Recognition of Herbivory-Associated Molecular Patterns

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During their long, approximately 350 million-year period of coexistence, plants, insects, and other arthropods evolved a variety of different interactions (Gatehouse, 2002). Some interactions can be beneficial for the plant, as in the case of insect-mediated pollination or seed dispersal, and others are deleterious, as in the case of attack by herbivorous insects (Fig. 1). To successfully combat aggressors, plants must be equipped with a sophisticated sensory system to perceive signals fast and efficiently from their environment and thereby detect potential enemies and subsequently translate and integrate such signals into appropriate biochemical and physiological responses. Thus, upon attack, a number of reactions are detectable in plant cells, including changes in ion flux and protein phosphorylation, formation of reactive oxygen species and oxylipins, as well as initiation of various defense reactions in the host plant (Kessler and Baldwin, 2002; Maffei et al., 2007b). Intriguing questions arising from these observations are how plants recognize the particular herbivores, what kinds of signals are involved, how such signals are perceived, and how they are converted into downstream signaling pathways involved in plant defense activation. Signal perception in the plant cell may rely on the presence of specific receptors for chemical signals or on general recognition processes based on localized tissue injuries. In principle, the feeding process combines two sites of the same coin: mechanical wounding of the infested tissue and introduction of oral secretions that are delivered from the feeding organism into the wounded tissue (i.e. the attacked plant is challenged by both a mechanical as well as a chemical stimulus). This *Update* introduces herbivore-derived metabolites, which represent serious candidates for signaling compounds; we will also discuss advances in herbivore recognition, namely, the perception of insect-derived signals by specific binding proteins. The properties of these binding proteins suggest their involvement in signal perception.

WHAT CAN WE LEARN FROM PLANT-PATHOGEN INTERACTIONS?

Up to now, our knowledge of recognition processes in plant-herbivore interactions has been very limited.

Thus, it seems justified to look first for similarities and related developments, rather than ignoring what we can learn from other systems. Signaling pathway studies revealed striking similarities in plant-pathogen and plant-herbivore interactions (Walling, 2000; Taylor et al., 2004; Garcia-Brugger et al., 2006; Maffei et al., 2007b). For instance, typical elements of the battery of pathogen-induced plant responses that are also initiated after attempted herbivore infestation include enhanced ion fluxes across the plasma membrane and, very close to the biting zone, collapse of membrane integrity of challenged plant cells (Maffei et al., 2004), activation of kinase cascades (Wu et al., 2007), and generation of reactive oxygen species (Maffei et al., 2006). These localized defenses occur at the site of attack and are restricted to the treated leaf tissue. Moreover, generation of the phytohormones jasmonic acid (JA), salicylic acid (SA), and ethylene (Walling, 2000; Kessler and Baldwin, 2002; Maffei et al., 2007b), activation of defense-related genes (Baldwin et al., 2001; Zavala et al., 2004), and synthesis of (volatile and nonvolatile) secondary compounds (Baldwin and Callahan, 1993; De Moraes et al., 1998; Kessler and Baldwin, 2001; Leitner et al., 2005) also can occur systemically. Thus, it seems tempting to speculate that the initial events concerning the recognition of pathogens or herbivores might be similar as well. Actually, widespread usage of the term "elicitor" for signaling compounds that can stimulate herbivore-induced responses in plants has been borrowed from the pathogen field.

Several models are conceivable to explain the basis of plant resistance against aggressors. Recognition of pathogens can be mediated either in a nonhost manner between a plant and pathogen species or in a gene-for-gene interaction between a particular plant cultivar and a pathogen race. The high degree of specificity in the latter case is indicative of the coevolution of the antagonists, host, and pathogen, respectively (Dangl and Jones, 2001; Jones and Dangl, 2006). Nonhost disease resistance envisions a ligand-receptor-like interaction. In this case, plant immunity relies on the perception of chemical cues, general elicitors, which are present either constitutively in the pathogens or generated during pathogen invasion, by specific cell surface-localized receptors (Jones and Dangl, 2006). Such general elicitors are believed to merit the classification as pathogen-associated molecular patterns (PAMPs), whereas the corresponding receptors are referred to as pattern recognition receptors (PRRs). Recently, the more general term, microbe-associated molecular patterns (MAMPs), is preferred (Bittel and Robatzek, 2007).
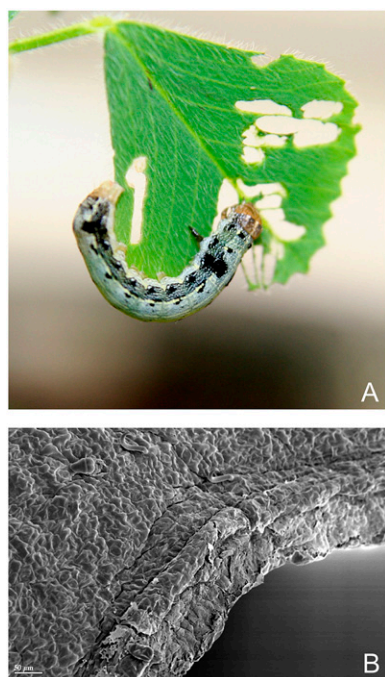
Figure 1. A, *S. littoralis* caterpillar feeding on a barrel medic (*Medicago truncatula*) leaf. B, Scanning electron micrograph of lima bean leaf damage zone after feeding of *S. littoralis* larvae (micrograph by G. Wanner, Munich).

MAMPs/PAMPs are perceived at low concentrations and act as inducers of defense reactions (Boller, 1995; Ebel and Mithöfer, 1998). MAMPs display diverse structures, including carbohydrates, (glyco)-proteins, lipids, peptides, and sterols (Boller, 1995; Ebel and Mithöfer, 1998; Nürnberger et al., 2004). They are constituents of the outer layers of the pathogens, such as chitin fragments (Yamada et al., 1993), β-glucan fragments (Ayers et al., 1976), Pep-13, a peptide of the cell wall transglutaminase of *Phytophthora* (Brunner et al., 2002), or flg22, a peptide of the bacterial flagellin (Felix et al., 1999), but also an *N*-acetylated peptide of the bacterial elongation factor (EF)-Tu (Kunze et al., 2004). For most of these MAMPs, the corresponding PRRs or binding sites have been genetically and biochemically identified (Bittel and Robatzek, 2007): for β-glucans, the extracellular glucan-binding protein (Mithöfer et al., 2000; Fliegmann et al., 2004); for chitin, the transmembrane LysM-containing receptor-like proteins, CEBiP (Kaku et al., 2006); and for flg22 and EF-Tu, the transmembrane Leu-rich repeat receptor-like kinases FLS2 (Gomez-Gomez and Boller, 2000; Chinchilla et al., 2006) and EFR (Zipfel et al., 2006), respectively. At least the FLS2 receptor can be internalized followed by subcellular redistribution and accumulation in endosomes (Robatzek et al., 2006).

Typically, MAMP-triggered host responses are elicited fast and transiently, a fact that holds true also for responses elicited during herbivory (Maffei et al., 2007b). Moreover, because MAMPs are structurally rather diverse and the variety of induced responses in plant-pathogen and plant-herbivore interactions seems to have similar characteristics, it is tempting to propose that the basic processes in herbivore recognition in plants might be related to the recognition of pathogens. In the following, we will summarize our knowledge on both herbivore-derived elicitors and their corresponding binding sites in plants.

HERBIVORE-ASSOCIATED MOLECULAR PATTERNS AS A NEW CLASS OF ELICITORS?

Oral secretions (OS) from feeding insects can contain herbivore-specific compounds with elicitor-like properties. According to the PAMP and MAMP classification, herbivore-derived elicitors will be denoted by herbivore-associated molecular patterns (HAMPs). This term will cover all herbivore-derived signaling compounds that might come into contact with the particular host plants during any stage of their life cycle and thereby elicit defense reactions. This can include, among others, components of OS, saliva, and oviposition fluid. Compared with the large number of MAMPs, up to now only few HAMPs have been isolated and their structures identified. Different proteins, such as Glc oxidase (Eichenseer et al., 1999) and alkaline phosphatase (Funk, 2001), have been shown to act as elicitor active compounds, and a β-glucosidase from larvae of the white cabbage butterfly (*Pieris brassicae*) triggered the release of volatiles from cabbage (*Brassica capitata*) leaves (Mattiacci et al., 1995). However, it is not clear whether or not certain peptide domains, rather than whole proteins, represent the active part of the elicitors. But besides proteins, low-M_r compounds that act as potent elicitors have also been identified. Peptide elicitors, the so-called inceptins, represent disulfide-bridged proteolytic fragments of the chloroplastic ATP synthase γ-subunit (Fig. 2). They were first isolated from OS of fall armyworm (*Spodoptera frugiperda*) larvae feeding either on cowpea (*Vigna unguiculata*) or maize (*Zea mays*; Schmelz et al., 2006). At very low concentrations (fmol leaf^{-1}), inceptins trigger ethylene production, increase the level of SA and JA, and mediate plant perception of herbivory through the induction of various defense reactions in cowpea, such as the emission of the volatile homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT). Due to the proteolytic cleavage in the insect gut, the amino acid sequences of inceptins can be slightly different. Thus, four different but still active inceptins have been isolated from larvae feeding on cowpea. Systematic substitution of the amino acids by Ala or even the deletion of the C-terminal amino acid revealed that the C terminus is much more important for bioactivity of this peptide than the N terminus (Schmelz et al., 2007). In spinach (*Spinacia oleracea*), an additional internal trypsin cleavage site causes inactivity of the peptide elicitor (Schmelz et al., 2007). Obviously, inceptins function as chemical cues that initiate specific plant responses upon insect attack. All these results strongly indicate that inceptins represent

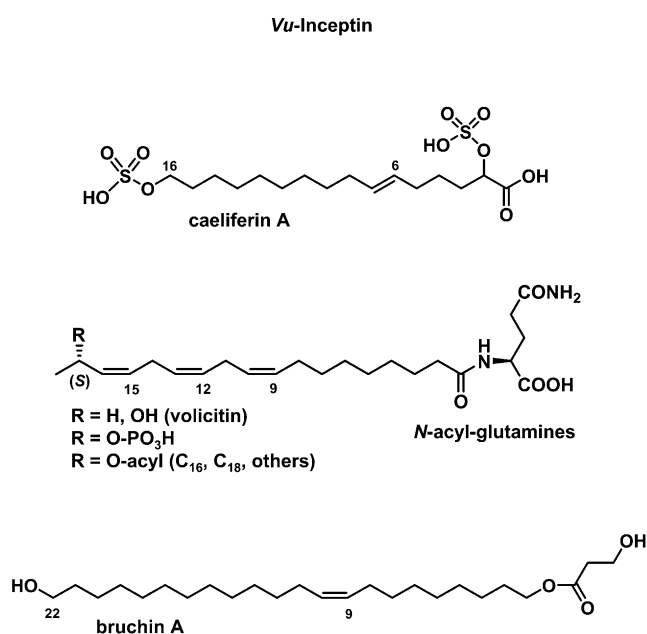*N*-Ile-Cys-Asp-Ile-Asn-Gly-Val-Cys-Val-Asp-Ala-*C*

Vu-Inceptin

Figure 2. Inceptins, Proteolytic peptides of the chloroplastic ATP synthase γ-subunit; Vu, *V. unguiculata*; caeliferins, caeliferin A16:1, (*E*)-2,16 disulfooxy-6-hexadecenoic acid; *N*-acyl amino acid conjugates, volicitin, *N*-(17-hydroxylinolenoyl)-ʟ-Gln; bruchins, bruchin A, (*Z*)-9-docosene-1,22-diol, 1-(3-hydroxypropanoate)ester.

true peptide elicitors with high structural specificity, comparable with flg22 or Pep-13. Their mode of action is very likely receptor mediated because they do not interact directly with membranes (Maischak et al., 2007) as, for example, alamethicin, a channel-forming peptide mixture from *Trichoderma viridae*, which has been shown to induce DMNT emission or JA accumulation in lima bean (*Phaseolus lunatus*; Engelberth et al., 2001). Whether or not insects other than *S. frugiperda* can generate inceptins remains to be elucidated.

Caeliferins are recently identified HAMPs composed of saturated and monounsaturated sulfated α-hydroxy fatty acids with 15 to 20 carbons where the ω-carbon is functionalized with either a sulfated hydroxyl or a carboxyl conjugated via an amide bond to Gly (Fig. 2; Alborn et al., 2007). These elicitors were isolated from regurgitate of the grasshopper species *Schistocerca americana* and are able to induce volatile emission in corn. Preliminary results suggest that caeliferins occur within the suborder Caelifera (e.g. grasshoppers), but not in the Ensifera (e.g. crickets), a second suborder in the order Orthoptera (Alborn et al., 2007). Thus, it will be interesting to learn whether these compounds are widespread insect-derived elicitors or are restricted to Caelifera.

The most prominent and recognized class of HAMPs is represented by volicitin, a *N*-(17-hydroxylinolenoyl)-ʟ-Gln (Fig. 2), which was first isolated from OS of beet

armyworm caterpillars (*Spodoptera exigua*; Alborn et al., 1997). Besides volicitin, other fatty acid (FA)-amino acid conjugates have been isolated from different noctuid and geometrid Lepidoptera larvae. The general structures of these compounds were determined as *N*-acyl-Glns, where the FA moiety is represented mainly by linolenic acid (C18:3), linoleic acid (18:2), and derivatives thereof (Alborn et al., 1997; Paré et al., 1998; Pohnert et al., 1999; Spiteller and Boland, 2003; Spiteller et al., 2004). The amino acid component was always Gln (Glu in *Manduca sexta*), but the FA moiety varied and apparently depended on the food plant (Paré et al., 1998). Recently, in three of 13 nonlepidopteran insects that have been investigated, the presence of such FA-amino acid conjugates has been proven (Yoshinaga et al., 2007). The gut contents of two closely related cricket species, *Teleogryllus taiwanemma* and *Teleogryllus emma* (Orthoptera: Ensifera: Gryllidae) both contained *N*-linolenoyl-ʟ-Glu as well as *N*-linoleoyl-ʟ-Glu. Two more probably hydroxylated derivatives of these Glu conjugates have been found and, additionally, *N*-linolenoyl-ʟ-Gln and a small amount of volicitin. In larvae of the fruit fly, *Drosophila melanogaster* (Diptera: Drosophilidae), *N*-linolenoyl-ʟ-Glu and *N*-linoleoyl-ʟ-Glu have been detected in larval extracts, whereas hydroxylated Glu conjugates, volicitin and *N*-linolenoyl-ʟ-Gln, were found only as trace components (Yoshinaga et al., 2007). Most of the *N*-acyl-Glns exhibited high volatile-inducing activities or herbivory-specific responses in general when added to various plant species that have been wounded only once (Alborn et al., 1997; Landolt et al., 1999; Halitschke et al., 2001; Schmelz et al., 2001; Maffei et al., 2004; Wu et al., 2007). However, *N*-acyl-Glns are not generally active as, for example, in lima bean and cotton (*Gossypium hirsutum*), where no induction of volatiles was detected (Spiteller et al., 2001). Interestingly, this finding corresponds to the high level of solely mechanically induced volatiles in these species (Maffei et al., 2007a).

Another class of elicitors has been isolated from pea weevil (*Bruchus pisorum*) and cowpea weevil (*Callosobruchus maculatus*). These elicitors, bruchins, are long-chain α,ω-diols, which are mono- and diesterified with 3-hydroxypropanoic acid (Fig. 2; Doss et al., 2000). If, during oviposition, female pea weevils come into contact with their host plant, pea (*Pisum sativum*), bruchins initiate neoplastic growth on pods in certain pea genotypes at the site of egg attachment. This growth of nondifferentiated callus cells lifts the eggs above the oviposition site. As a consequence, this hinders larval entry into the pod tissue and presents the larvae to enemies and promotes desiccation (Doss et al., 2000). Interestingly, formation of neoplasms strongly depends on the presence of the dominant wild-type allele, *Neoplastic pod* (*Np*), in the host plant genotype (Dodds and Matthews, 1966). Moreover, application of bruchins initiated the induction of *CYP93C18*, a putative isoflavone synthase gene, and the formation of the isoflavonoid phytoalexin pisatin (Cooper et al., 2005). Synthetic analogs have been prepared and examined to evaluate structural requirements for callus induction.

The chain length is important with an optimum of C-22 to C-24, whereas unsaturation within the chain is relatively unimportant. For maximal bioactivity, difunctionality is required; however, the α,ω-diols themselves are inactive. More critical is the ester portion of the molecules: 3-hydroxypropanoate esters are more active than any analogs (Oliver et al., 2002).

From which organisms the various HAMPs originate still remains to be solved. Obviously, inceptins are plant-derived peptides originally from the chloroplastic ATP synthase γ-subunit but processed in the insect gut. Concerning the FA-amino acid conjugates, there is an ongoing debate whether the insects themselves or bacteria of the insect gut generate these compounds. For N-acyl-Glns from the lepidopteran larvae, it has been demonstrated that bacterial isolates are capable of producing N-acyl amino acids when supplied with linolenic acid and Gln (Spiteller et al., 2000). In addition, a novel type of DNA-binding protein from starved cell proteins isolated from the *S. exigua* gut bacterium *Microbacterium arborescens* SE14 has been shown to hydrolyze and synthesize N-acyl amino acids (Ping et al., 2007). In contrast, the insect host also possesses a gut enzyme that is able to catalyze conjugation of food-derived FA with Gln (Lait et al., 2003). For bruchins and caeliferins, data on their biosynthesis are not available yet and need to be generated. Moreover, for all HAMPs, it is necessary to elucidate how the host plants recognize them to finally understand their particular modes of action: whether, for example, they act directly on the plant membranes or whether they interact with specific binding sites/receptors of the plants that subsequently mediate these signals. Interestingly, in aphids, no HAMPs have been identified so far; however, in some plant species, there is good genetic evidence for R-gene-mediated (gene-for-gene) resistance to phloem-feeding insects (for review, see Walling, 2008 [this issue]).

BINDING SITES FOR THE HAMP VOLICITIN

Recent progress in isolation of pure elicitors makes possible investigation of binding proteins, which might function as receptors in signal transduction pathways that ultimately activate defenses. However, up to now, such a study has been performed only for volicitin (Truitt et al., 2004). Using a tritiated volicitin as radioligand, in corn leaves the existence of a receptor-like binding site for volicitin has been demonstrated. The binding site is localized at the plasma membrane. It is heat and protease sensitive, and slightly (3-fold) inducible with methyl jasmonate, indicating its proteinaceous nature. The binding is of high affinity (K_d approximately 1.3 nM), saturable, reversible, and exhibits high ligand specificity. In particular, competition analysis revealed that the binding must be specific because the binding protein can discriminate between L- and D-volicitin enantiomers (i.e. conjugates with L- and D-Gln, respectively); only the biologically active L-volicitin competes with the radioligand (Truitt et al., 2004). All these parameters represent typical properties of classical receptors, which strongly suggests that, at least in corn, the volicitin effects are mediated by receptor-connected processes. Next, the corresponding gene of the putative receptor needs to be cloned. In the future, the cognate receptors for the different HAMPs must be identified.

WOUNDING AS SIGNAL?

Mechanical wounding of plant tissues is an inevitable consequence of herbivory. However, both intensity and extent of damage might be different and may vary with the mode of feeding (e.g. sucking [spider mites] or chewing [caterpillars]). The impact of injuries on the initiation of plant defense reactions has been underestimated for a long time. In almost all studies that investigated the effects of insect feeding, HAMPs, or both on the emission of, for example, volatiles, the corresponding control experiments have been performed using plants wounded by scratching (Turlings et al., 1990; Schmelz et al., 2001; Spiteller et al., 2001), crushing (Reymond et al., 2000), or puncturing leaves (Halitschke et al., 2001). Such types of mimicked herbivory resulted in induction of genes corresponding to different defense strategies but also in up-regulation of activities addressing changes in primary metabolism (Cheong et al., 2002; Reymond et al., 2004; Major and Constabel, 2006). Strikingly, induction of herbivory-related volatile emission was not observed. Although it was obvious that wounding affected gene expression and physiological responses in planta, the question of whether or not such treatments are adequate to mimic insect feeding remained open. Herbivorous insects feed on leaves by continuously clipping off pieces of tissue, a long-lasting series of mechanical injuries. Thus, it is conceivable that plants are able to discriminate mechanical wounding that occurred only once and continuously sustained damage. This hypothesis has been proven by the deployment of MecWorm, a computer-controlled mechanical caterpillar that simulates herbivory in a much more realistic mode (Mithöfer et al., 2005). For instance, on lima bean leaves, nearly the same blend of volatiles has been induced by long-lasting mechanical wounding with MecWorm as was known to be induced by herbivore damage (Mithöfer et al., 2005). However, not all plant species investigated so far responded to MecWorm treatment with the emission of the whole set of herbivory-related volatiles (Maffei et al., 2007a), suggesting that the relative contributions of both mechanical wounding and chemistry in the form of HAMPs can differ and is not unique among plant species.

A still-unknown phenomenon that needs to be identified is the nature of the initial wounding signal that launches wound- or herbivory-induced reactions in plants. The observed increase of the wounding signal JA or other phytohormones after minutes (León

et al., 2001; Kessler and Baldwin, 2002; Maffei et al., 2007b) is certainly a consequence, rather than the initial startup of subsequent responses. The same holds true for C-6 green leaf volatiles (Matsui, 2006), although they (1) are released very fast and much earlier than other compounds (Turlings et al., 1998) by existing enzymatic equipment; (2) have the ability to induce defense-related responses (Bate and Rothstein, 1998; Arimura et al., 2002); and (3) are necessary for a successful defense against herbivores (Vancanneyt et al., 2001; Halitschke et al., 2004; Shiojiri et al., 2006). Thus, other chemical factors, which are immediately set free within seconds after the first bite, or physical factors, such as electrical pulses, are much more likely the missing link between wounding and already-known wounding responses. A compound that is generated fast upon herbivory and thus represents an alternative to green leaf volatiles is H_2O_2 (Maffei et al., 2006). However, in this case, the methods to analyze the kinetics for H_2O_2 generation need to be improved to detect the very early phases of the wounding process. Electrical waves that propagate from the point of injury through the tissue are more likely early and fast signals (Maffei et al., 2007b). Such signals are indeed able to trigger defenses, for example, the up-regulation of proteinase inhibitor genes (Wildon et al., 1992; Stankovic and Davies, 1997; Herde et al., 1998), a response obviously following wounding (Graham et al., 1986). Recently, action potentials in plants have been correlated with defense responses upon herbivore attack (Maffei et al., 2004) and they also have been demonstrated to be released by various compounds (e.g. Glu, γ-aminobutyric acid, Ca^{2+}; Felle and Zimmermann, 2007). However, other electrical signals, such as variation potentials, might be involved as well. It will be interesting to figure out which kind of electrical signals are able to transport herbivory-induced information in principle and how such a signal can mediate specific information.

FUTURE PERSPECTIVES

Recent studies have contributed to our understanding of the mechanisms by which plants might recognize herbivores and subsequently initiate defense responses. Two areas where future efforts might result in major breakthroughs are related to signal molecule recognition and downstream signal transduction. The identification and use of purified or synthetic HAMPs will make possible the identification of corresponding binding proteins and their encoding genes in plant cells. The future challenge for research in this area will be to demonstrate that these binding proteins might be true receptors and are thus involved in launching signal transduction pathways and later on defenses. One approach to achieving this goal might be the use of plant mutants that are unresponsive to a particular signal. Characterization of such mutants will result in the identification of genes encoding HAMP receptors, proteins acting downstream in signal transduction,

and regulation of the defense response. There is also a need to identify putative components of signal transduction pathways by using biochemical and genetic methods to study their possible interactions and to analyze causal relationships with specific signal perception mechanisms. Such studies will not only unravel individual signaling pathways, but also could establish links in a network of alternative routes regulating the multitude of inducible plant defenses.

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