Arthropod-Inducible Proteins: Broad Spectrum Defenses against Multiple Herbivores[1]

Keyan Zhu-Salzman, Dawn S. Luthe, and Gary W. Felton*

Department of Entomology, Texas A&M University, College Station, Texas 77843–2475 (K.Z.-S.); and Department of Crop and Soil Sciences (D.S.L.) and Department of Entomology (G.W.F.), Penn State University, University Park, Pennsylvania 16802

Our understanding of the role of plant proteins in defense against herbivores lags behind that of proteins involved in defense against pathogens. However, recent microarray and proteomic approaches have revealed that a broader array of proteins may be involved with defense against herbivores than previously appreciated (Felton, 2005). Here, we discuss defense proteins that function postingestively, some of which are directly toxic while others exert their defense by impairing nutrient utilization. Our purpose is not to provide an extensive review of the topic but to highlight recent findings and suggest new avenues for research. We refer the reader to reviews that provide more extensive coverage (for review, see Carlini and Grossi-de-Sa, 2002; Kehr, 2006; Shindo and Van Der Hoorn, 2008). Because arthropods possess a diverse range of feeding habits and styles, including chewing as well as phloem- or xylem-feeding species, arthropod-inducible proteins (AIPs) may be regulated by multiple signaling hormones, including jasmonic acid (JA), salicylic acid, and/or ethylene.

PLANT DEFENSE

A sudden burst of insect speciation during the Cretaceous period undoubtedly presented a strong selection pressure on plants to develop an array of defenses to ward off attack. One well-conserved defense signaling pathway involves JA. A group of JA-regulated proteins plays a critical role in postingestive plant defense by targeting the insect digestive canal to impair its digestive and absorptive processes (Felton, 2005). Microarray studies have revealed that scores of genes encoding these proteins are up-regulated by herbivory. The defense-related transcriptome and proteome responses of several plant species to chewing (e.g. Lepidoptera) and sucking arthropods (e.g. aphids) are summarized in Tables I and II. Also included are proteins found by proteomics to remain stable in the insect gut (Chen et al., 2005, 2007). Furthermore, herbivory-induced posttranslational protein modifications may regulate their defensive function and enhance their stability in the gut (Lippert et al., 2007).

THE DIGESTIVE SYSTEM AND NUTRITION

Arthropods possess nutritional requirements similar to humans, including the need to obtain the 10 essential amino acids from their diets. For an arthropod feeding on plants with suboptimal amino acids, the efficient digestion of plant tissue is a necessity. Their capacity to digest major leaf proteins such as Rubisco is more efficient than previously recognized, as this protein cannot be detected in the midgut fluids of *Manduca sexta* after feeding on tomato (*Solanum lycopersicum*; Chen et al., 2005). Nevertheless, many ingested proteins survive intact in the gut (Chen et al., 2007) and may move across the gut wall into the hemolymph (Jeffers et al., 2005). Knowing how protein structure and posttranslational modifications contribute toward stability in the herbivore gut would assist in predicting toxicity and mechanism of action. As exemplified in a lectin from *Griffonia simplicifolia*, a single amino acid change could expose a protein's "weak site" for proteolytic degradation, resulting in loss of anti-insect functionality (Zhu-Salzman et al., 1998). Alternatively, anti-insect activity of a toxic but proteolysis-susceptible protein can be improved by simultaneously administering a protease inhibitor (PI), which can prevent degradation of the toxic protein and allow it to exert its defensive function (Amirhusin et al., 2004). This protein-stabilizing strategy has been recommended for producing insect resistant plants (Kiggundu et al., 2006).

The activity of AIPs against arthropods depends upon the chemical milieu of the arthropod's gut, which can vary among species. The main insect digestive organ is the midgut, generally a long tubular structure where digestive enzymes are released and many digested compounds are absorbed. The midguts display a remarkable breadth in their physicochemical properties of pH, redox potentials, surfactantcy, oxy-

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* Corresponding author; e-mail gwf10@psu.edu.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Gary W. Felton (gwf10@psu.edu).

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Table I. *Potential antinutritional proteins revealed by microarray and proteomic studies*

Putative Defense Gene or Protein	Plant Species	Herbivore Species	Reference(s) and Experimental Approach
Arginase	Tomato	*M. sexta*	Chen et al. (2005); proteomics of midgut contents
Asc oxidase	Arabidopsis	Aphids: *Myzus persicae, Brevicoryne brassicae*	Kusnierczyk et al. (2007)
Germin-like protein (oxalate oxidase)	Tomato	*M. sexta*	Chen et al. (2007); proteomics of insect midgut contents/frass
PIs	*Sorghum bicolor*	*Schizaphis graminum* (aphid)	Zhu-Salzman et al. (2004); microarray
	Arabidopsis	*Pieris* spp. (oviposition)	Little et al. (2007); microarray
	Tomato	*Tetranychus urticae* (spider mites)	Kant et al. (2004); microarray
	Tomato	*M. sexta*	Chen et al. (2005, 2007); proteomics of insect frass
	Nicotiana attenuata	*M. sexta*	Hui et al. (2003); microarray
	Hybrid poplar	*Malacosoma disstria*	Ralph et al. (2006); microarray
LOXs	Arabidopsis	*Pieris rapae*	Reymond et al. (2000); microarray
		Spodoptera littoralis	Reymond et al. (2004); microarray
		Bemisia tabaci (whitefly)	Kempema et al. (2007); microarray
		Aphids: *M. persicae, B. brassicae*	Kusnierczyk et al. (2007)
	Cucumis sativus	*T. urticae* (spider mites)	Mercke et al. (2004); microarray
	Solanum nigrum	*M. sexta*	Schmidt et al. (2005); microarray
	N. attenuata	*M. sexta*	Hui et al. (2003); microarray
	Hybrid poplar	*M. disstria*	Ralph et al. (2006); microarray
Peroxidases	Arabidopsis	*B. tabaci* (whitefly)	Kempema et al. (2007); microarray
	Hybrid poplar	*M. disstria*	Ralph et al. (2006); microarray
PPOs	Tomato	*M. sexta*	Chen et al. (2005); proteomics of midgut contents
	N. attenuata	*M. sexta*	Schmidt et al. (2005)
	Hybrid poplar	*M. disstria*	Ralph et al. (2006); microarray
TDs	Tomato	*M. sexta*	Hui et al. (2003); Giri et al. (2006); microarray/proteomics
	Tomato	*M. sexta, Trichoplusia ni*	Chen et al. (2005, 2007); proteomics of insect midgut contents/frass

gen levels, etc. (Johnson and Felton, 1996; Harrison et al., 2001). These properties may affect the toxicity of proteins by altering their enzymatic activity, solubility, protein-protein interactions, protein folding, digestibility, etc. The midgut pH is strongly regulated and can range, depending upon species, from a low of 5.0 to as high as 12.0. Such a wide pH range affects the activity of many ingested proteins, especially their enzymatic properties. The gut pH, in part, determines the type of protease that may predominate in the digestive tract. A range of digestive proteases are found in herbivores, including Asp-, thiol-, Ser-, and metalloproteases, each with differing substrate specificities that will determine how (and if) ingested proteins un-

Table II. *Potential toxic proteins revealed by microarray and proteomic studies*

Putative Defense Gene or Protein	Plant Species	Herbivore Species	Reference and Experimental Approach
Chitinases	*Sorghum bicolor*	*Schizaphis graminum* (aphid)	Zhu-Salzman et al. (2004); microarray
	Tomato	*Tetranychus urticae* (spider mites)	Kant et al. (2004); microarray
	Hybrid poplar	*Malacosoma disstria*	Ralph et al. (2006)
Cys proteases	Arabidopsis	*Pieris rapae, Spodoptera littoralis*	Reymond et al. (2004); microarray
		Pieris spp. (oviposition)	Little et al. (2007); microarray
	Nicotiana attenuata	*M. sexta*	Schmidt et al. (2005); microarray
Hevein-like protein (chitin binding)	Arabidopsis	*P. rapae*	Reymond et al. (2000); microarray
		Pieris spp. (oviposition)	Little et al. (2007); microarray
		Bemisia tabaci (whitefly)	Kempema et al. (2007); microarray
		Myzus persicae	Moran et al. (2002); microarray
Lectins	Arabidopsis	*P. rapae, S. littoralis*	Reymond et al. (2004); microarray
		Pieris spp. (oviposition)	Little et al. (2007); microarray
	Hybrid poplar	*M. disstria*	Ralph et al. (2006)
Leu aminopeptidase	Tomato	*T. urticae* (spider mites)	Kant et al. (2004); microarray
	Tomato	*M. sexta*	Chen et al. (2007); proteomics of insect frass
	Solanum nigrum	*M. sexta*	Schmidt et al. (2005); microarray

dergo proteolysis. Caterpillars generally have an alkaline midgut, and in some species the midgut lumen may be nearly anaerobic (Johnson and Felton, 2000); thus, the activity of O_2-dependent oxidases could be minimized in the guts of these insects. Defensive proteins do not necessarily need to remain active or stable in the herbivore's gut. Some enzymes may rapidly catalyze reactions at the feeding site.

AIPS: ROLES IN ANTINUTRITIVE PLANT DEFENSE

Inhibiting dietary proteolysis through PIs may decrease access to essential amino acids. PIs are categorized according to the proteases they inhibit, and inhibitors of all the above-mentioned protease classes have been identified in plants (Ryan, 1990). PIs are found in high concentrations in plant storage organs or tissues, such as seeds and tubers, where their protection is crucial for fitness. Evidence for the defensive function of PIs was furnished by the seminal work by Green and Ryan (1972), which showed both local and systemic PI induction in leaves following herbivory. This protective function is attributed to the formation of stable complexes between inhibitors and the catalytic clefts of specific proteases that block protein degradation.

The coordinated action of multiple AIPs appears to target various nutritional vulnerabilities in arthropods (Kessler and Baldwin, 2002; Zhu-Salzman et al., 2004; Kempema et al., 2007). An arthropod's ability to acquire amino acids involves multiple proteins working in concert. This is best illustrated by tomato defense proteins (see Table I). PIs constitute one part of the defense machinery; however, the JA-induced arginase and Thr deaminase (TD2) disrupt insect digestion by degrading existing amino acids necessary for insect growth (Chen et al., 2005), likely synergizing PI activity. Tomato and potato (*Solanum tuberosum*) use two different TDs for Ile biosynthesis (the housekeeping isoform TD1) and defense (Thr-degrading isoform TD2 that is stable in the insect midgut), whereas most other plants appear to have one TD. Interestingly, TD2 (but not TD1) is induced by wounding treatment, and its activity was improved by herbivore-mediated removal of a C-terminal domain that confers negative feedback regulation by Ile. TD2 and arginase are controlled by the JA signaling pathway, but unlike PIs, these proteins act catalytically. PIs must be abundant to exert their defensive function, while enzymatic defense molecules such as TD2 are effective at lower concentrations. Other enzymes involved in amino acid metabolism (e.g. Arg decarboxylase, Try decarboxylase, etc.) are frequently induced by insect feeding and merit further examination for their potential in restricting amino acid utilization.

One of the most thoroughly studied defenses is the myrosinase-glucosinolate system found in Brassicaceae (Halkier and Gershenzon, 2006). Upon tissue disruption, glucosinolates are hydrolyzed by myrosinase to form toxic products, including isothiocyanates, which act as electrophiles capable of reacting with nucleophilic centers of amino acids. Consequently, essential amino acids are lost and/or protein functions are impaired. The amount of isothiocyanates formed depends upon whether the arthropod can metabolically divert the myrosinase reaction toward less toxic products or prevent myrosinase action entirely (Ratzka et al., 2002; Wittstock et al., 2004).

Another group of enzymes that may impair nutrition through forming electrophiles are oxidases such as polyphenol oxidases (PPOs; and some peroxidases), which oxidize mono- or dihydroxyphenolics. The oxidation of *o*-diphenols forms reactive *o*-quinones, which are potent electrophiles capable of polymerizing or forming covalent adducts with the nucleophilic groups of proteins (e.g. -SH or ε-NH2 of Lys; Felton et al., 1992). PPOs are widespread in plants and are inducible by wounding, herbivory, and JA (Constabel et al., 2000; Thaler et al., 2001). There is evidence using transgenic plants that PPO functions in resistance to caterpillars (Wang and Constabel, 2004). PPO requires activation via proteases and remains active in the herbivore's digestive system (Felton et al., 1989; Wang and Constabel, 2004), but see Barbehenn et al. (2007). The role of lipoxygenases (LOXs) in producing precursors for JA biosynthesis and subsequent defense against insects is well established (Royo et al., 1999; Kessler et al., 2004). However, multiple LOXs occur, and not all are involved in JA signaling. Most experiments have not adequately separated the signaling role of LOX products from their possible direct role in plant defense. The hydroperoxides formed by the oxidation of linolenic/linoleic acids potentially have dual action. First, the fatty acids are essential nutrients for insects. Second, peroxides (or other lipid oxidation products) are potent electrophiles that can react with nucleophilic groups of dietary amino acids (Felton et al., 1994). Alternatively, the oxylipin products may react with insect proteins such as digestive enzymes or directly with insect tissues such as the midgut epithelium.

In addition to these oxidases, other enzymes can disrupt the arthropod redox status (Table I). Disturbances in gut redox state may cause proliferation of oxyradicals that damage proteins, lipids, and DNA. Enzymes that produce a superoxide radical (e.g. NADH oxidase) or hydrogen peroxide (e.g. oxalate oxidases, polyamine oxidases, peroxidases, etc.) could function as defensive proteins in the herbivore gut. Depending upon redox conditions, enzymes could impose either oxidative or reductive stress. Arthropods rely upon ascorbate (Asc), reduced glutathione (GSH), and NADH/NADPH as reductants, and enzymes that deplete any of these reductants or result in a surfeit of any single reductant could disrupt the normal redox state. Asc is an essential nutrient for arthropods and several enzymes regulate its abundance. Asc oxidase oxidizes L-Asc to dehydro-L-ascorbic acid. This enzyme remains stable in the *Helicoverpa zea* digestive system, where it may deplete Asc, disrupt

redox status, and reduce protein nutritional quality (Felton and Summers, 1993). Alternatively, the enzyme dehydroascorbate reductase (which requires GSH) is stable in the insect gut (Chen et al., 2005), where it could deplete GSH, produce excess Asc, and disrupt redox balance.

AIPs may impair the utilization of other nutrients such as phosphate, which has been overlooked as a nutrient (Woods et al., 2002). Vegetative storage proteins (VSPs) are best known as reservoirs for amino acids in vegetative tissues that facilitate source-sink interactions in a number of plants (Staswick, 1994). Arabidopsis (*Arabidopsis thaliana*) VSPs (AtVSPs) are induced by JA application, insect feeding, and other environmental stresses (Berger et al., 1995; Stotz et al., 2000; Reymond et al., 2004). There is a positive correlation between AtVSP expression and insect resistance (McConn et al., 1997; Stotz et al., 2000; Ellis and Turner, 2001). It was shown that AtVSP is potent against several insect species (Liu et al., 2005). Because many VSPs have enzymatic functions, AtVSP was evaluated for phosphatase activity, and site-directed mutagenesis indicated that this activity is the basis for the anti-insect function (Liu et al., 2005). Although the targets of AtVSP2 in insect digestive tract are not yet clear, it is reasonable to assume that AtVSP2 interferes with herbivores' phosphate metabolism.

AIPS: DIRECT ATTACK ON THE INSECT DIGESTIVE SYSTEM

The midgut is often lined with a protective layer called the peritrophic matrix (PM), which is composed of a chitin and protein matrix. The PM protects the midgut epithelium against food abrasion, toxins, oxidative stress, and microorganisms, and maintains compartmentalization of digestive enzymes. PM disruption may interfere with normal digestive and absorptive functions and predispose the insect to pathogens and toxins. Lectins are an important group of proteins that bind to certain sugar moieties with high specificity (Table II). Lectins that are resistant to proteolysis and possess an GlcNAc binding site often have anti-insect activity (Zhu-Salzman et al., 1998). They may readily bind the chitin components of the PM and disrupt its morphology (Fitches and Gatehouse, 1998; Zhu-Salzman et al., 1998). For instance, ingestion of wheat germ agglutinin by European corn borer (*Ostrinia nubilalis*) caused hypersecretion of an unorganized PM in the anterior midgut lumen, disintegration of microvilli of the epithelium, and cessation of feeding (Hopkins and Harper, 2001). The disruption of the PM allowed passage of food particles into the ectoperitrophic space and penetration into the microvillar brush border, and threatened the integrity of epithelium.

Plant proteases have been implicated in antiherbivore defense as some are induced by herbivory (Table II). For many years it was thought that the papaya (*Carica papaya*) latex protease, papain, protected the plant against insect feeding. This hypothesis was confirmed when the effects of feeding either papaya or fig (*Ficus carica*) leaves to lepidopteran pests were tested (Konno et al., 2004). To assess the role of latex proteases, latex was removed by washing, or the leaves were treated with the specific Cys PI E64. In all cases, the insects reared on washed or E64-treated leaves grew significantly larger than those reared on untreated leaves. The growth of larvae reared on artificial diet containing commercial papain, ficin, or bromelain were significantly reduced, although mortality was low.

Maize (*Zea mays*) lines genetically resistant to numerous lepidopteran pests accumulate a unique 33-kD Cys protease (Mir1-CP) in the whorl in response to feeding (Pechan et al., 2000). In vivo studies (Pechan et al., 2002) showed that Mir1-CP attacks the PM and disrupts its structure, whereas in vitro studies indicated that purified Mir1-CP permeabilized the PM, probably by directly degrading PM proteins (Mohan et al., 2006). Dose-response analysis demonstrated that Mir1-CP had LC_{50} values ranging from 0.6 to 8 $\mu g\ g^{-1}$ against several lepidopteran pests, and these values were the same order of magnitude as those of Bt-CryIIA (S. Mohan and D. Luthe, unpublished data). It has been shown that Mir1-CP accumulates in the thick-walled sieve elements in the maize phloem and root vascular tissue 24 h after foliar feeding by fall armyworm (*Spodoptera frugiperda*) larvae (Lopez et al., 2007). Removal of the roots prior to larval feeding prevented foliar accumulation and suggests that Mir1-CP may move through the vascular system in response to herbivory.

Leu aminopeptidase A accumulates in the chloroplasts of the spongy and palisade mesophyll cells in response to wounding, chewing insects, certain pathogens, and exogenous JA application (Chao et al., 1999; Narváez-Vásquez et al., 2008). Although much is known about the biochemical properties of Leu aminopeptidase A, its function in response to herbivory remains elusive. Its high pH optimum and stability in the lepidopteran midgut (Chen et al., 2005) suggests that it is capable of functioning in the alkaline midgut, where it possibly releases Arg from the N terminus of peptides and/or damages the gut (Felton, 2005). The Arg could then be degraded by arginase (Chen et al., 2005). Alternatively, a role in downstream JA signaling pathways has been proposed (Walling, 2006).

Chitinases are frequently induced as a response to pathogen infection and occasionally by arthropod feeding (Kant et al., 2004; Zhu-Salzman et al., 2004), but their role in defense against herbivores has not been well established. However, in one example, an inducible chitinase of poplar (*Populus* spp.) WIN6 was shown to be active against insects (Lawrence and Novak, 2006), presumably via direct action on the insect PM.

INSECT COUNTER-DEFENSES

Facing an onslaught of AIPs, arthropods employ a variety of tactics to avoid the effects of these defenses.

Insect herbivores can avoid inducing some defenses by certain salivary components (Musser et al., 2002; Bede et al., 2006). Alternatively, larvae may simply move to avoid locally induced defenses (Paschold et al., 2007), or adult insects may avoid ovipositing on induced plants (Bruinsma et al., 2007). Insects may even "eavesdrop" on JA or salicylic acid by up-regulating their detoxication systems in advance of induced defenses (Li et al., 2002).

One of the best studied counter-defenses is the response to PIs, in which insects compensate by overconsumption (De Leo et al., 1998; Cloutier et al., 2000) and/or by adjusting their digestive enzyme complements to become resistant to a variety of PIs (Mazumdar-Leighton and Broadway, 2001; Brunelle et al., 2004). The plasticity and wide diversity of insect digestive proteases are remarkable, as they not only digest dietary proteins but also play a role in counter-defense. Many insects modulate transcripts and protein products of major digestive protease isoforms, whose activity can be further regulated by posttranslational adjustment. This was shown in the cowpea bruchid *Callosobruchus maculates*, where more than 30 cDNAs encoding cathepsin L-like Cys proteases were isolated (CmCPs) (Zhu-Salzman et al., 2003). When fed on a diet containing the soybean (*Glycine max*) Cys PI scN, they selectively expressed a subset of CmCPs that have higher intrinsic proteolytic activity and exclusive scN-degrading activity and are more efficient in autocatalytic conversion of the latent proenzyme to the active mature protease (Ahn et al., 2004). Efficiency of these posttranslational events, particularly under inhibitor challenge, directly correlates with varied proteolytic activity of different isoforms (Ahn et al., 2007b).

Insect counter-defense reservoirs also contain enzymes that hydrolyze proteins using different catalytic mechanisms. Their quantity is generally small compared to major digestive enzymes under normal conditions (Xu et al., 2005; Vinokurov et al., 2006). Many insect species have midgut pH gradients—from acidic through neutral to alkaline in separate regions of the gut—that facilitate compartmentalized enzymatic function of particular classes of proteases (Ferreira et al., 1994; Vinokurov et al., 2006). The presence of multiple mechanistic classes of proteases broadens the spectrum of digestible proteins from their host plants. Functional redundancy resulting from multiple digestive enzymes could be a necessity to ensure amino acid supplies. Further, coordination between different classes of proteases is also required for effective fragmentation of plant defense proteins (Brunelle et al., 1999; Zhu-Salzman et al., 2003).

How insects sense PIs and transduce signals for differential expression of counter-defense-related genes is largely unknown. Promoter analyses of a cathepsin B-like Cys protease (CmCatB) gene led to the discovery of a chicken ovalbumin upstream promoter-transcription factor homolog in cowpea bruchids (Ahn et al., 2007a). CmCatB was the most highly induced gene in a microarray study designed to identify scN-regulated genes in cowpea bruchid alimentary tracts. Chicken ovalbumin upstream promoter-transcription factors are orphan nuclear receptor family members, previously known to play important roles in neurogenesis, organogenesis, and embryogenesis in various animals (Kerber et al., 1998; Mouillet et al., 1999). In the bruchid midgut, this transcription factor acts as a repressor of CmCatB expression under normal growth conditions. The repression, however, is released when insects are challenged by inhibitors, resulting in activation of CmCatB (Ahn et al., 2007a).

Functional genomic and proteomic studies may reveal the identities of interlinked counter-defense protein genes that facilitate insect adaptation to dietary challenges. Targeting transcription factors that interact with common cis-elements of these counter-defense-related proteins could be an attractive approach in biotechnology-based insect control. Because direct inhibition of digestive proteases has met with limited success, inhibition of these upstream regulators could potentially be more effective, as they control expression of a larger subset of genes involved in counter-defense. Further, the ability to fragment plant proteins sometimes backfires, as shown in the cowpea (*Vigna unguiculata*)-fall armyworm interaction (Schmelz et al., 2006), where such fragmentation elicits a plant defensive response.

CONCLUSION AND PERSPECTIVES

Microarray data indicate that a large set of genes are up-regulated in response to herbivory, but thus far very few gene products have been shown to play a direct role in plant defense. Undoubtedly, this list will increase as more functional analyses are completed, yet there is the need to view defense as an emergent property, not just the sum of the individual gene products (Duffey and Stout, 1996). The new challenge "is to understand plant defense as resulting from suites of interacting traits with predictable properties and measurable mechanisms of action, and to relate these properties and mechanisms to ecological outcomes. Traditional analytical, experimental, and statistical approaches are perhaps not adequate for the challenge" (p. 29; Duffey and Stout, 1996). With the advent of genomics, we will gain a more comprehensive understanding of the breadth of interacting traits. The challenge in the era of systems biology is to use the massive amounts of quantitative data that will be acquired from both plant and herbivore data sets to construct and validate predictive models for defense against specific herbivores.

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