Interaction of Plant Defense Compounds with the Insect Gut: New Insights from Genomic and Molecular Analyses

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Short title: Action of Plant Defense Compounds with the Insect Gut

Summary

The co-evolutionary conflict between insect herbivores and their host plants is profoundly influenced by biochemical reactions associated with passage of toxin-laden plant material through the herbivore digestive canal. Insect herbivores provide excellent models in which to understand the mechanistic interplay between nutrition and detoxification, how plant defense compounds hijack these processes, and how insects adapt to host defense chemistry. Expanding genome sequence information and genetic approaches to manipulate gene function in both interacting partners are providing new insights into the genetic underpinnings of host preference and plasticity in gut physiology. Fundamental knowledge gained from these studies has practical application in understanding how insects evolve resistance to pesticides, and may also inform efforts to better understand how plant chemicals impact human health.

Introduction

The growth and development of arthropod herbivores depends on their ability to acquire a wide range of nutrients from host plant tissues. Among the most abundant primary plant products consumed and metabolized in the insect gut are proteins, lipids, complex carbohydrates, nucleic acids, and porphyrins. Ingested plant material is also a main source of vitamins, trace elements, and water. The conversion of plant material into new insect tissue occurs in several stages, including tissue maceration and ingestion, digestion by hydrolytic enzymes in the gut lumen, transport of nutrients across the gut epithelium, and subsequent *de novo* synthesis of macromolecules for use in construction of insect tissues. These processes have evolved over hundreds of millions of years and presumably are tailored to both the chemical composition of plant tissue and physiochemical conditions in the gut [1,2]. Larval feeding habits are also tightly coordinated with various developmental programs and behaviors, including avoidance of predators and parasitoids [3,4].

A multitude of highly specific reactions between plant- and insect-derived molecules occur within the confines of the gut. Although many of these interactions are directed toward nutrient acquisition on the part of the insect, selection pressure imposed by herbivory has spurred the evolution of myriad plant defense compounds that target digestive processes. The production of host defense compounds may be constitutive or, as is often the case in leaves, induced via the jasmonate (JA) signaling pathway in response to herbivore feeding [5,6,7]. As a consequence of induced defenses, feeding larvae may encounter progressive deterioration in diet quality as the concentration of defense compounds increases during feeding. Not surprisingly, arthropod herbivores use innovative strategies to avoid, detoxify, or sequester plant defense chemicals, thereby driving a biochemical “arms race” for control over nutrition and toxicity (Figure 1). Although deciphering the complex connections between nutrient acquisition and detoxification at a mechanistic level is a daunting task, new technologies and experimental approaches are providing new inroads to progress. Here, we briefly highlight some of these recent advances and discuss promising future directions for the field.

Equality for both partners: genes and genomes galore

A major challenge in understanding plant-insect biology at the molecular level is to identify host compounds that exert direct physiological and behavioral effects on the herbivore, and to define the mode of action of bioactive plant compounds in target cells. Progress toward this goal is often confounded by the biochemical complexity of plant tissues and the behavioral complexity of the plant consumer. Like all areas of biology, advances in DNA sequencing technology are revolutionizing the field by providing a comprehensive “parts list” of genes that predict function. In addition to the rapidly expanding list of sequenced plant genomes, new initiatives are also deciphering the secrets of arthropod genomes [8]. Notable examples include genome sequences for the silkworm (*Bombyx mori*) [9], pea aphid (*Acyrtosiphon pisum*) [10], mountain pine beetle (*Dendroctonus ponderosae*) [11], and the polyphagous two-spotted spider mite (*Tetranychus urticae*) [12]. These efforts are revealing unprecedented information about the organization, genetic polymorphism, and evolution of herbivore gene families that serve central roles in all aspects of gut physiology. Such inventories provide a foundational resource and launch pad for functional studies at the cellular, biochemical and molecular levels. It is now possible to integrate functional analyses with evolutionary and ecological questions to address the true richness of plant-herbivore biology. This integrative approach will benefit from use of ecologically relevant plant-herbivore model systems for which there are genetic and genomic resources for both interacting partners. An excellent example is recent work by Gloss and coworkers, who combined biochemical and structural studies of a detoxification enzyme with phylogenetic models to provide new insight into the evolution of herbivory in the genus *Scaptomyza* [13**].

Querying gut physiology through genetically controlled variation in whole-plant diets

Despite the importance of artificial diet-based experiments in defining insect nutritional requirements and regulatory aspects of nutrition [3,14], a welcome methodological advance in recent years is the use of whole-plant diets that preserve the natural food matrix, bioavailability, and synergy among bioactive plant compounds [15,16]. Host-switch experiments, for example, provide useful insight into the extent to which polyphagous herbivores reconfigure gut physiology upon encountering a new host [12,17*]. Genetic variation within the same host plant species, including natural or bred lines that differ in a defined defense trait, can also be exploited as a tool to determine how insect herbivores respond to that trait [18*,19**]. A more refined approach involves the use of isogenic plants in which the abundance of one or more co-regulated

suites of defense compounds is altered through appropriate genetic manipulation [17*,20,21]. In the model plant *Arabidopsis*, a plethora of well-characterized mutants affected in the composition of lipids, cell wall, starch, and trace metal abundance are available to assess the impact of nutritional traits on the plasticity of gut function [22]. New genome editing technologies such as CRISPR/Cas [23] provide an unprecedented opportunity to extend the use of isogenic host-plant comparisons to a broad range of plant-insect interactions. In keeping with this concept, Martin and colleagues have effectively advocated for the use of isogenic plant foods to better understand how plant-based diets impact human health [24].

Gut transcriptional responses provide a new window into plant-insect interactions

Just as plant metabolism is reconfigured in response to biotic attackers, so too do arthropod herbivores rapidly adjust their gut physiology to changes in the abundance of plant-derived nutrients and defense compounds [3,25-27]. These changes in gut physiology occur at the transcriptional level and, importantly, may be triggered by specific dietary compounds. Well studied examples of gut plasticity include induced expression of insect digestive proteases in response to plant proteinase inhibitors [28] and xenobiotic-induced expression of cytochromes P450 (CYPs) that neutralize a wide range of plant defense chemicals [26,29]. Genome-wide transcription profiling provides a powerful “guilt by association” approach with which to identify candidate insect genes that participate in detoxification or other aspect of gut function [17*-21]. Studies of *Trichoplusia ni* (cabbage looper) and the leaf miner *Scaptomyza flava* reared on *Arabidopsis*, for example, identified candidate glutathione-S-transferases (GSTs) that participate in detoxification of glucosinolates [17*,21]. Functional studies provided compelling evidence that specific GST isoforms in *S. flava* are involved in an ancient pathway for glucosinolate detoxification [13**].

Transcript profiling of the herbivore gut is particularly powerful when combined with genetic analysis of candidate detoxification genes. Deep transcriptional sequencing of the *Helicoverpa armigera* midgut led to the identification of *CYP* genes whose expression is induced by gossypol, a defensive sesquiterpene produced in glandular trichomes of cotton [30,31]. Plant-mediated RNAi silencing of one of these genes (*CYP9A14*) reduced the ability of *H. armigera* to tolerate gossypol, suggesting a role for this enzyme in gossypol detoxification. Transcript profiling in *Manduca sexta* and *Myzus persicae* has been used to identify *CYP* genes involved in

detoxification of the alkaloid nicotine [20,32]. Kumar and coworkers employed plant-mediated RNAi to establish a role for CYP6B46 in tolerance of *M. sexta* to nicotine and, interestingly, to also show that *M. sexta* larvae employ CYP6B46 for nicotine-mediated protection against predatory wolf spiders [33**,34].

A significant recent finding to emerge from transcriptional profiling experiments is that the capacity of arthropod herbivores to adjust their gut physiology to the presence of toxic plant defense compounds is mechanistically linked to the development pesticide resistance. Dermauw et al. [35*] found that transcriptional signatures associated with host switching of spider mites from bean to tomato closely resemble transcript patterns affiliated with mite resistance to chemical pesticides. Adaptation of pesticide-susceptible spider mites to the chemically rich defense system of tomato enhanced mite resistance to synthetic acaricides [35*]. Transcript profiling in the cotton bollworm (*Helicoverpa armigera*) identified *CYP* genes that are induced both by gossypol and the widely used insecticide deltamethrin [19**]. This study employed plant-mediated RNAi to establish that an inducible *CYP* isoform (CYP9A14) is required for larval resistance to deltamethrin. These and other recent studies [36,37] indicate that herbivore detoxification systems, which evolved to counter the negative effects of natural plant products, have recently been re-purposed for detoxification of pesticides. The notion that multiple environmental stresses converge on common detoxification responses may inform research to identify *cis*-regulatory elements and transcription factors that coordinate these transcriptional responses [38,39]. Detailed knowledge of transcriptional control of detoxification responses in the in genetically tractable *Drosophila* gut may assist these efforts [40].

Consumption of toxin-laden plant tissues may result in metabolic tradeoffs between nutrient acquisition and detoxification processes in the gut. Have plants evolved defense mechanisms to exploit these tradeoffs? Evidence in support of this hypothesis comes from comparative analysis of gut responses of *T. ni* larvae to defense compounds in *Arabidopsis* and tomato [17*]. Whereas the response of *T. ni* to glucosinolates in *Arabidopsis* was characterized by strong expression of CYPs, GSTs, and other Phase I and II detoxification genes, *T. ni* responses to anti-nutritional proteinase inhibitors and other JA-regulated defenses in tomato were dominated by upregulation of digestive proteases and, strikingly, strong repression of detoxification enzymes. These observations raise the hypothesis that metabolic costs associated with *de novo* synthesis of massive quantities of digestive enzymes may constrain the ability of

the insect to mount a robust response to toxic metabolites, which are abundant in tomato leaves and trichomes [41,42]. This interpretation is consistent with the view that the efficacy of plant chemical defense depends on synergistic interactions between defense compounds [15,43,44].

An expanding arsenal of gut-active plant defense proteins

In addition to defensive secondary metabolites, plant proteins also target multiple facets of gut physiology (Table 1; Figure 1) [2,45]. The well-characterized Mir1 Cys protease from maize [46] and a variety of carbohydrate-binding proteins (e.g., lectins) [47] disrupt the integrity of the peritrophic matrix that surrounds the food canal and protects midgut epithelial cells. Other defense proteins exploit a major nutritional vulnerability of obligate herbivores; the low protein content of plant tissues creates a nutritional “Achilles heel” for insects that must obtain essential amino acids from their diet [48,49]. In tomato, a JA-regulated suite of proteins acts in the gut to decrease the availability of essential amino acids [2,45]. This group of co-regulated proteins includes proteinase inhibitors (PIs) that disrupt the action of digestive proteases and amino acid-degrading enzymes, including arginase (ARG2) and threonine deaminase (TD2; Figure 1) [50,51]. Leu amino peptidase (e.g., LAP-A) may also participate in this post-ingestive defense system [50,52,53], although a role for LAP-A in wound signaling has also been proposed [54].

Several common features of plant anti-insect proteins provide insight into general mechanisms of protein action in the gut and, importantly, may be exploited to identify novel defense proteins in host plants for which there is genomic sequence information. Because gut-active host proteins tend to be highly stable and therefore enriched during passage of the food bolus through the gut, mass spectrometry-based proteomic analysis of midgut or fecal (i.e., frass) material provides an unbiased approach to identify candidate defense proteins [17*,50,52]. It is also evident that the expression of many, if not most, plant anti-insect proteins is strongly elicited via the JA pathway in response to tissue damage or insect feeding [2,45,50,55-57]. Comparative proteomic analysis of digested gut content from insects reared on wild-type *versus* JA-deficient plants provides an additional filter to identify candidate proteins [50]. Mass spectral data obtained from this type of experiment can also be searched against cognate insect sequence databases to gain insight into how the genotype of the host plant alters the composition of insect proteins in the gut lumen [17*].

Another feature of many plant anti-insect proteins is their activation by proteolysis during tissue damage [50,57]. This process presumably limits autotoxicity of the protein in plant tissues and thus is analogous to compartmentalized two-component defense systems, including the so-called mustard and cyanide bombs [58,59]. For example, proteolytic removal of the regulatory domain of tomato TD2 results in production of a highly stable catalytic domain that efficiently degrades Thr without feedback inhibition by high levels of Ile in the gut (Figure 2). That proteolytic processing of TD2 is catalyzed by an insect digestive protease ensures that this defense is activated post-ingestively [51]. Proteolytic activation of TD2 thus provides an example of how a plant defense has co-opted an essential aspect of insect digestive physiology.

In contrast to knowledge of the role of plant secondary metabolites in defense against herbivory, relatively little is known about the extent to which primary plant metabolites (i.e., compounds produced in all plants) participate in gut-localized defense. A recent study provided evidence that chlorophyll, a plastid-localized major dietary component of all folivores, is part of a two-component anti-insect defense system in which a non-plastidic form of chlorophyllase (CLH1) catabolizes chlorophyll to a toxic derivative (chlorophyllide) during leaf damage but not in intact leaves [60*]. A role for CLH1 as a post-ingestive defense is consistent with patterns of chlorophyll breakdown in the gut of generalist and specialist herbivores [61]. It remains to be determined how chlorophyll degradation products exert toxic effects in the gut. Interestingly, the *CLH1* gene in Arabidopsis was initially identified on the basis of its high expression in response to wounding and JA elicitation [62], a feature shared by many other defense proteins that act in the gut (Table 1). It will be interesting to further develop the hypothesis that these proteins evolved from enzymes used for primary metabolism through gene duplication and recruitment of neofunctionalized orthologs into the JA-response network [51].

Summary and perspectives

A major challenge in plant-herbivore biology is to determine the mechanisms by which plant compounds exert physiological and behavioral effects on the herbivore, and how these effects shape the ecology and evolution of plant-herbivore relationships. Herbivores from all reaches of the animal kingdom adapt their eating habits and physiology to the nutritional landscape of available food. In the case of humans, there is compelling evidence that whereas some plant chemicals are toxic, others reduce the risk of several major chronic diseases in humans; yet,

surprisingly little is known about the beneficial physiological effects and mechanism of action of these compounds in humans [63]. To what extent can the study of plant-insect interactions at the biochemical and molecular level be used to inform knowledge of how phytochemicals impact human health? Plant-based diets pose many of the same nutritional and toxicological challenges to insect and mammalian herbivores, and specialized plant compounds often target conserved biochemical functions, as exemplified by the action of cardenolides on Na^+K^+ -ATPases [64]. Although it is clear that specialized plant compounds have major effects on gene expression programs and associated physiological processes, much more work is needed to elucidate the underlying signal transduction pathways and mechanisms by which perception of specific plant molecules is linked to changes in animal gene expression. New advances in genomic science make this a particularly exciting time to address these questions through the use of genetically tractable model systems. These efforts may benefit from a better understanding of how the midgut epithelium and other tissue types interact biochemically with specific components of the plant diet. Functional studies of key insect gene families, such as ABC transporters that promote the uptake and sequestration of plant toxins, will be an important step in this direction [65]. Likewise, as knowledge of CYP structure-function relationships continues to expand, it may be possible to combine *CYP* gene expression profiles with sequence-based information to refine hypotheses concerning the biochemical function of the many uncharacterized CYPs [66,67]. There is also a need to better understand how the microbiome community of the insect gut influences nutrient acquisition and detoxification processes [68], particularly in light of the recent discovery the horizontal gene transfer from bacteria to an arthropod herbivore confers resistance to host plant toxins [69**].

Acknowledgments

We apologize to those whose work we could cite owing to space limitations. We acknowledge research support from Agriculture and Food Research Initiative Competitive Grant no. 2007-35604-1779 from the USDA National Institute of Food and Agriculture, a fellowship (HE5949/1-1) from the Deutsche Forschungsgemeinschaft (supporting MH), and the Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences of the U.S. Department of Energy (DEFG02-91ER20021). We also acknowledge support from the Michigan AgBioResearch Project MICL02278 and the Rackham Foundation.

Table 1. Plant defense proteins that act in the insect gut.

Defense protein	Proposed insect target or mode of action	Reference
<i>Antinutritional proteins</i>		
Protease inhibitors	Binding to digestive proteases	[70]
Vegetative storage protein / phosphatase	Phosphate metabolism?	[56]
Threonine deaminase	Threonine catabolism	[51]
Arginase	Arginine catabolism	[50,53]
Leucine aminopeptidase	Amino acid catabolism?	[52]
<i>Toxic proteins</i>		
Lectins, chitin-binding proteins, chitinases	Disrupt peritrophic matrix	[47,71]
Mir1 protease	Disrupt peritrophic matrix	[46]
Cyclotides	Membrane disruption	[72]
Ribosome-inactivating protein	Unknown	[57]
Chlorophyllase	Chlorophyll catabolism	[60*]
Myrosinase	Glucosinolate hydrolysis	[73]
β -Glycosidase	Cyanide production	[59]

Figure legends

Figure 1. Schematic depiction of representative plant defense compounds and strategies for insect counter defense. Examples of plant defense mechanisms and their respective targets in the insect are shown with red arrows and corresponding strategies of the insect to attenuate these effects are shown with blue arrows. From left to right: cyclotides disrupt gut membrane function [72]; inhibition of gut proteases by proteinase inhibitors (PIs) is counteracted with the production of PI-resistant proteases (res. prot.) [28]; threonine deaminase 2 (TD2) degrades the essential amino acid threonine; the toxic alkaloid nicotine is subject to detoxification by a cytochrome P450 enzyme (CYP6B46) [34]; the maize cysteine protease (Mir1) affects the integrity of the peritrophic matrix, which may be compensated for by the insect by new synthesis of the structure (refill) [18]; insects use multiple strategies to neutralize toxic glucosinolates, including metabolism by sulfatase [74].

Figure 2. A defensive isoform of tomato threonine deaminase starves insect herbivores of an essential amino acid. (A) Plant threonine deaminase (TD) catabolizes Thr as the committed step in the biosynthesis of Ile. The enzyme is subject to feedback inhibition by Ile (red line). Most plants contain a single *TD* gene for use in Ile biosynthesis. Certain species of *Solanum* (e.g., cultivated tomato), however, contain a duplicated *TD* paralog (*TD2*) whose expression is highly induced by the defense hormone JA [52]. (B) Schematic diagram showing cleavage of tetrameric TD2 by an insect gut protease (catalytic domain, blue circle; regulatory domain, gray oval). During attack of tomato plants by caterpillars, a chymotrypsin protease of insect origin removes the regulatory domain of TD2, resulting in a truncated protein (pTD2) that efficiently degrades Thr without being inhibited by Ile. In this manner, pTD2 depletes a nutrient (Thr) that is essential for insect growth and development [50,51].

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Figure 1

Figure 2