s	3.30	1.34	0.93	1.08	1.32	1.04	1.30	1.17	1.08
sucrose-phosphate synthase	L. e.	PM	775	s	3.13	1.48	1.00	1.13	1.02	0.94	1.48	1.24	1.46
unknown	N. a.	UN	99	s	3.13	1.31	1.32	1.58	1.12	1.49	0.94	1.07	1.68
wound stimulated protein Sn-1	N. a.	SR	23	s	1.73	1.22	0.69	1.16	1.83	1.43	1.35	2.19	1.31
flower-specific thionin	N. a.	SM	37	s	3.15	1.43	4.55	3.69	1.68	1.28	0.61	1.16	4.17
wound stimulated protein Sn-1	N. a.	SR	343	s	1.55	1.18	1.12	1.02	1.51	1.58	1.05	1.42	1.11
γ-thionin	N. a.	SM	400	s	3.34	1.41	1.78	1.73	1.60	0.76	0.49	1.33	2.37
***T. notatus*-specifically down-regulated genes**													
tetraubiquitin	N. a.	NC	365	s	0.81	0.44	0.52	0.55	0.60	0.41	0.45	0.55	0.33
unknown	N. a.	UN	484	s	0.21	0.55	0.41	0.53	0.57	0.41	0.43	0.81	0.72
phosphoglycerate kinase	N. t.	PM	625	s	0.74	0.41	0.46	0.62	0.64	0.46	0.69	0.49	0.48
metallothionein-like protein	N. a.	SR	634	s	0.67	0.33	0.31	0.39	0.44	0.38	0.32	0.32	0.80
large subunit of rubisco	N. t.	PS	638	s	0.83	0.51	0.34	0.31	0.40	0.32	0.37	0.29	0.40
unknown	N. a.	UN	83	u	0.81	0.56	0.50	0.58	1.21	0.96	0.60	0.80	0.51
unknown	N. a.	UN	84	u	0.72	0.41	0.29	0.43	1.20	1.05	0.54	0.75	0.38
lipid transfer protein (out)	N. a.	SR	384	s	0.99	0.46	0.44	0.58	0.88	0.54	0.58	0.48	0.48
induced stolon tip protein	N. a.	NC	396	s	0.74	0.46	0.52	0.53	0.89	0.64	0.65	0.62	0.35
mitogen-activated protein kinase 2	N. t.	NC	717	s	0.72	0.58	0.51	0.75	0.45	0.50	0.55	0.44	0.61
pathogenesis-related protein P6	L. e.	PD	756	s	0.70	0.55	0.55	0.82	0.56	0.67	0.65	0.63	0.84
metallothionein-like protein	N. a.	PD	189	s	0.73	0.65	0.52	0.73	0.88	0.98	0.78	0.75	0.74
transketolase	S. t.	PM	655	s	0.71	0.63	0.72	0.83	0.78	0.63	0.94	0.71	0.58
unknown	N. a.	UN	60	s	0.68	0.50	0.72	0.77	0.97	1.17	0.61	0.57	0.78
unknown	N. a.	UN	53	s	0.98	0.62	1.36	1.34	1.00	1.35	0.78	0.79	1.10
ADP/ATP translocator	N. a.	PM	452	s	1.00	0.62	0.88	0.70	0.91	0.73	0.86	0.77	0.52
5-epi-aristolochene synthase	N. a.	SM	160	s	1.02	0.60	0.91	0.83	non	0.89	1.63	1.17	3.27
GAP-dehydrogenase	N. t.	PM	547	s	0.72	0.64	0.89	0.82	0.91	0.87	0.96	0.79	0.71
***T. notatus*-specifically up-regulated genes**													
unknown	N. a.	UN	425	u	0.77	2.01	0.45	0.57	0.75	1.21	1.30	1.25	0.84
unknown	N. a.	UN	486	s	1.40	1.57	1.28	1.07	0.70	1.31	0.95	1.06	0.90
unknown	N. a.	UN	503	s	1.21	1.54	1.39	1.19	0.79	1.04	0.97	1.08	1.18
anthranilate synthase α-2 chain	N. a.	PM	633	s	0.37	1.60	1.18	1.40	1.10	1.05	1.12	1.25	0.89
polyphenol oxidases	S. t.	SM	745	s	1.38	1.75	0.83	1.04	0.81	0.90	1.08	1.22	0.90
xyloglucan endo-transglycosylase B1	N. a.	SM	432	s	2.46	2.64	0.81	0.35	1.02	0.72	0.81	1.42	1.18
WRKY (inas)	N. a.	SI	525	s	1.12	1.67	0.85	0.40	0.70	0.78	1.02	1.39	1.02
xyloglucan endotransglycosylase (inas)	N. a.	SM	532	s	1.39	2.35	0.83	0.35	0.75	0.49	0.79	0.99	0.95

Table 2 continued

xyloglucan endotransglycosylase (inas)	N. a.	SM	534	s	1.06	2.48	0.73	0.26	0.34	0.79	0.89	0.99	1.02
wound-induced protein kinase	N. t.	SR	785	s	1.08	2.25	0.81	0.42	1.02	1.25	1.00	0.87	1.00
unknown	N. a.	UN	430	u	0.80	1.79	1.10	0.76	1.13	1.74	0.96	0.97	0.62
molybdopterin synthase sulphurylase	N. a.	PM	341	s	1.61	1.53	1.33	1.18	1.05	1.60	1.43	1.33	1.32
peroxidase C	N. t.	SM	623	s	0.95	2.10	1.04	0.95	1.16	1.83	0.96	0.69	1.19
NPR1	N. t.	PD	608	s	1.86	2.25	1.33	1.00	1.42	1.17	0.86	1.60	0.96
nitrate reductase	L. e.	PM	721	s	1.39	1.54	1.29	1.38	1.14	1.32	1.40	1.52	1.25
allene oxide synthase 2	N. a.	SI	272	s	2.73	1.61	1.36	0.97	1.22	1.76	1.41	1.53	1.65
α-amylase (Amy21)	N. a.	PM	405	s		2.94	1.56	1.40	0.70	1.26	1.26	1.95	2.10
asparagine synthetase	N. t.	PM	539	s	1.05	10.36		2.77	0.75		1.19	1.04	1.46
lipoxygenase 3 (inas)	N. a.	SI	280	s		2.38	1.67		1.11	1.33	1.19	1.40	2.03
unknown	N. a.	UN	42	s	2.42	2.12	1.76	1.70	0.87	1.33	1.10	1.13	2.52
threonine deaminase	N. a.	PM	288	s	33.85	19.51	5.28	4.41	0.90	1.11	1.90		10.98
flavae 3-β-hydroxylase	N. a.	SM	397	s	1.09	2.47	1.80	2.28	0.87	1.10	1.64	1.38	1.58
unknown	N. a.	UN	477	u		6.17	3.82	3.20	1.07	1.37	1.40	3.25	5.08
α-dioxygenase (out)	N. a.	SI	619	s	4.64	1.59	2.14	1.74	1.21	1.40	1.36	1.18	1.79
Genes predominantly regulated by sequential herbivory (M. sexta followed by T. notatus (4M) or vice versa (4T))													
Photosystem II D1 protein	S. n.	PS	757	s	1.36	1.40	0.53	0.41	0.45	0.53	0.97	0.52	0.29
Photosystem II D1 protein	S. n.	PS	758	s	0.99	0.83	0.28	0.26	0.21	0.31	0.68	0.27	0.17
rubisco large subunit	L. e.	PS	761	s	0.87	0.74	0.25	0.41	0.23	0.43	0.77	0.40	0.40
rubisco large subunit	L. e.	PS	762	s	0.84	0.70	0.25	0.42	0.26	0.58	0.74	0.43	0.40
RALF precursor	N. t.	SI	760	s	1.01	1.03	0.48	0.61	0.49	0.70	0.87	0.68	0.52
fructokinase	N. t.	PM	567	s	0.70	0.71	0.66	0.59	0.87	0.84	0.93	0.76	0.51
blp5 luminal binding protein	N. a.	SR	20	s	1.20	1.14	0.55	0.53	0.60	0.48	0.66	0.90	0.90
WRKY6 (inas)	N. a.	SI	528	s	0.97	1.21	0.44	0.23	0.98	1.19	0.74	1.28	0.89
α-tubulin	N. a.	CS	72	s	0.75	0.78	0.63	0.72	0.92	0.90	0.74	0.55	0.61
tropie reductase II	S. t.	NC	662	s	0.47	0.84	0.41	0.78	0.72	0.73	0.84	0.94	0.66
lipid transfer protein (inas)	N. a.	SR	383	s	1.11	0.73	0.46	0.75	1.02	0.64	0.79	0.63	0.77
unknown	N. a.	UN	149	s	0.71	0.86	0.63	0.98	0.65	0.43	0.69	1.27	1.08
unknown	N. a.	UN	305	s	0.73	1.07	0.65	0.90	0.65	0.56	0.69	1.26	1.30
3-hydroxy-3-methylglutaryl-CoA reductase 2	N. a.	SM	24	s	0.78	1.04	0.53	0.98	0.86	0.48	0.93	1.13	1.05
unknown	N. a.	UN	169	u	0.74	0.96	0.46	1.02	0.76	0.48	0.78	1.09	1.14
cathepsin D inhibitor	S. n.	SM	689	s	0.78	0.90	0.52	0.93	0.88	0.64	0.94	1.00	1.01
unknown	N. a.	UN	6	s	0.72	1.07	0.66	0.98	0.54		1.12	0.90	1.24
dehydration induced RD22-like protein	N. a.	SR	9	s	0.53		0.57	1.24	0.80	0.83	1.07	1.45	1.03
unknown	N. a.	UN	51	s	1.01	1.62	0.29		1.35	1.25	1.07		0.89
unknown	N. a.	UN	55	s	0.74	1.06	0.46	1.14	0.86	0.78	0.93	1.31	1.16
unknown	N. a.	UN	96	u	0.32	0.44	0.42			0.70	0.72		0.84
basic form of pathogenesis-related protein 1	N. a.	PD	130	s	0.71	0.78	0.46	0.91	0.86	1.12	1.02	0.87	1.02
unknown	N. a.	UN	136	u	0.97	1.24	0.36	0.97	1.97	1.18	0.85	1.05	0.89
unknown	N. a.	UN	148	s	0.74	0.81	0.59	0.83	0.92	0.68	0.76	0.76	0.89
unknown	N. a.	UN	163	u	0.98	1.34	0.43	0.88	1.00	0.91	1.21	1.20	1.01
unknown	N. a.	UN	301	s	0.72	1.22	0.62	1.05	0.77	0.70	0.93	1.10	1.04
unknown	N. a.	UN	311	s	1.33	1.15	0.60	1.17	1.32	1.43	1.48	1.46	1.00
defensin	N. a.	PD	386	s	0.78	0.70	0.61	0.77	0.88	0.92	0.84	0.92	0.79
unknown	N. a.	UN	435	s	0.85	1.38	0.54	0.80	0.85	1.01	1.14	1.18	1.07
unknown	N. a.	UN	448	s	0.81	0.96	0.58	0.68	1.12	0.82	1.00	1.08	0.89
unknown	N. a.	UN	453	s	0.92	1.20	0.55	0.90	0.77	1.13	0.76	1.05	0.89
fructose 1,6 bisphosphatase precursor	S. t.	PM	614	s	0.74	0.95	0.62	0.81	0.88	0.85	0.96	0.90	0.74
pathogenesis-related protein P6	L. e.	PD	755	s	0.91	0.98	0.54	0.81	0.87	0.67	0.94	0.99	0.89
SNF1-related protein kinase	S. t.	NC	773	s	0.71	0.94	0.65	0.93	0.78	0.74	0.88	0.95	1.00
(E)-β-farnesene synthase	M. p.	SM	782	s	0.69	1.05	0.62	0.89	0.86	0.77	0.96	0.99	0.91
Hsc70 gene	N. a.	SR	118	s	0.81	0.77	1.55	0.84	1.12	0.98	0.85	0.96	0.91
small GTP-binding protein	N. a.	NC	371	s	1.45	1.33	1.62	1.39	1.24	1.42	1.21	1.16	0.96
3-hydroxy-3-methylglutaryl-CoA reductase	N. a.	SM	667	s	1.38	1.14	1.54	0.76	1.10	0.81	1.15	1.44	1.31
3-hydroxy-3-methylglutaryl-CoA reductase	N. a.	SM	139	s	1.31	1.46	1.90	0.82	0.95	0.96	1.26	1.43	1.62
TMV response-related gene	N. a.	PD	76	s	1.22	1.32	0.74	0.62	1.13	0.99	0.92	0.81	1.10
cytosolic GAP-dehydrogenase	N. a.	PM	140	s	0.70	0.68	0.74	0.64	1.24	0.90	0.74	0.67	0.75
unknown	N. a.	UN	226	s	1.54	1.35	1.20	0.53	0.74	1.00	0.80	0.92	1.40
unknown	N. a.	UN	261	s	0.77	0.94	0.76	0.65	0.69	0.83	0.76	0.85	0.92
alternative oxidase (inas)	N. a.	SI	274	s	1.05	1.22	0.97	0.61	1.02	1.06	0.91	1.05	0.98
alternative oxidase (out)	N. a.	SI	275	s	1.34	1.37	1.13	0.56	0.78	0.95	0.82	1.10	1.27
unknown	N. a.	UN	456	s	2.43	1.47	0.99	0.44	0.82	0.98	0.76	1.17	0.97
WRKY2	N. a.	SI	501	s	0.62	1.46	1.02	0.41	0.73	0.95	0.79	1.49	0.96
WRKY3 (out)	N. a.	SI	526	s	1.61	1.40	1.03	0.42	2.38	0.83	0.88	1.50	0.89
xyloglucan endotransglycosylase (XET)	L. e.	SM	790	s	0.69	0.96	0.98	0.63	1.05	0.70	0.95	0.80	0.56
unknown	N. a.	UN	443	s		3.05	0.71	0.31	0.74	0.45	0.59	1.39	1.41
elicitor-inducible cytochrome P450	N. t.	SM	553	s	0.51	0.99	1.28	0.56	0.83	0.86	1.12	1.20	1.68
SNF1-related protein kinase α-subunit	N. a.	SI	1	s	1.25	1.22	1.29	1.62	1.57	1.64	1.35	1.28	0.95
unknown	N. a.	UN	4	pa	1.02	1.26	0.91	1.57	2.13	1.20	1.32	1.53	1.39
Genes exclusively regulated by simultaneous herbivory from M. sexta and T. notatus (7M+T)													
cytochrome f	N. a.	PS	28	s	0.86	0.92	0.92	1.07	0.71	1.05	0.80	0.82	0.56
chloroplast gene	N. a.	PS	110	s	0.70	0.71	0.88	0.97	1.05	1.10	0.77	0.78	0.62
unknown	N. a.	UN	113	s	0.79	0.73	0.95	0.86	0.90	1.01	0.71	0.68	0.63
chloroplast gene	N. a.	PS	181	s	0.80	0.79	0.90	0.95	0.75	0.94	0.77	0.75	0.49
chloroplast gene	N. a.	PS	222	s	0.82	0.85	0.89	1.08	0.90	1.09	0.92	0.80	0.50
SNF1-related protein kinase β-subunit (out)	N. a.	SI	264	s	0.79	0.90	0.75	1.16	0.87	0.93	1.03	1.02	0.53
lipoxygenase 2	N. a.	SI	278	s	1.40	0.97	0.74	0.90	1.02	1.24	0.76	1.01	0.64
GAL83	N. a.	SI	345	s	0.92	1.10	0.99	1.37	1.03	1.29	1.30	1.10	0.64
putative preprocysteine proteinase	N. a.	NC	370	s	1.25	0.78	1.14	1.08	1.30	1.12	0.90	1.09	0.56
unknown	N. a.	UN	416	s	0.81	1.10	0.80	0.85	1.19	1.11	0.96	0.95	0.56
unknown	N. a.	UN	511	u	0.97	0.97	1.11	0.93	1.41	0.87	0.85	1.45	0.61
ubiquitin carrier protein	N. a.	NC	517	s	0.98	0.84	0.78	0.79	1.09	0.97	0.87	0.84	0.64
caffeic acid O-methyltransferase	C. a.	SM	543	s	0.95	1.15	0.77	0.70	1.16	0.94	0.73	0.68	0.64
Cucumber Mosaic Virus coat protein	CMV	NC	550	s	0.84	0.98	0.92	1.05	0.84	1.14	0.97	1.19	0.61
ethylene response element binding protein 2	N. t.	SI	558	s			0.25	0.63		1.12	1.23		0.18
ferulate-5-hydroxylase	L. e. X L. pl	SM	564	s	0.89	1.18	0.70	0.71	1.18	1.09	1.03	0.85	0.59
germin	L. e.	SR	573	s	0.80	0.86	1.00	0.92	0.94	0.87	1.19	1.14	0.59
germin	N. l. X N. s.	SR	577	s	0.87	1.21	0.68	0.77	0.96	1.11	1.05	1.10	0.65
lipid transfer protein	T. a.	SR	597	s	0.72	1.10	0.90	1.00	0.86	1.03	1.14	1.02	0.66
phytochrome A	N. t.	SI	616	s	0.17	1.01	0.99	0.82	1.12	0.85	0.94	0.77	0.54
ethylene insensitive 3 homolog (TEIL)	N. t.	SI	652	s	0.92	0.91	1.14	1.08	1.05	0.91	1.05	0.96	0.61
catalase	L. e.	SR	688	s	0.73	0.72	0.72	0.76	0.94	0.91	0.92	0.92	0.52
unknown	N. a.	UN	242	s	1.48	1.33	0.90	0.93	1.26	0.54	1.13	1.35	1.51
unknown	N. a.	UN	497	s	1.50	1.45	1.06	0.98	0.95	1.30	0.97	1.14	1.95
5-epi-aristolochene synthase	N. t.	SM	780	s	0.83	1.30	0.86	1.32	0.90	0.91	1.09	1.36	1.64
Late response genes type I (respond after attack has already ceased)													
5-epi-aristolochene synthase	N. a.	SM	659	s	non	0.61	0.87	0.56	0.50	0.61	0.85	0.87	
unknown	N. a.	UN	446	u	0.49	0.48	0.10	0.46	0.50	0.46	0.52	0.80	0.78
Alfalfa Mosaic Virus coat protein	AMV	NC	538	s	0.51	0.79	0.33	0.80	0.45	0.73	0.54	0.80	0.68
chloroplast gene	N. a.	PS	235	s	0.79	0.74	0.93	1.03	0.65	0.95	0.73	0.85	0.55
unknown	N. a.	UN	332	s	0.63	0.95	0.87	0.71	0.65	0.94	0.75	0.82	0.93
unknown	N. a.	UN	337	s	0.77	1.09	0.91	0.84	0.62	1.04	0.82	0.93	1.13
unknown	N. a.	UN	451	u	0.57	1.41	0.39	0.37	0.55	0.85	0.74	0.92	0.90

Table 2 continued

NADH GOGAT	A. t.	PM	601	s			0.35		0.47	0.54	1.05	1.46	0.45
TMK1 protein kinase	A. t.	NC	657	s	1.13	1.18	0.42	0.86	0.63	0.83	1.05	1.06	0.80
putative phospholipase A2	N. t.	SI	743	s	0.72		0.29		0.46			0.48	
translation initiation factor 4A-15	N. a.	PT	5	s	1.12	1.00	1.07	1.02	1.92	1.18	1.07	1.12	1.13
oxysterol-binding protein	N. a.	NC	13	s	1.29	1.30	1.26	1.01	1.53	1.20	1.16	1.22	1.11
potato virus Y coat protein	PVY	NC	636	s	0.46	1.24	0.74	0.78	1.99	1.13	1.19	1.20	1.04
sedoheptulose-1,7-bisphosphatase	L. e.	PM	643	s	0.67	0.98	0.86	0.78	0.77	0.65	1.08	0.56	0.72
WRKY	S. t.	SI	788	s	0.63	0.67	0.59	0.69	1.04	0.47	0.65	0.99	0.65
unknown	N. a.	UN	253	s	0.80	1.19	0.76	0.91	0.94	0.65	1.10	1.07	1.06
chalcone synthase 2	N. a.	SM	364	s	0.70	1.12	0.73	0.86	0.89	0.63	0.84	0.84	0.72
unknown	N. a.	UN	437	u	0.95		0.32	0.46	1.65	0.50	1.34	1.77	0.85
unknown	N. a.	UN	38	s	0.83	0.99	1.13	0.99	1.02	1.58	1.10	0.88	0.97
unknown	N. a.	UN	40	u	1.29	1.22	0.78	1.15	1.09	1.66	1.08	0.82	1.53
unknown	N. a.	UN	97	s	0.98	0.90	0.83	1.18	1.11	1.60	0.88	1.15	1.12
germin homolog	N. a.	SR	293	s	1.37	1.31	1.20	1.06	1.24	1.59	1.20	1.08	1.26
unknown	N. a.	UN	299	s	0.94	0.98	1.20	1.27		1.56	1.10	0.85	0.84
unknown	N. a.	UN	303	s	0.78	1.21	0.84	1.09	0.93	1.51	1.15	1.41	1.19
unknown	N. a.	UN	317	s	1.07	1.31	1.05	0.97	1.23	1.57	1.31	1.25	0.99
ethylene response element binding protein 3	N. t.	SI	559	s	0.85	1.36	0.69	0.88		1.89	1.28	1.15	0.87
invertase	N. t.	PM	585	s	1.22	1.45	1.09	1.15	1.18	1.54	1.39	0.99	1.08
tomato spotted wilt virus capsid protein	TSWV	NC	664	s			0.98	0.52	0.69	1.60	1.02	0.98	0.81
Late response genes type II (respond late but under ongoing attack)													
myb1 gene	N. a.	SI	457	s	7.66	1.47	1.03	0.87		1.02	0.64	1.02	0.58
germin	L. e.	SR	570	s	0.41	0.56	0.35	0.67		0.64	0.53	0.84	0.79
unknown	N. a.	UN	327	s	1.02	1.27	0.67	0.79	1.48	1.07	1.52	1.30	0.93
isopropylmalate dehydrogenase	N. a.	PM	379	s	0.84	1.01	1.02	1.11	0.99	1.14	1.57	0.99	0.97
germacrene C synthase	L. e.	SM	594	s	0.74	1.20	0.56		0.44		1.86	1.66	1.52
TAF Gbox binding factor	N. t.	SI	651	s	1.06	1.29	1.31	1.15	1.47	1.26	1.79	0.81	0.70
aluminium induced protein	N. a.	SR	401	s	0.82	0.73	0.73	0.76	1.07	0.88	0.80	0.66	0.38
SKP1-like protein	N .c.	NC	645	s	1.02	0.78	0.89	0.85	1.02	0.78	1.01	0.65	0.63
sucrose synthase	N. t.	PM	650	s	0.77	1.03	0.95	0.74	1.21	0.88	0.95	0.58	0.48
potato virus X coat protein	PVX	NC	635	s			0.33	1.51	0.35	0.59	3.22	1.72	
GAL83 protein	S. t.	SI	695	s	1.33	1.39	1.05	1.28	1.07	1.07	1.35	1.50	1.01
GAL83 protein	S. t.	SI	696	s	0.96	1.23	0.95	1.16	1.11	0.94	1.14	1.52	1.06
acidic PR-1 protein	L. e.	PD	747	s	2.23	1.00	0.83	0.42	0.52	1.34	1.13	1.56	0.93

Table legend

Abbreviations		Oligonucleotide origin				Category	
ER = expression ratio		A.b.	*Atropa belladonna*	N.l.	*Nicotiana langsdorffii*	PM	primary metabolism
MSS = mean sum of signal strength for Cy3 and Cy5		A.t.	*Arabidopsis thaliana*	N.p.	*Nicotiana plumbaginifolia*	SM	secondary metabolism
Criteria		AMV	alfalfa mosaic virus	N.s.	*Nicotiana silvestris*	PS	photosynthesis
ER>1.5 + ER≠1 + MSS>1000	up	BCTV	beet curly top virus	N.t.	*Nicotiana tabacum*	SI	signaling
ER<0.67 + ER≠1 + MSS>1000	down	C.a.	*Capsicum annuum*	PVX	potato virus X	PD	pathogen defense
at least one of the criteria not fullfilled	non	CMV	cucumber mosaic virus	PVY	potato virus Y	CS	cytoskeleton
no data available	empty	L.e.	*Lycopersicon esculentum*	S.a.	*Solanum americanum*	PT	protein translation
oligonucleotide orientation on microarray		L.pl.	*Lycopersicon perennellii*	S.n.	*Solanum nigrum*	TR	transcription
sense	s	L.pv.	*Lycopersicon peruvianum*	S.t.	*Solanum tuberosum*	SR	stress response
potentially antisense (one strand spotted)	pa	M.p.	*Mentha piperita*	T.a.	*Triticum aestivum*	UN	unknown
undecided (both strands spotted)	u	N.a.	*Nicotiana attenuata*	TRV	tobacco rattle virus	NC	not classified
ribosomal RNA (unreliable)	r	N.c.	*Nicotiana clevelandii*	TSWV	tomato spotted wilt virus		

for treatment descriptions (1M, 1T, 4M, 4T, 5M, 5T, 6M, 6T, 7M+T) see manuscript III

and (4) microarrays, regardless whether they consist of sequences from 'ask the plant approaches' or sequences from the 'shelf of the most likely induced transcripts', speed up the candidate gene discovery process by simultaneously monitoring many genes under many conditions. Recent developments like geniom®one, a benchtop microarray facility (Febit AG, Mannheim, Germany) which automates the design and fabrication of customized arrays, the injection of DNA samples, hybridizations, and fluorescence detection, will facilitate these type of analyses and make them standard procedures.

4.3. Transcriptomics of Plant-Herbivore Interactions – What Comes Next?

Using DDRT-PCR, SHMB, and microarray analyses, a handful of genes with interesting expression patterns after insect herbivory have been identified; among them many of unknown function. More primer combinations in DDRT-PCR as well as more binary comparisons with different driver and tester mRNAs in SHMB would certainly increase the number of target genes for future studies. Taken into account the relatively low number of 'right positives' returned by these procedures, another approach currently in progress may

be faster: some of the treatments described in manuscript III (24 hour *M. sexta* attack, 24 hour *T. notatus* attack, 24 hour *M. sexta* attack followed by four days of *T. notatus* attack, five days of *T. notatus* attack, five days of *T. notatus* and *M. sexta* attack) have been repeated with three biological replicates each in order to hybridize RNA to an array produced by the Institute of Genomic Research (TIGR, Rockville, Maryland, USA). This cDNA array contains about 10,000 validated potato Expressed Sequence Tags (ESTs) and has been successfully hybridized to samples from other solanaceous species, such as tomato, eggplant, tobacco, petunia, and *N. benthamiana* (http://www.tigr.org/tdb/potato/). The analysis will reveal whether the patterns found with the biased, small scale arrays are repeatable and identify more genes regulated by insect herbivores in general and by insect species or members of insect feeding guilds in particular.

Questions. Accumulating this knowledge, how can it be used in addressing the questions of ultimate interest? We want to understand why these genes are regulated after herbivore attack and are looking for answers on the mechanistic and the functional level. Mechanistically, we are interested in questions such as: which oxylipin signals generated by the jasmonate cascade mediate herbivore resistance? Which enzyme within an enzyme family plays a role in resistance mechanisms? Which enzymes catalyze committed steps in secondary metabolism pathways? How are mechanical damage and herbivore-derived elicitors perceived in plants? What is the nature of a systemic signal? Which signals do plants use to communicate with other trophic levels? Functionally, we are interested in questions such as: How many of the elicitations observed are adaptive responses and how many are just a consequence of pleiotropic effects or epistatic interactions? Do these changes in gene regulation increase plant fitness or are they indicative of a manipulation of plant metabolism by the herbivore and as a result increase the herbivore's fitness? Why are plants resistant against certain herbivores but susceptible to others? How do herbivores overcome resistance? How does plant resistance influence the structure of its herbivore community? What are the costs of resistance? Is there a trade-off between resistance and growth? Do plants cope with herbivore attack by resistance or tolerance traits (or both) depending on the attacking herbivore? Can the effects of direct defenses interfere with the 'success' of indirect defenses? What are the benefits of communicating herbivore attack within a plant or to other plants?

Gene knock-outs. A way of examining gene function on either level of analysis is by studying plants lacking the expression of these genes. The *Arabidopsis* research community permanently generates mutants exhibiting single or multiple defects in every aspect of plant

metabolism in order to explain gene function. Transgenic technology also offers the possibility to reduce endogenous gene expression by antisense or inverted repeat expression of the respective gene. Examples, where antisense-mediated gene silencing has revealed functional aspects of tomato prosystemin (Orozco-Cardenas et al. 1993), potato lipoxygenase H3 (Royo 1999), potato hydroperoxide lyase (Vancanneyt et al. 2001), and a tobacco P450 hydroxylase (Wang et al. 2001) were cited in manuscript I. Each of these genes was shown to be involved in herbivore resistance, since herbivore performance was increased on the antisense lines. In *N. attenuata*, plants are being transformed with antisense and inverted repeat constructs of genes with putative roles in plant defense (proteinase inhibitors, putrescine-N-methyltransferase - PMT, lipoxygenase - LOX, allene oxide synthase - AOS, hydroperoxide lyase - HPL, lipid transfer protein, prosystemin) or genes which were cloned in herbivore-elicitation experiments (threonine deaminase - TD, α-dioxygenase, xyloglucan-endotransglycosylase - XTH, germin, SNF-1 interacting protein, thionin) to study the function of these genes.

Examples from N. attenuata – the LOX story. From the *N. attenuata* lipoxygenase family, LOX3 showed a rapid but transient amplification after wounding and the application of *M. sexta* regurgitate (R), suggesting that LOX3 mediates the wound-induced JA burst (Halitschke et al. 2003). Moreover, LOX3-derived oligonuclotide and cDNA probes indicated LOX3 induction after *M. sexta, T. notatus, H. virescens, S. exigua*, and *M. nicotianae* attack (manuscripts III-IV). Three independent antisense LOX lines had reduced wound-induced and R-induced JA levels, but the wound-induced emission of green leaf volatiles (GLV) remained unchanged. Thus LOX3 supplies hydroperoxides for the synthesis of JA rather than GLV. *M. sexta*-induced nicotine accumulation was reduced in antisense plants but could be restored by methyl jasmonate (MeJA) application. *M. sexta* induced TPI activity was lowered in only one antisense line, but MeJA-induced wild type levels could not be restored in MeJA-treated antisense plants indicating that TPI activity is not regulated by JA alone. Regurgitate-induced cis-α-bergamotene emission was lacking in antisense LOX3 plants, but cis-α-bergamotene levels in MeJA-treated wild type and transgenic plants did not differ. Gene expression analysis using the cDNA array revealed a role for LOX3-derived oxylipins in up-regulating defense genes (TPIs, XTH, TD, HPL) and suppressing both down-regulated growth genes (Rubisco small subunit, photosystem II peptide) and up-regulated oxylipin genes (α-dioxygenase). In addition to establishing a mechanistic role for LOX3 in gene expression as well as hormone and defense metabolite biosynthesis, herbivore resistance was found to be compromised in transgenic plants. *M. sexta* larvae

gained more weight and consumed more leaf area feeding on antisense plants and MeJA treatment did restore resistance as well as eliminated differences in leaf area consumption (Halitschke and Baldwin 2003). The lack of *M. sexta*-induced cis-α-bergamotene emission, unchanged wound-induced GLV levels, and high caterpillar weight gain were also measured with field grown antisense LOX3 plants. Moreover, in a comparison with antisense HPL, antisense AOS, and wild type plants, antisense LOX3 plants exhibited the highest herbivore damage and a significantly higher proportion of antisense LOX3 plants as compared to the other genotypes was attacked by herbivores. The same pattern (higher damage on antisense LOX3 plants and higher number of antisense LOX3 plants attacked) was observed with an herbivore not previously found on *N. attenuata* – the leaf hopper *Empoasca* spp. Moreover, *Empoasca* nymphs were only found on the antisense LOX3 line, indicating a clear choice made by the ovipositing females. In choice tests, *Empoasca* adults as well as *Diabrotica undecimpunctata* beetles, another novel leaf chewing herbivore on *N. attenuata*, showed a clear preference for antisense LOX3 over wild type plants. Again, array results demonstrated a complex LOX3-dependent regulation of primary and secondary metabolism genes in response to attack by *Empoasca* leafhoppers (Kessler et al. in press).

Examples from N. attenuata – the PMT story. Plants transformed with inverted repeat PMT constructs (irPMT) had dramatically reduced constitutive and MeJA-induced nicotine accumulations. Because of an excess of nicotinic acid, anatabine levels were increased in the irPMT lines, which otherwise did not differ from wild type plants. *M. sexta* larvae reared on irPMT plants gained more mass and changed instars faster than larvae reared on wild type plants. Moreover, laboratory and field choice test revealed a preference for ir PMT leaves over wild type leaves by *M. sexta* as well as *Diabrotica undecempunctata*. Another occasional visitor of *N. attenuata*, *Trichobarus mucorea* did not distinguish between leaves from both genotypes. In a field plantation, unelicited irPMT plants lost more than double the amount of leaf area to herbivores than unelicited wild type plants, which is likely to result into a fitness loss for irPMT plants. MeJA elicitation restored the damage levels received by irPMT plants to those of wild type plants, indicating the induction of other efficient defense traits by MeJA. Survival of first instar *M. sexta* larvae that had either fed on irPMT or wild type plants before being placed on *N. attenuata* plants in the field did not differ, in other words, the predation pressure exerted by *Geocoris pallens* bugs was the same. Survival times of *Geocoris* were not influenced when bugs were fed *M. sexta* larvae which themselves had been fed irPMT leaves, wild type leaves, or wild type leaves with enhanced nicotine levels. These findings demonstrate that *M. sexta* is not able to sequester

nicotine for its own defense against its most significant insect predator. Therefore nicotine functions as an efficient direct defense without interfering with the indirect defense, which is mediated by the release of volatile signals to attract predators of the plant's herbivores (Steppuhn et al. in press).

Examples from N. attenuata – synthesis. Taken together, the study of these two 'species' of transgenic *N. attenuata* plants revealed important insights into (1) the modes of action of two defense genes (LOX3, PMT) and (2) the significance of their inducibility by herbivores (LOX) or constitutive expression (PMT) for herbivore performance and the structure of the herbivore community. Another set of experiments with low and high TPI genotypes revealed the costliness of TPI production in the absence of *M. sexta* herbivory, but a fitness benefit for the plant in its presence (J. Zavala, PhD thesis). Furthermore, the role of TD, which is highly up-regulated after *M. sexta/S. exigua/H. virescens/T. notatus* (manuscripts III, V), is currently being studied with antisense TD plants (J-H. Kang, unpublished results). A suite of unknown genes with interesting expression patterns across 200 cDNA/oligonucleotide array hybridizations will be engineered into inverted repeat constructs and transformed into plants to investigate their role in plant resistance. If the mirid-specific elicitation of asparagine synthetase was independently confirmed (e.g. by Northern analysis), an inverted repeat AS line would be an especially interesting genotype to examine in herbivory experiments.

4.4. Rubisco Activase Knock-Out Plants – To What End?

So far, most genes studied with transgenic plants show increased expression after herbivory and are related to signaling and defense pathways. However, herbivore-induced changes also comprise down-regulations and changes in primary metabolism, exemplified by the reduced expression of the Rubisco activase (rca) gene (manuscript III).

Rca – the facts. Rubisco activase is a member of the AAA^+ (ATPases associated with diverse cellular activities) protein family representing a novel type of molecular chaperones that act as disruptors of molecular and macromolecular structures. It facilitates the release of inhibitory sugar phosphates such as ribulose bisphosphate (RuBP) and carboxyarabinitol-1-phosphate (CA1P) from non-carbamylated and carbamylated Rubisco active sites, respectively, by re-opening the catalytic site and thereby maintaining Rubisco activity. In some species (spinach, *Arabidopsis*, barley, rice) Rubisco activase consists of two isoforms generated by alternative splicing of a pre-mRNA arising from one nuclear gene. Barley has another gene encoding only a single and divergent isoform and the different isoforms of

cotton are encoded by multiple genes. Some species, among them tobacco, may express only the shorter isoform. At ADP/ATP ratios typical of the dark (1:1), rca appears to have minimal activity; at ratios typical of the light (1:2, 1:3), rca exhibits less than half of its maximal activity. Rca's sensitivity to the ADP/ATP ratio allows the reduction of Rubisco activity when adequate sinks for reduced carbon are not available, which at the chloroplast level (among other things) is expressed by lowered ATP/ADP ratios. Thioredoxin-f-mediated reduction of a disulfide formed by two cysteine residues in the large isoform allows the fine tuning of the activase and the Rubisco activation state to changing light intensities by redox-regulation. It is not clear how light modulation of Rubisco is achieved in plants that lack the redox-sensitive larger rca isoform. In several species (maize, wheat, cotton) new isoforms appear in response to heat stress, suggesting a role for rca in the acclimation of photosynthesis to high temperatures. Thermal denaturation of rca was found to begin long before that of phosphoribulokinase or Rubisco. However, rca denaturation starts at higher temperatures than those where the first signs of inhibition of Rubisco activation occur – excluding heat liability of rca as the only cause for heat deactivation of Rubisco. Experiments with rca deficient plants have shown that activase is in excess of that required for maintaining steady-state photosynthesis at normal temperatures and that only 20% of wild type levels are sufficient to recover photosynthesis after heat stress to almost the same degree (90%) that it was recovered to in wild type plants. Interestingly, a gibberillin-binding protein in rice was identified as being homologous to the activase and gibberillin-dependent phosphorylation of the protein was reported suggesting an additional role for rca beyond Rubisco regulation (references in Portis 2003).

Herbivore-induced down-regulation of rca – potential models. Several scenarios are conceivable for the down-regulation of rca after herbivory:

1. Rca down-regulation primarily serves as a mechanism to reduce CO_2 fixation by reduced Rubisco activation and Rubisco down-regulation serves to reduce CO_2 fixation by reduced Rubisco synthesis.

2. The production of Rubisco is down-regulated to make resources, which otherwise would be sequestered in this highly abundant enzyme, available for defense-related processes and the down-regulation of rca is an inevitable consequence of Rubisco down-regulation (e.g. by a common transcription factor). Both enzymes seem to be in excess and upon herbivore attack extra production may be cancelled in favor of other processes, while the smaller amount of Rubisco may be compensated for by higher Rubisco activity mediated by less abundant but more effective rca. If in addition to reduced production of

Rubisco (induced by both *M. sexta* and *T. notatus,* Table 2*)*, resources (nitrogen, carbon skeletons) would be made available by proteolysis of already existing Rubisco (induced by *T. notatus* but not by *M. sexta*, to be proven), *T. notatus* up-regulated asparagine synthetase could serve to re-assimilate the developing ammonium. However, Rubisco degradation by proteases is suggested to be inhibited by CA1P binding (Khan et al. 1999) and a down-regulation of rca which removes this protective metabolite would contradict the 'increased proteolysis hypothesis'.

3. Given the diverse function of AAA^+ proteins and the gibberellin-binding activity of an rca homolog from rice, rca may have an as yet undiscovered function obscuring its role in herbivore resistance.

Anticipated antisense rca phenotypes. With plants that have their endogenous Rubisco activase expression silenced the role of herbivore-induced rca down-regulation will be addressed in future experiments. If rca deficiency leads to impaired carbon acquisition, what consequences will this have on resource channeling into growth as compared to constitutive and induced defenses? Antisense rca plants may grow more slowly, have lower constitutive defenses, and the signature of induced responses seen in wild type plants (up-regulation of signaling and defense related transcripts and down-regulation of growth related transcripts) may be different in these plants. The inducibility of defense traits may be constrained in undersupplied plants and/or the proportion of nitrogen-based defenses in carbon-limited plants may increase (for a controversial discussion of C/N theory see Nitao et al. 2002). Antisense rca *N. tabacum* plants grew slower in CO_2-enriched atmospheres, but eventually attained the same height and number of leaves as wild type plants. Moreover, rca-deficient plants had reduced CO_2 assimilation rates, normal contents of chlorophyll and soluble leaf protein, and much higher Rubisco contents (particularly in older leaves), leading to a delay in the usual developmental decline in Rubisco content seen in wild type leaves (He et al. 1997). Hence, rca deficiency may lead to a compensatory response resulting in increased Rubisco levels, which in turn may constrain a Rubisco decline upon herbivore attack. In other words, the sequestering of resources in Rubisco is needed to maintain normal photosynthesis and there is a smaller degree of freedom to channel them into defenses.

Outlook. A deep knowledge on (1) Rubisco and rca regulation, (2) the interplay of processes like photosynthesis and nitrogen metabolism, and (3) theories on defense allocation in plants as well as (4) a thorough characterization of rca deficient plants and (5) the additional use of similarly well characterized antisense Rubisco lines are necessary to

understand herbivore-induced down-regulation of rca in particular and the down-regulation of primary metabolism genes in general.

4.5. Microarrays in Ecology – An Ongoing Story

Entitling an article 'Microarrays in Ecology and Evolution – A Preview', Gibson (2002) reviews the great potential of microarray technology in these areas. For example, with species with short generation times such as yeast or *Drosophila*, the evolution of gene expression under various selection regimes or as a result of neutral divergence could be investigated. Moreover, microarray analyses offer the possibility to estimate fundamental parameters of gene expression variation, including the additivity, dominance, and heritability of transcription, as well as to study the effects of individual variation, genotype, sex, age, microenvironment, population structure, and geography on gene expression. Transcriptional responses in host-symbiont and host-parasite interactions can be examined in both players and it should be possible to identify modifiers of infectivity, virulence, and pathogenicity by contrasting gene expression in different strains/isolates (Gibson 2002).

This thesis used microarrays to examine the role of plant gene expression in herbivore-induced plant vaccination (manuscript III), whether differences in constitutive expression of defense genes within a plant correlate with an aphid's feeding preference for certain plant parts (manuscript IV), whether there are transcriptional signatures of an aphid-induced manipulation of plant metabolism (manuscript IV), and whether a plant's transcriptome changes in relation to lepidopteran elicitor profiles (manuscript V). Because each of the array studies (III-V) was tailored to test special hypotheses, a global comparison of *N. attenuata*'s response using similar elicitation kinetics for the different herbivores is missing and would be a valuable analysis to perform in the future.

While the studies compiled in this thesis characterized the plant's transcriptional plasticity, monitoring gene expression in herbivores would significantly enhance the understanding of plant-insect interactions. Kinetics of gene expression during *Manduca* larval development would reveal how larval gene expression is tailored to the plant's induced defenses. For example, one could (1) test, whether different proteinases are induced in early as compared to late larval instars in response to the induction of plant proteinase inhibitors, (2) detect the induction of detoxification enzymes, such as P450 oxygenases, to cope with toxic plant secondary metabolites, or (3) monitor the expression of elicitors and elicitor-producing or degrading enzymes to examine if herbivores increase or decrease the production of elicitors during infestations. Furthermore, one could figure out, whether the

glutamine- and glutamate-based FAC chemotypes found in *Manduca* (Roda et al. submitted) are due to differences in expression of the FAC-conjugating or -cleaving enzymes (herbivore instead of microbial origin provided) or originate from different isoforms of these proteins. With 'ask the herbivore' approaches one could test whether *Manduca* responds with differential gene expression to high versus low nicotine plants and might identify genes that modify the nicotine molecule to facilitate its excretion or sequestration. In case of *T. notatus,* genes might be found, which catabolize the toxic acylsugars the mirid ingests from *N. attenuata*'s glandular trichomes.

The time it takes to establish the tools (e.g. EST databases) to conduct expression profiling with herbivores will be used to address more plant-related ecological questions. For example, a microarray analysis examining gene expression in black nightshade (*Solanum nigrum*) grown in plant communities with varying degrees of inter- and intra-specific competition (different levels of biodiversity) is currently underway (D. Schmidt, unpublished results). Arrays were also used to monitor gene expression in response to *M. sexta* regurgitate in the tetraploids *N. quadrivalvis* and *N. clevelandii,* which stem from hybridizations with an ancestral *N. attenuata* (Qu et al. 2004), to characterize the evolution of herbivore-induced transcriptional responses during allopolyploid speciation.

Hence, to agree with Gibson's 'preview' – the use of microarrays to examine *insect-induced changes in the transcriptome of a native tobacco plant*, as described in this thesis, is likely to be followed by more studies addressing ecological questions with molecular tools of which microarrays are only one.

5.1. Summary

Since plants are largely immobile, they have evolved a large degree of physiological plasticity to cope with fluctuating environments. Plastic responses to herbivore attack involve increases in hormone concentrations, enzyme activities, and secondary metabolite levels, anticipating a polygenic response and a large transcriptional reorganization upon herbivory. A comparative gene expression analysis examined whether plants adjust these polygenic responses to herbivores with different feeding habits, host breadth, and phylogeny.

Experiments were conducted with a native system – a wild tobacco species from the Great Basin Desert (*Nicotiana attenuata*), which is a host for the grazing larvae of *Manduca sexta*, *Spodoptera exigua*, and *Heliothis virescens* and the mesophyll-sucking *Tupiocoris notatus* bugs. Phloem-feeding aphids (*Myzus nicotianae*), though not found on *N. attenuata* in nature, easily colonized plants in the greenhouse. All these herbivores are major pests on either solanaceous or non-solanaceous crops. A large body of research on wound- and herbivore-induced responses of *N. attenuata* using ecological, analytical, and molecular approaches (Baldwin and Preston 1999, Baldwin 2001) preceded the work that is presented in the five manuscripts of this dissertation.

A review (**I**) of the mechanisms of transcriptional regulation in plants, the methods used in gene expression profiling, and studies examining plant transcriptional responses to herbivorous insects summarized already existing knowledge. The identification of *N. attenuata* genes whose expression presumably changes in response to *M. sexta* or *T. notatus* attack by two unbiased, differential screening procedures (**II**) provided an additional gene collection for a customized, oligonucleotide microarray (OA) used to compare gene expression under different herbivore-infestation regimes (**III**). In addition to comparing transcriptional signatures elicited by leaf-chewing hornworms and mesophyll-sucking mirids with an OA (**III**), local and systemic expression patterns induced by phloem-feeding tobacco aphids (**IV**) and transcriptional responses to attack from three Lepidopteran larvae with similar feeding habits but different host range (**V**) were investigated using a cDNA microarray enriched in *M. sexta*-responsive genes.

I. → Plants devote a substantial portion of their genome to transcription factors (e.g. ORCA), which through interactions with the *cis*-elements of gene promoters not only mediate specific responses, but also link the expression of several genes and thereby enable coordinated polygenic responses to environmental challenges such as herbivory.

→ Transcriptional studies confirm that herbivore attack frequently modifies the plant's wound response as shown by different elicitation kinetics as well as suppressions and enhancements of wound-induced transcripts. Stealthy feeders elicit less wound-induced (e.g. proteinase inhibitors, PIs) but more pathogenesis-related transcripts than grazing herbivores. Specific responses can be induced by attack from members of the same feeding guild, closely related species, or different developmental stages; they can be local or systemic.

→ Molecular techniques could serve to monitor and manipulate plant-herbivore interactions during the study of herbivore-mediated ecosystem processes. Such techniques would include fusions of herbivore- and wound-induced gene promoters to reporter genes (e.g. β-glucuronidase) and a genetic engineering of the expression of novel genes or the silencing of endogenous genes.

II. → Unbiased enrichment procedures (DDRT-PCR, SHMB) predicted 77 genes being differentially expressed after *M. sexta* and/or *T. notatus* herbivory. A minority (e.g. thionins) behaved as predicted, some genes (e.g. Rubisco activase) showed opposite than predicted regulation, and many genes showed no regulation. Despite the low yield of true differentials, these procedures have proven worthwhile because of (a) their repeatability (DDRT-PCR), (b) the cloning of false positive showing opposite than predicted regulation (an unexpected source of candidate genes), and (c) the cloning of genes unlikely to be found by biased procedures.

III. → Irrespective of feeding guild, insect attack induces an up-regulation of signaling and secondary metabolism genes and a down-regulation of primary metabolism and pathogenesis-related genes. This indicates an herbivore-induced switch from a growth-related to defense-related transcriptional phenotype.

→ A principal components analysis (PCA) revealed that *M. sexta* and *T. notatus* elicit distinct transcriptional imprints in *N. attenuata*'s transcriptome after one day but not after five days of continuous attack. The latter was primarily due to a relaxation response, since imprints from temporarily induced and continuously induced plants became similar with time. Sequential and parallel infestations with both herbivores led transcriptional signatures that imply synergistic and antagonistic gene regulations. PCAs with different subsets of genes revealed the total response to be driven by the expression of signaling and secondary metabolism genes. While a PCA allowed a global view on gene expression patterns, a reductionist analysis of these patterns

identified a handful of specifically responding genes, such as asparagine synthetase, whose expression increased tenfold in response to mirid attack.

→ Repetitive hybridizations with RNA from two biological replicates revealed a reproducibility of single gene changes of 60%. Although variation of experimental parameters may contribute to the variance in the data, microarray analyses still suffer from various sources of variation inherent to the technique. As a consequence, global patterns characterized by PCAs are likely more durable (reproducible) than single gene changes.

IV. → Consistent with previous studies, *M. nicotianae* aphids turned out to be stealthy feeders inducing a considerably smaller transcriptional response than grazers like *Manduca* or mesophyll feeders like *T. notatus*. In contrast to previous results, a local and systemic induction of PIs was found after aphid attack. An up-regulation of glutamate synthase may indicate that aphids enhance the nutritional quality of phloem sap by manipulating its amino acid concentration and composition. Constitutively higher levels of aphid-deterring transcripts (PIs and hydroperoxide lyase) in younger leaves do not prevent them from being the preferably attacked plant parts by aphids.

→ Alternative hybridizations targeted to different aspects of the same biological question can indirectly validate the expression patterns they suggest and thus serve as replicate hybridizations.

V. → The transcriptional signatures elicited by two generalist feeders – the noctuid larvae of *S. exigua* and *H. virescens* – are more similar to one another than to that elicited by a larva specialized to solanaceous hosts – the sphingid *M. sexta*. Interestingly, this pattern found within the plant's response to larval attack correlates with the fatty acid-amino acid conjugates (FAC) profiles found in larval regurgitate. Since for many Lepidopteran larvae it is the FACs by which plants tailor their responses to herbivory, a change in FAC composition may allow a caterpillar to manipulate plant metabolism and exploit host plants more efficiently than other herbivores and thus become a specialist. A qualitative manipulation of regurgitate FAC composition and a characterization of FAC perception in plants are necessary to test this hypothesis.

Future studies with an array carrying 10,000 *Solanum tuberosum* cDNAs will show whether differential screening procedures (DDRT-PCR, SHMB) captured the *M. sexta/T. notaus*-responsive 'defensome' or whether the polygenic response to these herbivores is

even larger. Reproducibility will be ensured by hybridizing RNA from replicate treatments to replicate arrays. The greater challenge however, is not to refine the characterization of herbivore-induced transcriptional signatures, but to determine what proportion of the polygenic responses is adaptive and which responses are simply due to pleiotropic effects or epistatic interactions. A powerful approach towards this goal is to knock-out herbivore-elicited genes, such as asparagine synthetase or Rubisco activase, and assess the consequences for both the plant and the herbivore during their interaction.

5.1. Zusammenfassung

Pflanzen kompensieren ihre Immobilität durch ein hohes Maß an physiologischer Plastizität und begegnen so den Schwankungen ihrer Umwelt. Plastische Reaktionen auf Herbivorie beinhalten eine erhöhte Hormon- und Sekundärstoffproduktion sowie gesteigerte Enzymaktivitäten, was auf vielfältige Änderungen in der pflanzlichen Genexpression schließen läßt. Die hier präsentierte, vergleichende Genexpressionsanalyse widmete sich der Frage, ob die Reaktion der Pflanze dem jeweiligen Herbivoren angepaßt ist, und zwar in Abhängigkeit von dessen Fraßverhalten (Nahrungsgilde), Abstammung (Phylogenie) und Wirtspektrum (Polyphagie).

Gegenstand der Untersuchungen waren in der Great Basin Wüste heimische Tabakpflanzen (*Nicotiana attenuata*), die von blattgewebe-fressenden Schmetterlingsraupen mit engem (*Manduca sexta*) oder weitem (*Spodoptera exigua*, *Heliothis virescens*) Wirtspektrum sowie von auf Solanaceen spezialiserten, mesophyll-saugenden Wanzen (*Tupiocoris notatus*) befallen werden. Phloem-saugende Blattläuse (*Myzus nicotianae*) gehören nicht zur natürlichen Herbivorengemeinschaft von *N. attenuata*, akzeptieren die Pflanze aber bereitwillig als Wirt. Bei den genannten Herbivoren handelt es sich außerdem um bedeutende Schädlinge von Kulturpflanzen. Eine umfassende, ökologische, analytische und molekulare Charakterisierung der durch Verwundung und Herbivorie induzierten Reaktionen von *N. attenuata* (Baldwin und Preston 1999, Baldwin 2001) ging den im folgenden zusammengefaßten Studien (**I-V**) voraus.

Ein Review (**I**) zu den Mechanismen pflanzlicher Genregulation, den in der Genexpressionsanalyse angewandten Methoden und Studien, in denen herbivor-induzierte Veränderungen der pflanzlichen Genexpression nachgewiesen wurden, ordnete bisherige Erkenntnisse. Mittels zweier unvoreingenommer, molekularer Techniken (DDRT-PCR, Subtraktive Hybridisierung) wurden Tabakgene kloniert, deren Expression sich durch Herbivorie von *M. sexta* und *T. notatus* vermutlich ändert (**II**). Diese Genkollektion wurde auf einem für die Untersuchung pflanzlicher Abwehrreaktionen maßgeschneiderten, auf Oligonukleotiden basierenden Biochip (OB) plaziert, welcher zur Analyse der Genregulation nach verschiedenen Herbivorenbehandlungen (**III**) verwendet wurde. Zusätzlich zum Vergleich der von *M. sexta* und *T. notatus* ausgelösten Expressionsmuster mittels OB (**III**), wurden die von Tabakläusen lokal und systemisch induzierten Muster (**IV**) sowie die pflanzliche Reaktion auf spezialisierte (*M. sexta*) und generalistische (*S. exigua*, *H. virescens*) Lepidopteren Raupen (**V**) mittels cDNA-basierter Biochips untersucht. Aus den durchgeführten Studien ließen sich folgende Ergebnisse ableiten:

I. → Im pflanzlichen Genom ist eine beachtliche Anzahl von Transkriptionsfaktoren codiert (z.B. ORCA), welche über Interaktionen mit den *cis*-Elementen von Genpromotoren nicht nur in der Lage sind, spezifische Reaktionen zu vermitteln, sondern auch die Expression verschiedener Gene zu koppeln und auf diese Weise komplexe Reaktionen auf Umweltreize wie Herbivorie ermöglichen.

→ Expressionsstudien bestätigen, daß herbivor-induzierte Reaktionen vielfach von Reaktionen auf mechanische Verwundung abweichen. Deutlich wird dies an unterschiedlichen Induktionskinetiken sowie der Unterdrückung bzw. Steigerung von Verwundungsreaktionen. Milde Herbivorie im Gegensatz zu aggressiver Herbivorie verursacht eine schwächere Induktion von Verwundungsgenen (Proteinase Inhibitoren, PIs) und eine stärkere Induktion von Pathogenabwehrgenen. Spezifische Reaktionen können von Mitgliedern derselben Nahrungsgilde, verwandten Arten und verschiedenen Entwicklungsstadien ausgelöst werden und lokal oder systemisch erfolgen.

→ Molekulare Techniken eröffnen neue Möglichkeiten des Monitorings und der Manipulation von Pflanze-Herbivor Interaktionen in Ökosystemstudien. Eingesetzt werden könnten Konstrukte aus herbivor-induzierbaren Promotern und Reportergenen (z.B. β-glucuronidase) und die gentechnische Expression neuer Gene bzw. das Ausschalten pflanzeneigener Gene.

II. → Mittels unvoreingenommener Verfahren wurden 77, durch *M. sexta* bzw. *T. notatus* vermutlich differentiell exprimierte Gene identifiziert. Eine Minderheit (z.B. Thionine) verhielt sich wie vorhergesagt, etliche Gene waren konträr zur Vorhersage reguliert (z.B. Rubisco-Aktivase) und viele Gene zeigten keine Regulation. Trotz der geringen Trefferquote haben sich diese Verfahren der Genanreicherung als lohnend erwiesen, da sie (a) reproduzierbare Ergebnisse lieferten (DDRT-PCR), (b) Gene, die entgegengesetzt der Vorhersage reguliert waren, trotzdem von Interesse für zukünftige Studien sind und (c) Gene kloniert wurden, die mittels voreingenommer Verfahren nur zufällig gefunden worden wären.

III. → Gilden-unabhängig induziert Herbivorie einen Anstieg in der Expression von Signal- und Sekundärstoffwechselgenen und einen Abfall in der Expression von Primärstoffwechsel- und Pathogenabwehrgenen. Dies läßt auf eine Umprogrammierung des pflanzlichen Stoffwechsels von Wachstum auf Verteidigung schließen.

→ Eine Hauptkomponentenanalyse (PCA) zeigte, daß die von *M. sexta* und *T. notatus* nach einem Tag im pflanzlichen Transkriptom ausgelösten Muster sehr verschieden

waren, aber nach fünf Tagen einander glichen. Letzteres war vor allem auf ein Abklingen der Reaktion zurückzuführen, da sich die Muster in temporär bzw. kontinuierlich induzierten Pflanzen ähnelten. Aufeinanderfolgende bzw. gleichzeitige Behandlung mit beiden Herbivoren führte zu Reaktionen, welche synergistische bzw. antagonistische Genregulationen vermuten lassen. PCAs mit verschiedenen Gruppen von Genen zeigten, daß das globale Muster primär durch die Expression von Signal- und Sekundärstoffwechselgenen bestimmt ist. Während PCAs ein globales Bild von Expressionsmustern zeichneten, führte eine reduktionistische Analyse dieser Muster zur Identifizierung von einem Dutzend spezifisch reagierender Gene, wie z. B. dem durch Wanzen zehnfach hochregulierten Asparagin-Synthetase Gen.

→ Unabhängige Hybridisierungen mit RNA biologischer Replikate ergaben eine Reproduzierbarkeit einzelner Genänderungen von 60%. Obwohl ein Teil der Varianz mit Veränderungen experimenteller Parameter erklärbar ist, kommen in der Biochipanalyse noch viele Quellen von Variabilität zum Tragen. Es ist daher wahrscheinlich, daß sich globale Muster eher reproduzieren lassen als einzelne Genänderungen.

IV. → Im Einklang mit früheren Studien bestätigte sich, daß Blattläuse „umsichtigere“ Herbivoren sind, die in ihren Wirtspflanzen eine bedeutend geringere Veränderung der Genexpression auslösen als aggressivere Herbivoren wie *Manduca* oder *T. notatus*. Im Gegensatz zu vorherigen Studien wurden eine lokale und systemische Induktion von PI Genen durch Blattläuse gefunden. Ein Anstieg der Glutamat-Synthase Expression kann als Indiz dafür gelten, daß Läuse den Nährstoffgehalt von Phloemsaft erhöhen, indem sie dessen Aminosäurekonzentration und –zusammensetzung manipulieren. Die konstitutiv höhere Expression von PIs und Hydroperoxid-Lyase in jüngeren Blättern bewahrt diese nicht vor einem bevorzugten Konsum durch die Läuse.

→ Alternative Hybridisierungen, die darauf gerichtet sind, unterschiedliche Aspekte desselben biologischen Problems zu beleuchten, können sich in ihren Ergebnissen indirekt bestätigen und so als replikate Hybridisierungen gelten.

V. → Die Expressionsmuster, welche von zwei nah verwandten Lepidopteren Raupen mit weitem Wirtspektrum (*S. exigua*, *H. virescens*) hervorgerufen wurden, waren ähnlicher zueinander als zu dem von *M. sexta* induzierten Muster. Interessanterweise korrelierten diese Ähnlichkeitsverhältnisse in der pflanzlichen Reaktion mit denen der FAK Profile im Speichel der beteiligten Raupen (FAK = Fettsäure-Aminosäure Konjugate). Da FAKs für Pflanzen Schlüsselfaktoren in ihrer Reaktion auf viele Le-

pidopteren Raupen darstellen, wäre es möglich, daß Raupen über eine Veränderung ihres FAK Profils den pflanzlichen Stoffwechsel manipulieren und ihre Wirtspflanze besser ausbeuten können als konkurrierende Raupen. Eine gezielte Manipulation der FAK Zusammensetzung von Raupenspeichel und die Aufklärung der FAK Perzeption in Pflanzen sind nötig, um diese Hypothese zu testen.

Eine anschließende Studie mit einem 10.000 Gene umfassenden *Solanum tuberosum* Biochip soll zeigen, ob die Reaktion der Pflanze auf *M. sexta/T. notaus* Herbivorie mittels der hier angewandten Verfahren (DDRT-PCR, SHMB) vollständig charakterisiert wurde oder eventuell noch umfangreicher ausfällt. Hybridisierungen mit RNA mehrerer biologischer Replikate sollen die Reproduzierbarkeit der Ergebnisse gewährleisten. Die größere Herausforderung besteht allerdings nicht in der verfeinerten Charakterisierung der herbivor-induzierten Genexpressionsmuster, sondern in der Aufklärung der (eigentlich) adaptiven Reaktionen im Gegensatz zu lediglich durch Genkopplung auftretenden Begleiterscheinungen. Ein vielversprechender Ansatz hierzu beinhaltet das Ausschalten herbivor-induzierter Gene wie Asparagine-Synthetase oder Rubisco-Aktivase und die Untersuchung der Folgen dieser Manipulation für Pflanze und Herbivor.

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8. Eigenständigkeitserklärung

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

Jena, den 8. April 2004

9. Curriculum vitae

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Education

09/79 – 08/89	Wilhelm Pieck School Kahla
09/89 – 07/91	Johannes R. Becher School Jena, "Abitur"

Professional training and experience

12/91 – 07/92	Westdeutsche Landesbank Düsseldorf Foreign-Exchange back office
08/92 – 06/94	Apprenticeship at West LB Düsseldorf, "Kaufmannsgehilfenbrief"
07/94 – 09/94	West LB Securities-Trading back office

Study

10/94 – 03/00	Friedrich Schiller University (FSU) Jena, "Diploma" in biology Major in ecology; Minors in botany, biochemistry, and environmental protection
01/99 – 01/00	Thesis at Max Planck Institute for Chemical Ecology (MPICE) Jena in the Molecular Ecology group of Prof. Ian T. Baldwin Title: "Ethylene-mediated ecological interactions between wild tobacco *Nicotiana attenuata* and its nicotine-adapted herbivore *Manduca sexta*"

Practical training

08/97 – 09/97	Volunteer at Jatun Sacha Biological Station in Ecuadorian rainforest
03/98 – 04/98	Excursion to Karoo desert, South Africa with focus on "Influence of grazing on soil and living communities around artificial waterholes"
11/99 – 12/99	Internship at Instituto Nacional de Biodiversidad San Jose, Costa Rica in the Bioprospecting department

Scientific career

02/00 – 12/00	Research associate at MPICE Jena, including 4 month-field work (04/00-07/00) at Lytle Preserve Southwestern Utah
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10. Publications

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11. Appendices

Supplementary Material (CD ROM)

Manuscript III

- ExpI-SupplMat1-array description
- ExpI-SupplMat2-array design
- ExpI-SupplMat3-data
- ExpI-SupplMat4-PCA matrix
- ExpII-SupplMat1-array description
- ExpII-SupplMat2-array design
- ExpII-SupplMat3-data
- ExpII-SupplMat4-extended version of table 2
- ExpII-SupplMat5-details for table 1
- ExpII-SupplMat6-PCA matrices
- ExpII-SupplMat7-additional PCA plots
- ExpII-SupplMat8-extended results
- Kessler and Baldwin 2004 (Vaccination Part I, pdf)

Manuscript IV

- SupplMat1-array description
- SupplMat2-array design
- SupplMat3-data
- SupplMat4-data

Manuscript V

- SupplMat-data

Publications (pdf files)