Constitutive plant toxins and their role in defense against herbivores and pathogens

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Most recent investigations have focused on induced, rather than constitutive, plant defenses. Yet significant research has helped to illuminate some of the principal characteristics of constitutive defenses, including mechanisms of action and synergistic effects, as well as strategies used by herbivores and pathogens to circumvent them.

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Abbreviations

cytochrome P450s	cytochrome-P450-dependent monooxygenases
DIBOA	2,4-dihydroxy-1,4-benzoxazin-3-one
DIMBOA	7-methoxy DIBOA

Introduction

Plants synthesize a broad range of secondary metabolites, including alkaloids and terpenoids, that are toxic to herbivores and pathogens, and so are believed to act as defense compounds. Classical examples of plants that are poisonous to humans, such as poison hemlock, foxglove, and aconite, demonstrate how well natural products can defend plants, at least against mammalian herbivores. Defensive chemicals have long been thought to be costly for plants because of the resources consumed in their biosynthesis, their toxicity to the plant itself or the ecological consequences of their accumulation [1,2] (see also Heil, this issue). One way for a plant to reduce these costs is to synthesize defense compounds only after initial damage by a herbivore or pathogen. This strategy is obviously risky because the initial attack may be too rapid or too severe for such damage-induced defenses to be deployed effectively. Consequently, plants that are likely to suffer frequent or serious damage may be better off investing mainly in constitutive defense, whereas plants that are attacked rarely may rely predominantly on induced defenses [3].

When applied to individual plant organs or developmental stages, the same considerations suggest that plant parts that are of high fitness value or that are under a high risk of attack may be best protected by constitutive defenses, whereas others may be better defended by induced responses. For example, a field survey has shown that the reproductive organs of wild parsnip (*Pastinaca sativa*) are attacked very frequently by herbivores. These organs accumulate high constitutive levels of the toxic furanocoumarin, xanthotoxin (Figure 1a), which are not increased by artificial damage. In contrast, the roots of wild parsnip are rarely attacked and have only low constitutive levels of xanthotoxin, but these increase readily upon wounding [4].

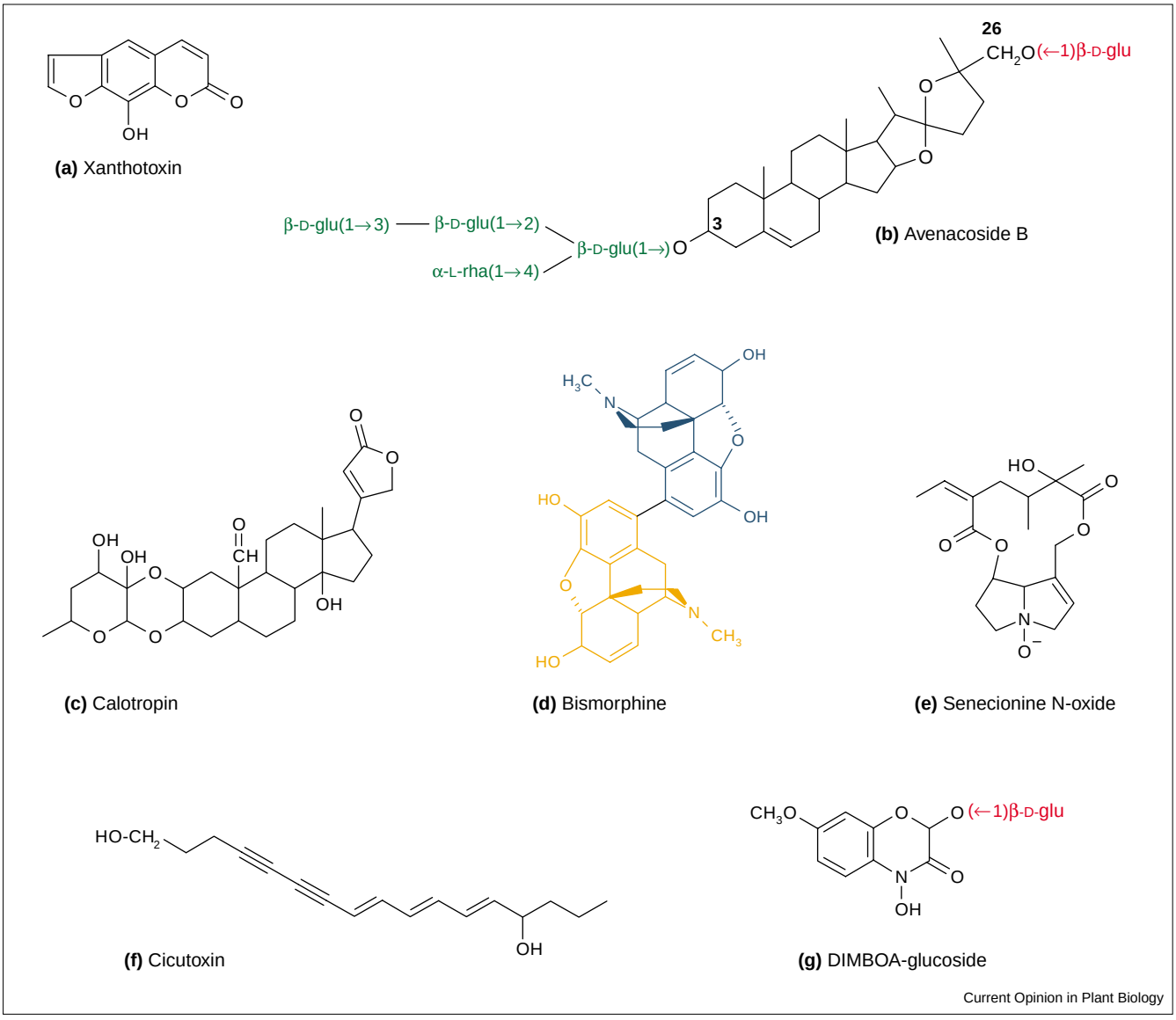
Thus, both constitutive and induced defenses may contribute to the optimal protection of a plant against its multitude of herbivorous and microbial enemies. Because of the large variety of elicitors available for triggering the accumulation of defense compounds and the development of molecular tools for studying differential gene expression, however, research in the past decade has largely focused on induced defense [5,6,7•]. In contrast, studies on the roles and mechanisms of constitutive chemical defense are rather rare because of the difficulty of manipulating constitutive compounds in experimental settings. This review discusses recent progress in our understanding of some general principles that underlie constitutive chemical defense, and explores the use of molecular tools to study its role in plants.

Plant toxins act through various mechanisms

All plant compounds that have negative effects on the growth, development or survival of another organism can be regarded as toxins. The mechanisms of action of some plant toxins are well known. For example, saponins (Figure 1b) disrupt cellular membranes [8], hydrogen cyanide released from cyanogenic glycosides (Figure 2) inhibits cellular respiration [9], and cardenolides (Figure 1c) are specific inhibitors of the Na^+/K^+-ATPase [10,11]. But the modes of action of many other toxins still await discovery.

In recent studies, the active principle of water hemlock (*Cicuta virosa*), the polyacetylene cicutoxin (Figure 1f), was shown to act by prolonging the repolarization phase of neuronal action potentials, presumably by blocking voltage-dependent potassium channels [12]. Thanks to this mechanism, water hemlock is one of the most poisonous plants of the Northern Hemisphere. The analgesic morphine from opium poppy (*Papaver somniferum*) has pronounced effects on the central nervous system owing to its binding to opiate receptors. A possible additional mode of action of morphine in defense against pathogens was recently described [13]. Upon mechanical damage, constitutive morphine is quickly metabolized to bismorphine (Figure 1d), which accumulates in the cell wall and becomes cross-linked to pectins, making them resistant to hydrolysis by pectinases. Bismorphine formation requires a pre-existing peroxidase and H_2O_2 that may arise from the oxidative burst triggered upon pathogen attack.

Figure 1

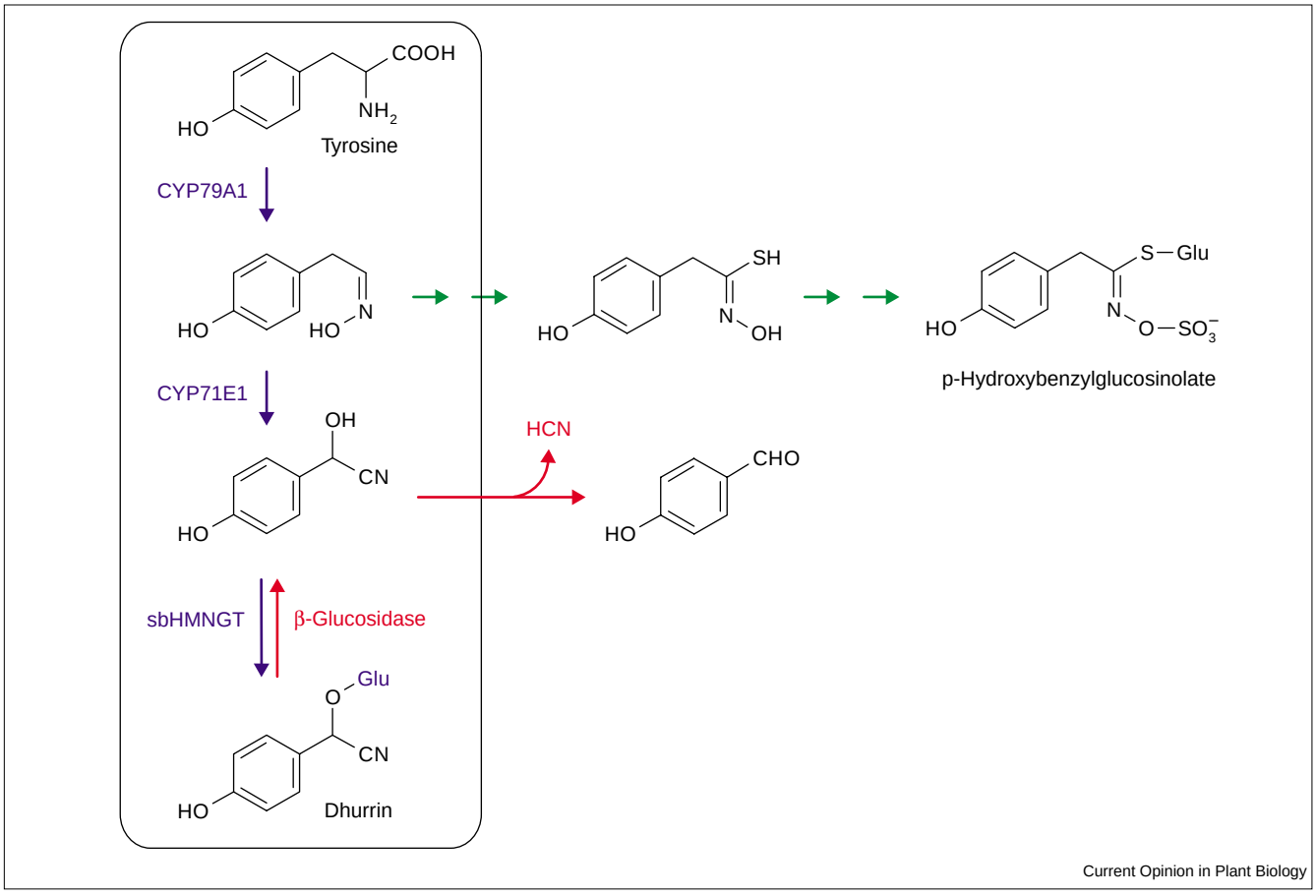


Examples of constitutive plant toxins. **(a)** A furanocoumarin from *Pastinaca sativa*, **(b)** a saponin from *Avena sativa*, **(c)** a cardenolide from *Asclepias currassavica*, **(d)** the product of wound-induced dimerization of the preformed alkaloid morphine from *Papaver somniferum* (morphine monomers shown in orange and blue), **(e)** a pyrrolizidine alkaloid from *Senecio jacobaea*, **(f)** a polyacetylene from *Cicuta virosa*, **(g)** a benzoxazinone glucoside from *Zea mays*. Sugar residues highlighted in red are released by endogenous plant enzymes upon tissue damage leading to the activation of the toxins. Sugar residues highlighted in green are released by glycosidases of pathogens that are able to overcome the plant toxins.

Recently, some compounds that are well known for their other functions in primary or secondary metabolism have also been found to be involved in plant defense [14,15]. For example, phytic acid, a strong cation chelator, whose salts serve as the major storage form of phosphorus in the seeds and fruits of many plants, has been suggested to function in antiherbivore defense on the basis of its ability to bind essential dietary nutrients. Larvae of the two lepidopterans, *Depressaria pastinacella* (which feeds on immature reproductive structures) and *Trichoplusia ni* (which feeds on foliage) all died when fed an artificial diet supplemented with 1% phytic acid. However, the seed-feeding *Heliothis virescens* were not killed by the same diet [15].

The ways in which plant toxins are stored are often crucial for their effectiveness. Certain plant species accumulate toxins in resin ducts, laticifers (Figure 3) [16•] or glandular trichomes (Figure 4) [17,18]. The toxins are released in large amounts as soon as these structures are ruptured by herbivore feeding, movement on the plant surface or the growth of pathogens.

Figure 2



Metabolic engineering of the biosynthetic pathway of the cyanogenic glucoside dhurrin into *Arabidopsis thaliana* [57••]. Upon the introduction of the three enzymes (shown in blue) catalyzing dhurrin biosynthesis in *Sorghum bicolor* into *A. thaliana*, *A. thaliana* accumulated the glycoside dhurrin (glucose residue highlighted in blue). The engineered plants were toxic to *Phyllotreta nemorum*, a specialized herbivore of crucifers, as hydrolysis of the glucoside upon tissue damage leads to the release of the metabolic poison hydrogen cyanide (shown in red). If just CYP79A1 is transferred to *A. thaliana*, endogenous *A. thaliana* enzymes (green arrows) convert the aldoxime intermediate into the respective glucosinolate, and the plants are readily consumed by *P. nemorum* [58].

The power of synergism: one plus one equals more than two

Plants are not only able to synthesize individual defense metabolites with diverse chemical structures but also produce complex mixtures of defense compounds, such as the terpenes of essential oils. Many of the individual constituents of essential oils are acutely toxic to insects [19] and pathogens [20]. However, the toxicity of these compounds can be potentiated in mixtures, so that the activity of the mixture is higher than would be expected by adding up the activities of its individual constituents. This phenomenon, known as synergism, has recently been demonstrated for mixtures that each contained two essential-oil constituents, which were fed to larvae of the generalist lepidopteran *Spodoptera litura* [21]. These mixtures were up to nine times more toxic than would have been expected from the simple additive effects of the constituents. Synergistic effects are also known for antimicrobial peptides. *In vitro* assays have demonstrated that snakin-1, a constitutive peptide from potato that is mainly expressed in tubers and reproductive organs, acts synergistically against *Clavibacter michiganensis* subsp. *sepedonicus* with the potato defensin PTH1, which has a similar expression pattern [22]. The growth inhibition caused by the combination of these two peptides exceeded their calculated additive effect by 100%. The mechanisms behind such synergisms are unknown, but may involve the ability of one component of a mixture to inhibit the detoxification of others or to enhance the absorption of others from the gut [23].

Plants must live with their own toxins

Many defense compounds are toxic to the plant itself, and so plants that rely on constitutive chemical defense must be able to synthesize and store these substances without poisoning themselves. One strategy is to store toxins as inactive precursors, for example as glycosides [24], separate from activating enzymes. For example, it has long been known that the glucosinolates found in plants of the order

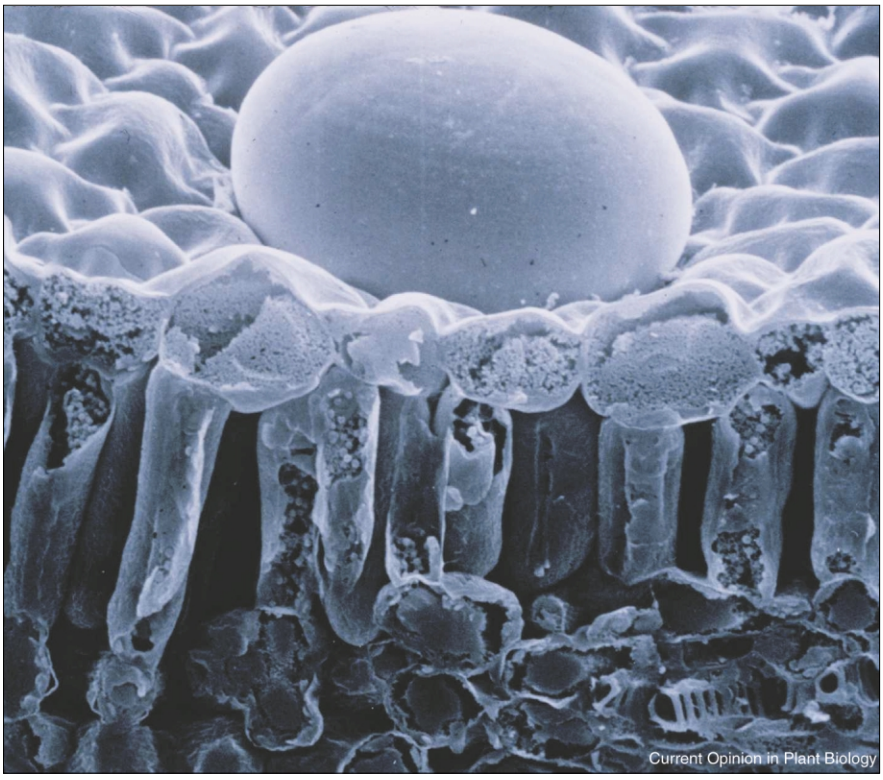
Figure 3

Larvae of some lepidopterans avoid intoxication by severing (i.e. 'trenching') the laticifers upstream of their intended feeding site; however, during trenching they may encounter potent doses of latex toxins. **(a)** Larva of *Erinnyis alope* starting to feed after severing a *Carica papaya* leaf. **(b)** Larva of *Trichoplusia ni* hanging immobilized and vulnerable to predators after ingesting the cardenolide-containing latex of *Asclepias currassavica*. Photographs by David Dussourd.

Capparales (Figure 5) [25–27] are compartmentalized separately from their activating enzyme, the thioglucosidase myrosinase. Glucosinolates are found in many plant tissues, whereas myrosinase is localized in scattered 'myrosin' cells that seem to be glucosinolate-free. In *Arabidopsis thaliana*, recent studies suggest that sulfur-rich cells (S-cells) that are situated between the phloem and the endodermis of the flower stalk contain high concentrations of glucosinolates [28], whereas myrosinase is localized in adjacent phloem parenchyma cells (Figure 5) [29]. Upon tissue damage, the glucosinolates contact myrosinase and are hydrolyzed irreversibly into an unstable aglycone. The aglycone rearranges into a variety of biologically active compounds, typically isothiocyanates and nitriles (Figure 4).

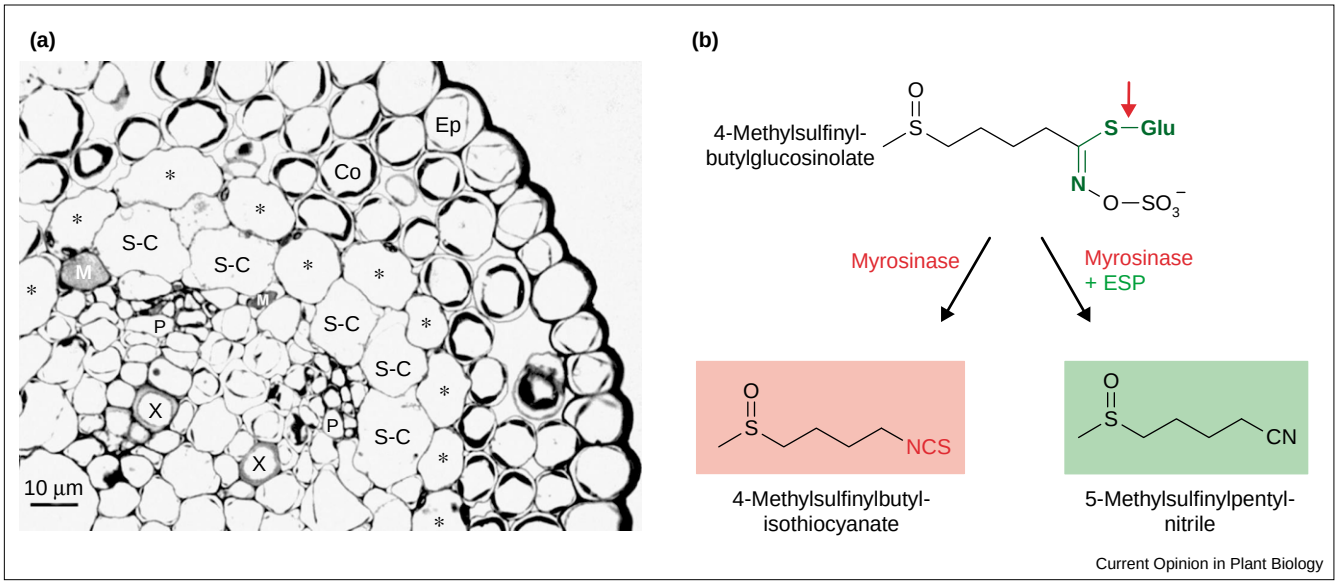
The defensive function of the isothiocyanates released upon glucosinolate hydrolysis ('the mustard oil bomb') became apparent in a recent study in which larvae of the generalist lepidopteran *T. ni* avoided *A. thaliana* ecotypes that produced predominantly isothiocyanates upon

Figure 4



In mints and many other plant species, toxins are accumulated in glandular hairs (glandular trichomes) that are found on the surfaces of leaves and other aerial parts of the plant. This scanning electron micrograph depicts a portion of a young peppermint (*Mentha* x *piperita*) leaf cut away to show the size of a peltate glandular trichome (approximately 100 μm in diameter) in relation to the other cell types of the leaf. In peppermint, glandular trichomes accumulate a mixture of metabolites dominated by monoterpenes. Photograph taken by the senior author in collaboration with R Croteau at the Washington State University Electron Microscope Center, V Franceschi, Director.

Figure 5



The glucosinolate-myrosinase system. **(a)** Compartmentalization of glucosinolates and myrosinase. In pedicels (i.e. flowering stems) of *A. thaliana* glucosinolates are thought to be present in sulfur-rich S-cells (S-C) localized separately from the hydrolyzing enzyme, myrosinase, which is stored in immediately adjacent cells (M). A transverse section of a pedicel of *A. thaliana* (ecotype Wassiljewskija) is shown in which epidermis (Ep), cortex (Co), starch sheath (asterisks), and two vascular bundles containing xylem (X) and phloem (P) can be seen. The pedicel was fixed in a glutaraldehyde-formaldehyde mixture followed by osmium tetroxide, and embedded in epoxy resin. Section staining as described in [29]. Microphotograph by Lise Bolt Jørgensen and Erik Andréasson. **(b)** Influence of epithiospecifier protein (ESP) on the myrosinase-catalyzed hydrolysis of 4-methylsulfinylbutylglucosinolate. Upon hydrolysis, the presence of functional ESP results in the formation of 5-methylsulfinylpentylnitrile instead of 4-methylsulfinylbutylisothiocyanate. Larvae of the generalist *Trichoplusia ni* prefer to feed on *A. thaliana* ecotypes that express functional ESP [30••]. –CN indicates the nitrile moiety; -NCS indicates the isothiocyanate moiety.

glucosinolate hydrolysis, preferring to feed instead on ecotypes that produced nitriles [30••]. Mapping of the locus that controlled hydrolysis product formation (which was virtually superimposable on the previously described *TASTY* locus [31]) led to the identification of an epithiospecifier protein whose presence results in the formation of nitriles instead of isothiocyanates upon myrosinase-catalyzed hydrolysis of glucosinolates. Following tissue damage, it may be necessary for plants to detoxify residual active compounds. However, it is not known how glucosinolate-containing plants detoxify isothiocyanates and other glucosinolate hydrolysis products. Nitrilases and methyltransferases have been suggested to catalyze the *in planta* detoxification of nitriles and thiocyanate, respectively [32–34].

Benzoxazinoids, found mainly in Gramineae, are another class of glycosides that are activated upon tissue damage [35]. The reversible hydrolysis of inactive D-glucoside precursors leads to the generation of the phytotoxic aglycones 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and its 7-methoxy derivative DIMBOA (Figure 1g). In maize plants, DIBOA and DIMBOA are detoxified by the reformation of D-glucosides, which is catalyzed by two glucosyltransferases encoded by the *BX8* and *BX9* genes [36•]. Dicotyledonous species that encounter benzoxazinoids that are released by neighboring grasses can detoxify the phytotoxic DIBOA-decomposition product benzoxazolin-2-one (BOA) by hydroxylation and N-glycosylation [37].

Specialized herbivores and pathogens overcome toxic plant compounds with diverse biochemical and behavioral strategies

Herbivores and pathogens have developed a variety of mechanisms to circumvent plant toxins. Recent studies have improved our understanding of both metabolic detoxification and behavioral mechanisms for avoiding toxins. Extensive research on the detoxification of plant compounds by insects has been carried out on the furanocoumarins of the Apiaceae and Rutaceae (Figure 1a), which are metabolized by cytochrome-P450-dependent monooxygenases (cytochrome P450s) of the CYP6B-subfamily in larvae of the lepidopteran genera *Papilio* and *Helicoverpa* [38,39]. Several of the more than 30 identified members of this subfamily (http://drnelson.utmem.edu/P450db.html) are induced by the furanocoumarin xanthotoxin [40,41]. However, the basal and inducible expression levels of the individual cytochrome P450s differ depending on the degree of specialization of the insect herbivore [42•].

Examples of the detoxification of plant toxins by pathogens include plant-pathogenic fungi that are able to metabolize the saponins of their hosts [8,43]. Recent work

Figure 6

Larvae of the specialist *Tyria jacobaeae* are able to completely defoliate their host plant, *Senecio jacobaea*, even though it contains pyrrolizidine alkaloids. The larvae detoxify the alkaloids and sequester them for their own defense against predators. Their conspicuous coloration is thought to serve as a warning for predators of their unpalatability. Photograph by Thomas Hartmann.

has elucidated the detoxification enzymes of pathogenic isolates of the fungus *Stagonospora avenae* growing on *Avena sativa*. The antifungal 26-desglucoavenacosides released from the steroidal saponins avenacoside A and B upon pathogen attack of *Avena sativa* are sequentially hydrolyzed by three fungal enzymes, one α-rhamnosidase and two β-glucosidases (Figure 1b), resulting in a strong reduction in antifungal activity [44].

Sawflies that induce galls on willow trees overcome the chemical defense of their host plants by altering the chemical composition of gall tissue compared to that of the rest of the plant [45]. Galls induced by six different *Pontania* species on six chemically diverse willow species contained significantly smaller concentrations of most of the 36 individual phenolic compounds analyzed compared to ungalled leaves.

Lepidopteran larvae of the subfamily Plusiinae (Noctuidae) are able to avoid contact with defensive secretions stored in laticifers and oil ducts by severing ('trenching') these structures upstream of the intended feeding site, thereby preventing the influx of toxic latex or oil into the tissue they feed upon (Figure 3a) [46]. However, this adaptation does not allow feeding on the cardenolide-containing plant *Asclepias curassavica* (Asclepiadaceae), which severely poisons insects with latex and surface cardenolides that are encountered during trenching (Figure 3b) [16•].

Once herbivores have developed biochemical mechanisms that enable them to feed on a formerly toxic host with impunity (Figure 6), they often use the toxins as cues in the search for a suitable host plant [47], or sequester toxins for their own defense against predators or as pheromone precursors [48,49]. For example, considerable research has been performed on the detoxification and sequestration of pyrrolizidine alkaloids (Figure 1e, Figure 6) [50]. Another classical example of sequestered plant toxins are the cardenolides acquired by larvae of the Monarch butterfly (*Danaus plexippus*) from their *Asclepias* host plants. Monarch butterfly larvae, which sequester cardenolides to become unpalatable to predators, are insensitive to cardenolides because of a single amino acid substitution at the ouabain-binding site of their Na^+/K^+-ATPase [51].

Gene discovery provides tools for studying constitutive plant defense

The past few years have witnessed a surge of reports describing the identification of biosynthetic genes and transcription factors that are involved in the formation of plant defense compounds [9,27,52,53,54••,55,56], as well as the discovery of genes that encode the detoxifying enzymes of herbivores or pathogens (see above). These discoveries provide numerous opportunities for investigators to manipulate the interactions between plants and their enemies under experimental conditions, and such work has already begun. Among the most notable examples is the engineering of *A. thaliana* to express the entire biosynthetic pathway of the tyrosine-derived cyanogenic glucoside dhurrin by introducing three biosynthetic genes (two cytochrome P450s and one glucosyltransferase) from *Sorghum bicolor* (Figure 2) [57••]. The specialist crucifer flea beetle *Phyllotreta nemorum*, which normally accepts the glucosinolate-containing *A. thaliana* as a food plant, consumed up to 80% less of transgenic leaf discs containing high concentrations of dhurrin than of controls. Furthermore, the accumulation of dhurrin in the transgenic leaves reduced leaf mining by the flea beetle larvae and increased larval mortality. Interestingly, *A. thaliana* plants transformed with just the first gene of the pathway, which encodes the cytochrome P450 CYP79A1 that converts tyrosine to p-hydroxyphenylacetaldoxime, are readily consumed by the flea beetle [58]. In these plants, p-hydroxyphenylacetaldoxime is channeled into the glucosinolate biosynthesis pathway, resulting in a four-fold

increase in the total glucosinolate content because of the accumulation of p-hydroxybenzylglucosinolate that is not found in wildtype plants [59]. These two studies with *P. nemorum* demonstrate that specialized insects have evolved powerful strategies to cope with the chemical defenses of their typical host plants. However, the production of an entirely new class of toxins in the host plant may defend the plant against specialist enemies that have not adapted to this new defense.

On the pathogen side, glucosidases have been suggested to be involved in fungal detoxification of α-tomatine, a steroidal glycoalkaloid of tomato fruits, by the cleavage of sugar residues attached to the basic alkaloid skeleton. Recent investigations using a tomatinase-deficient mutant of the fungus *Septoria lycopersici* revealed that although the targeted replacement of the tomatinase gene made the fungus more sensitive to α-tomatine, the mutant fungus was still able to grow on high α-tomatine concentrations. These findings indicate that other detoxification mechanisms must be involved in the α-tomatine resistance of this pathogen [60].

Conclusions

As the discovery of plant defense genes seems set to continue at its present rapid pace, the studies discussed above likely represent just the beginning of a long series of investigations on plant defense using genetically modified plants or plant enemies. Given the chemical complexity of plants, the ability of the experimenter to manipulate their phenotypes or the phenotypes of their enemies precisely is crucial to demonstrating the actual role that postulated defense adaptations or counteradaptations play in plant–herbivore or plant–pathogen interactions.

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