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4.08 Chemical Defence and Toxins of Plants

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4.08.1 Chemical Defense against Herbivores

4.08.1.1 Introduction

An important component determining plant growth and reproduction is the defense against herbivores. Direct lines of defense against herbivores have been well documented. In addition, several methods of indirect defense have been noted in which the herbivores' natural enemies are exploited.¹⁻⁵ Initially, it was thought that both direct and indirect defenses were constitutive in nature. However, evidence is accumulating that both direct and indirect defenses can be either constitutive or inducible by herbivores. Furthermore, exposure to volatiles emitted by neighboring plants also induces plant defense against herbivores (priming). Here, direct chemical defense and indirect chemical defense of plants against herbivores are reviewed. A complete review is not provided, but rather the processes of some examples are outlined.

4.08.1.2 Direct Defense of Plants against Herbivores

Plants contain a large variety of secondary metabolites that are not involved in primary metabolisms necessary for maintaining life activities and survival. Over the last several decades, a number of studies, including the pioneering study by Feeny⁶ on the ecological roles of plant tannins, have reported that many of the plant secondary metabolites play important roles in plant defense activities against herbivores, including insects, mammals, and snails. By 1980s, major groups of defense chemicals such as alkaloids, nonprotein amino acids, cyanogenic glycosides, glucosinolates, terpenoids, coumarins, cardenolides, iridoid glycosides, tannins, phenolics, flavonoids, and phytoecdysteroids became well known, and their defensive roles against insects have been well established.^{4,5,7,8} Since then, the discovery of totally new groups of secondary metabolites is rare; however, there is considerable progress in our understanding of the modes of action of toxicity, target sites in herbivores, precursors of toxic molecular species and enzymatic activation, plant tissues specialized in defense and localization of secondary metabolites, inducible defense and induction of secondary metabolites in plants, evolution of the synthetic pathway of defense chemicals, synergism of defense chemicals, and molecular-level adaptive mechanisms in specialist herbivores. The progress made is mainly due to new experimental methods, especially molecular biology techniques. Furthermore, it has become more apparent that plant defense does not depend fully on secondary metabolites and that various defense proteins are also important. Such recent progresses as well as important basic knowledge on plant defense based on plant ingredients both chemicals and proteins are discussed in this section.

4.08.1.2.1 Secondary metabolites

4.08.1.2.1(i) Alkaloids Alkaloids are a diverse group of secondary metabolites and more than 12 000 alkaloids have been reported from 150 families of plants and more than 20% of all plant species contain alkaloids.^{9–11} According to Pelletier an alkaloid is “a cyclic compound containing nitrogen in a negative oxidation state”;¹² however, recently other nitrogen-containing compounds not classified into other groups of compounds, such as amino acids, amines, and cyanogenic glycoside, are sometimes classified as alkaloids.¹⁰ Alkaloids are quite diverse in structure and are classified into several groups: tropane alkaloids (e.g., atropine), quinolizidine alkaloids (e.g., lupinine), pyridine alkaloids (PAs) (e.g., nicotine), indole alkaloids (e.g., harman), pyrrolizidine alkaloids (e.g., senecionine-*N*-oxide), isoquinoline alkaloids (e.g., morphine), purine alkaloids (e.g., caffeine), and polyhydroxy alkaloids (e.g., 1-deoxynojirimycin (DNJ), 1,4-dideoxy-1,4-imino-*D*-arabinitol, and castanospermine), and other groups (Figure 1). Most alkaloids are derived from amino acids (predominantly ornithine, lysine, tyrosine, phenylalanine, and tryptophan), apart from those that involve insertion of nitrogen into preformed skeletons, notably diterpene and steroidal alkaloids.¹³ This is also the case for sugar-mimic alkaloids such as DNJ, at least in bacteria.¹⁴

The modes of action of different alkaloids are diverse. For example, nicotine binds to and affects nicotinic acetylcholine receptors and shows toxicity. A recent molecular 3D model suggests that both acetylcholine and nicotine bind to the same pocket formed in a nicotinic acetylcholine receptor.¹⁵ Morphine binds to and activates opioid receptors, transmembrane-spanning G protein-coupled receptors, in the central nervous system of humans.¹⁶ Caffeine, which is structurally similar to adenine, inhibits cyclic AMP phosphodiesterase activity and inhibits the degradation of cAMP, thus exerting a toxic effect on insects;¹⁷ in human beings, binding of caffeine to the adenosine A2A receptor induces wakefulness.¹⁸ Atropine binds to muscarinic acetylcholine receptors, competing with acetylcholine, and blocks neurotransmission.¹⁹

Although many alkaloids exert their toxic effects by affecting the nervous system, there are other alkaloids that show toxicity in completely different ways. Colchicine binds to tubulin and inhibits its polymerization, and thus inhibits the formation of microtubules in cells.

A new and interesting group of alkaloids is polyhydroxy alkaloids, such as DNJ and 1,4-dideoxy-1,4-imino-*D*-arabinitol (Figure 1), which are also called sugar-mimic alkaloids or iminosugars. Because polyhydroxy alkaloids resemble the structures of sugars, they are very potent inhibitors of sugar-metabolizing enzymes.²⁰ The IC₅₀ values (the concentration necessary for inhibiting 50% of enzyme activities) of polyhydroxy alkaloids for disaccharidases such as amylase, sucrase, and trehalase from human beings and many insects are as low as 10^{−4}–10^{−6} mol l^{−1}.^{20,21} However, some plants contain polyhydroxy alkaloids in very high concentrations. For example, the seeds of *Castanospermum australe* contain 0.06% of castanospermine,^{20,21} and the latex of mulberry trees (*Morus* spp.) contains up to 2.5% (0.2 mol l^{−1}) of 1,4-dideoxy-1,4-imino-*D*-arabinitol and DNJ in

Figure 1 Alkaloids.

combination.²² The sugar-mimic alkaloids in mulberry latex inhibit both sucrase in the midgut, thereby inhibiting sugar digestion, and trehalase in various tissues, thereby inhibiting the utilization of trehalose, the major blood sugar in many insects, and thus mulberry trees are well protected from herbivores.²³ However, the silkworm *Bombyx mori*, a specialist of mulberry leaves, is tolerant to sugar-mimic alkaloid because they have developed sucrase and trehalase that is insensitive to sugar-mimic alkaloids.²³

Pyrrolizidine alkaloids are unique among alkaloids in that evolution of synthetic process, toxicology, ecological impacts on herbivorous insects, adaptation, detoxification, sequestration, and utilization as defense chemicals by insects are all well studied (Figure 2).^{11,24–26} The synthesis of necine base, the characteristic structure of PA, starts from the synthesis of homospermidine, catalyzed by homospermidine synthase (HSS), using primary metabolites spermidine and putrescine.²⁴ Sequence data indicated that HSSs from Boraginaceae, Asteraceae, and Orchidaceae have high homologies with deoxyhypusine synthase (DHS), an enzyme involved in the post-transcriptional activation of eukaryotic initiation factor 5A (eIF5A), and have evolved several times independently from eIF5A.^{24,25} Thus in this case, the key enzyme involved in the synthesis of a secondary metabolite has evolved from an enzyme necessary for a basic biological function by change of function. After several steps, necine base is synthesized. PA has two molecular forms: the protoxic free base and the nontoxic *N*-oxide.²⁶ In most plants, PA is stored as *N*-oxide produced by oxidation of free base. When herbivores feed on a PA-containing plant, *N*-oxide is reduced into free base automatically and is then converted by cytochrome P-450 monooxygenase of herbivores into an unstable toxic pyrrolic intermediate that alkylates cellular nucleophiles (i.e., –OH, –NH₂, –SH residues of proteins and nucleic acids); therefore, PAs show toxicity to herbivores.²⁶ Insects specialized in feeding on PA-containing plants have developed physiological mechanisms to avoid the toxicities of PAs.²⁶ For example, PAs absorbed in the form of the protoxic free base in the larvae of the arctiid moth *Tyria jacobaeae* feeding on the host plant *Senecio jacobaea* (Asteraceae) were converted in hemolymph by a specific NADPH-dependent monooxygenase into *N*-oxide form and then stored in the larval

Figure 2 Synthetic pathway and activation process of pyrrolizidine alkaloid.^{24–26}

body, with about 75% in the integument. A chrysomelid leaf beetle, *Oreina cacaliae*, feeding on the PA-containing plant *Adenostyles alliariae* (Asteraceae) is able to suppress the autoreduction of *N*-oxide in the gut lumen and then absorb and store it in the body. The stored *N*-oxide functions as a reservoir for exocrine defense glands. Any absorbed PAs in free base form are efficiently detoxified by glucosylation. A neotropical beetle, *Platyphora boucardi*, has developed a completely different strategy to cope with and sequester PAs. The beetle absorbs PAs in protoxic free base. Different from *T. jacobaeae*, the beetle does not have the ability to convert free base into *N*-oxide; however, in *P. boucardi*, the protoxic free base is efficiently pumped into the exocrine defense gland and the concentration of the free base in hemolymph is always low. Furthermore, females of *Utetbesia ornatrix* transmit PAs that have been transmitted by males during copulation to eggs for protection of eggs.²⁷

4.08.1.2.1(ii) Nonprotein amino acids Nonprotein amino acids are amino acids other than the 20 amino acids incorporated in protein (Figure 3). More than 900 nonprotein amino acids have been reported from a wide variety of plants including Leguminosae, Liliaceae, Sapindaceae, Cycadaceae, Compositae, Rubiaceae, and Lecythidaceae.^{28–30} However, nonprotein amino acids are most often found in legumes and are mainly abundant in seeds.

The typical toxic mechanism of nonprotein amino acids is that they function as mimics of 20 protein amino acids and are mistakenly incorporated in protein in the place of the corresponding protein amino acids similar in structure, thereby leading to the production of unnatural proteins that cannot function properly. It is the case with azetidine-2-carboxylic acid found in *Convolvallaria majalis* (Liliaceae), which is a mimic of proline, and a legume toxin canavanine that occurs in the seeds of jackbean, *Canavalia ensiformis*, and *Dioclea megacarpa* in very high concentrations (up to 6 and 10%, respectively), which is a mimic of arginine.⁸

Other nonprotein amino acids exert toxicity in different ways. For example, 3,4-dihydroxyphenylalanine (L-DOPA), which is found in high concentrations (6–9%) in *Mucuna* seeds, shows strong toxicity to insects, but not to mammals, presumably by affecting tyrosinase activity, which is important in the hardening of insect cuticle.⁸ Mimosine, which was first isolated from *Mimosa pudica* and which exists in the seeds and leaves of *Leucaena leucocephala* in high concentrations (8–10% dry weight in leaves), causes loss of hair in mammals when fed.³⁰ This phenomenon is explained by the fact that mimosine prevents initiation of DNA replication.³¹ The fruit of *Blighia sapida* is consumed especially in Jamaica. The unripe fruits, however, are toxic, because hypoglycine, which exists in unripe fruit arils in relatively high concentrations (0.1% dry weight), induces severe hypoglycemia or very low blood sugar concentrations.^{29,30} In mammals, hypoglycine is degraded into α -methylenecyclopropylacetic acid and this compound completely blocks fatty acid oxidation; thus, sugar is consumed intensively as the sole energy source and as a result all glycogen in the liver is exhausted, leading to hypoglycemia.^{29,30,32}

The above toxic nonprotein amino acids serve as defense against mammals and insects in general; however, the herbivorous insects specialized in feeding on plants containing nonprotein amino acids have developed physiological adaptation to nonprotein amino acids. For example, the larvae of the bruchid beetle *Caryedes brasiliensis*, a specialist feeder of *D. megacarpa* seeds rich in canavanine, are able to avoid misincorporation of canavanine into proteins, because the arginyl-tRNA synthetase of the beetle can discriminate between arginine and canavanine.^{30,33}

4.08.1.2.1(iii) Cyanogenic glycosides Cyanogenic glycosides are nitrogen-containing secondary metabolites that have an ability to produce highly toxic hydrogen cyanide when degraded by plant enzymes. More than 75 different cyanogenic glycosides have been reported from at least 2650 plants from 130 families, including Euphorbiaceae, Rosaceae, Asteraceae, Passifloraceae, Fabaceae, and Poaceae.^{34–36} These compounds are typically *O*- β -glycoside of α -hydroxynitriles (cyanohydrins) and are kept in plant tissues. Linamarin and

Figure 3 Nonprotein amino acids.

Figure 4 Cyanogenic glycosides and release of cyanide.

lotaustralin are the two most commonly found cyanide glycosides and are reported from many plant families, whereas other cyanide glycosides such as amygdalin common in Rosaceae plants and dhurrin often found in Poaceae plants are more confined in distribution (**Figure 4**). Cyanide glycosides are synthesized from amino acids.

Cyanide glycosides function as defense chemicals because they produce hydrogen cyanide, which is highly toxic to most living organisms because of its ability to inhibit the electron transport system by binding to cytochromes. When plant tissues are damaged by herbivory, β-glucosidases that are kept in other tissues separate from cyanogenic glycosides come in contact with and cleave sugars from cyanide glycoside and, as a result, cyanohydrins (hydroxynitriles) are formed (**Figure 4**).^{34–36} Then, the cyanohydrins are degraded by a second enzyme, hydroxynitrile lyase, into hydrogen cyanide and ketones (or aldehydes) (**Figure 4**), although the last degradation occurs spontaneously but slowly.^{34–36} The details of compartmentation differ among plant species. In the shoot of sorghum, *Sorghum bicolor*, dhurrin is contained in the vacuoles of epidermal cells, whereas β-glucosidase is kept in the chloroplasts of mesophyll cells and hydroxynitrile lyase is kept in the cytosol of mesophyll cells.³⁷ In cassava, *Manihot esculenta*, linamarin and lotaustralin are widely distributed among tissues,³⁸ whereas linamarase is concentrated in laticifer and latex³⁹ and hydroxynitrile lyase is concentrated in the cell wall of leaf tissues but absent in the root.⁴⁰ As hydrogen cyanide is highly toxic to most animals and insects, cyanide glycoside-borne defense systems are effective against most herbivores. Nonadapted insects and mammals have limited ability to detoxify hydrogen cyanide by means of the enzyme rhodanese or β-cyanoalanine synthase, which converts cyanide ion into less toxic thiocyanate. Moreover, insects specialized in feeding on plants with cyanide glycoside-borne defense systems can tolerate the toxicity, and some highly specialized insects such as *Heliconius* butterflies can even sequester cyanide glycosides for their own defense from predators. For example, the larvae of *Heliconius sara* sequester up to 1.5 mg CN dwg^{−1} of cyanide glycoside, mostly epivolkenin, from their host plant *Passiflora auriculata*.⁴¹ The same larvae can further convert a substantial amount of epivolkenin into the corresponding thiol compound sarauriculatin by enzymatic replacement of the nitrile group by a thiol group.⁴²

4.08.1.2.1(iv) Glucosinolates Glucosinolates are reported from a few plant families including Brassicaceae, Capparaceae, Tovariaceae, and Caricaceae, most of which belong to the order Brassicales in APG II system of plant taxonomy. In spite of their rather limited distribution, glucosinolates have been studied intensively because Brassicaceae includes many important crops such as cabbage and rapeseed and an important model plant species, *Arabidopsis thaliana*.

Glucosinolates such as sinigrin are molecules that consist of a β-thioglucose moiety, a sulfonated oxime, and a variable side chain derived from various amino acids (**Figure 5**).^{43,44} Glucosinolates themselves are not toxic to herbivores and are widely distributed in plant tissues. On the contrary, the enzyme called myrosinase or thioglucosidase is distributed in myrosin cells that do not contain glucosinolates. In the flower stalk of

Figure 5 Enzymatic activation of glucosinolates (a) and adaptation of specialist herbivores (b).

A. thaliana, sulfur-rich cells (S-cells) that exist between the phloem and the endodermis contain high concentrations of glucosinolates, and myrosinase exists in adjacent phloem parenchyma cells.⁴⁵ When plant tissues are broken by herbivory, myrosinase cleaves the β -thioglucose moiety. The aglycones are unstable and further degrade in most cases into either isothiocyanates or nitriles depending on pH and other conditions (**Figure 5(a)**), although in rarer cases depending on R groups, thiocyanates and epithionitriles are formed.^{43,44} Isothiocyanates, which are also called mustard oils, are highly toxic and even lethal to most unadapted generalist herbivores, especially to herbivorous insects,^{46,47} whereas nitriles are less toxic.⁴⁶ Chemical conditions have been shown to be important in determining whether isothiocyanates or nitriles are formed: acidic conditions and existence of Fe^{2+} favor nitrile formation and alkaline conditions favor isothiocyanate formation (**Figure 5(a)**).^{43,44} Recently, however, particular plant proteins associated to myrosinase are found to determine the final product, nitriles or isothiocyanates. The epithiospecifier protein (ESP) found in a certain line of *A. thaliana* promotes the production of nitriles, whereas another myrosinase-associated protein EPITHIOSPECIFIER MODIFIER1 (ESM1) recently found in certain *A. thaliana* lines represses nitrile formation and favors isothiocyanate production (**Figure 5(a)**).⁴⁸ The herbivory by the larvae of generalist lepidopteran species *Trichoplusia ni* or *Spodoptera littoralis* is greater on *A. thaliana* lines expressing ESP than on those not expressing ESP, and is greater on lines not expressing ESM1 than on those expressing ESM1.⁴⁸ It is interesting that the larvae of small white butterfly, *Pieris rapae*, a specialist on Brassicaceae plants, adapt themselves to glucosinolate-borne defense by modifying the final product to nitriles and inhibiting the production of isothiocyanates by the activity of nitrile specifier protein (NSP) in the larval gut, and as a result isothiocyanates are not formed and less toxic nitriles are excreted in feces (**Figure 5(b)**).⁴⁹ The larvae of diamondback moth, *Plutella xylostella*, another specialist species, adapt themselves with gut glucosinolate sulfatase (GSS), which cleaves the sulfate moiety (sulfonated oxime) of glucosinolates and forms desulfo-glucosinolates (**Figure 5(b)**). The plant myrosinase cannot cleave the glucose moiety any more from desulfo-glucosinolates, and thus mustard oils (isothiocyanates) are not formed (**Figure 5(b)**).⁵⁰

4.08.1.2.1(v) Coumarins Coumarins are chemicals that have a 2*H*-1-benzopyran-2-one structure. Coumarins are grouped into simple coumarins, which have only two rings, such as coumarin, and those with an additional ring on benzopyran-2-one ring. Those with an additional furan ring attached at the 6,7 positions are called linear furanocoumarins, such as xanthotoxin, and those with an additional furan ring attached at the 7,8 positions are called angular furanocoumarins, such as angelicin (Figure 6).

Simple coumarins are toxic or deterrent to insects and are widely distributed among plant families, being found in more than 79 families of angiosperms, whereas furanocoumarins are more restricted to a few plant families such as Rutaceae and Umbelliferae.⁵¹ Furanocoumarins have a unique phototoxicity, which occur under UV radiation. Under UV radiation, furanocoumarins crosslink both strands of DNA by binding to pyrimidine bases. The crosslinking inhibits DNA replication and transcription, and thus furanocoumarins are toxic to generalist insects under UV radiation.⁵¹ To avoid phototoxicity, some insects roll the leaves and feed on the inside of the leaves, thereby avoiding UV radiation.⁵² Specialist insects feeding on furanocoumarin-containing plants (Umbelliferae and Rutaceae) such as swallowtail butterflies detoxify them by P-450 oxygenases. Angular furanocoumarins are more difficult to detoxify than linear ones,⁵³ and some specialists can feed only on linear furanocoumarin-containing plants. Further, in Umbelliferae plants, chemicals that inhibit the detoxification of furanocoumarins and enhance the toxicity of furanocoumarin by inhibiting P-450 activity coexist with furanocoumarin.⁵³

4.08.1.2.1(vi) Phenolics and tannins Phenolics, including tannins, are found in plant tissues in very large amounts. The concentration of tannin in oak reaches 5% dry weight in late summer and autumn.^{6,8} Tannins bind to proteins noncovalently, precipitate dietary proteins, and inhibit many digestive enzymes, and therefore inhibit the digestive processes and growth of herbivorous mammals and insects.⁸ Herbivorous mammals adapted to tannin-rich diets secrete several kinds of tannin-binding proteins, such as proline-rich proteins that precipitate with tannin as an adaptation to tannin.⁵⁴

Phenolics not only exert negative growth effects by noncovalent interactions, but also by covalent interactions. Oxidative enzymes such as polyphenol oxidases (PPO) and peroxidases (POD) oxidize phenolics, monophenols, and diphenols into reactive molecular species such as quinones and quinone methides, which covalently bind to nucleophilic residues in dietary proteins such as $-SH$, $-NH_2$ residues of nutritionally important amino acids such as cysteine and lysine, thereby decreasing the nutritive values of dietary proteins (Figure 7).⁵⁵

4.08.1.2.1(vii) Terpenoids Terpenoids are chemicals that basically consist of isoprene (C₅) units. Despite this similarity in basic units, terpenoids are extremely diverse in their structures and biological activities and include a large number of chemicals; at least 15 000 terpenoids are found in plants. Because of this diversity, terpenoids are divided into several groups according to their structures and biological functions. For example, monoterpenoids (C₁₀) include volatile terpenoids rich in conifer resins, essential oil, and exudates of glandular trichome such as α -pinene, β -pinene, limonene, and menthol (Figure 8), and confer defense on plants.^{56,57} Iridoids are also included in monoterpenoids. Sesquiterpenoids (C₁₅) include sesquiterpene lactones, phytojuvenile hormones, and others; diterpenoids (C₂₀) include clerodanes, tiglanes, daphnanes, and others; and triterpenoids (C₃₀) include cardenolides, cucurbitacins, limonoids, phytoecdysteroids, saponins, and others. As it is not possible to describe all these diverse terpenoids, some characteristic groups of terpenoids are briefly described here.

4.08.1.2.1(vii)(a) Hormone analogues: Phytojuvenile hormones and phytoecdysteroids Insect development is controlled by several hormones, for example, juvenile hormone (JH) and two closely related steroid

Figure 6 Coumarins.

Figure 7 Activation of phenolics by oxidative enzymes such as polyphenol oxidases (PPO) and peroxidases (POD) and reaction of activated phenolics with protein.

Figure 8 Volatile monoterpene hydrocarbons often found in conifer resins, essential oils, and glandular trichomes that function as defense chemicals of plants against insect herbivores.

molting hormones, ecdysone and 20-hydroxyecdysone (commonest in insects). JH III is important for maintaining juvenile forms during molting and 20-hydroxyecdysone is important in molting and metamorphosis (Figure 9).

From the balsam fir tree *Abies balsamea*, a sesquiterpenoid, juvabione (Figure 9), was isolated as a compound that has a JH activity that causes the last instar larvae of European linden bug, *Pyrrhocoris apterus*, to molt into nymphal-adultoid forms.^{58,59} Since then, a number of phytojuvenile hormones have been detected⁵⁶ such as juvonicene I and II from the sweet basil *Ocimum basilicum*.^{60,61}

A compound that closely resembles the structure of molting hormone 20-hydroxyecdysone was first reported from the leaves of *Podocarpus naka* in large quantities and was called ponasterone A (Figure 9).⁶² Starting from this discovery, more than 200 ecdysteroids have been found, most of which are from plants, most often in ferns and conifers.^{59,63} These phytoecdysteroids have molting hormone activity and disturb the normal development of insects and in many cases the insects die. In the flowers of *Serratula inermis*, 20-hydroxyecdysone is found in very high concentrations of up to 2%.⁶⁴ In spinach, the amount of phytoecdysteroids increases in response to herbivory.^{63,65} Both the induction of phytoecdysteroids in response to insect herbivory and the insect-specific toxicity of phytoecdysteroids support the idea that phytoecdysteroids are defense chemicals against insect herbivory.

4.08.1.2.1(vii)(b) Cardenolides Cardenolides are a group of cardiac-active steroids that have a five- or six-membered lactone ring and, in most cases, a sugar moiety, and are found mainly in plants belonging to Asclepidaceae and Apocynaceae, such as ouabain (Figure 10) from an African plant, *Acokanthera ouabaio*

Figure 9 Insect hormones and plant analogues.

Ouabain

Figure 10 Ouabain, a cardenolide found in *Acokanthera ouabaio* (Apocynaceae).

(Apocynaceae), but also in plants belonging to Moraceae and other families.⁶⁶ The latex of several plants belonging to Asclepidaceae and Apocynaceae often contains very high concentrations of cardenolides.^{66,67} The toxicity of cardenolides resides in their inhibitory activity against $\text{Na}^+ - \text{K}^+$ ATPase (or $\text{Na}^+ - \text{K}^+$ pump) involved in maintenance of ion concentrations in cells and neurotransmission, and thus cardenolides are generally toxic to animals, including insects.^{66,68} Cardenolides bind specifically to membrane extruding part of $\text{Na}^+ - \text{K}^+$ ATPase; however, several insect species specialized in feeding on cardenolide-containing plants have developed cardenolide-insensitive $\text{Na}^+ - \text{K}^+$ ATPase, which commonly have single amino acid substitutions in target sites (cardenolide-binding sites).⁶⁹

4.08.1.2.1(vii)(c) Iridoid glycosides Iridoid glycosides are a group of compounds that have a structure related to iridodial (**Figure 11**).⁷⁰ Almost 600 iridoid glycosides have been described from 57 families of plants.⁷⁰ Iridoids have been regarded as defense chemicals against herbivores and pathogens, since iridoid glycosides generally have bitter taste and have antifeedant and growth inhibitory activities against insects.⁷⁰ The observation of induction of iridoid glycosides in *Lonicera implexa* leaves bearing naturally laid eggs of the specialist herbivore *Euphydryas aurinia* and the observation that iridoid glycosides such as secologanic acid are present in these leaves in high concentrations (15-fold the total concentration, which is 15% of dry weight) compared to other leaves⁷¹ support the idea that iridoid glycosides play a role in plant defense.

Iridoids are ecologically very interesting, because several insect herbivores from different families specialized in feeding on iridoid glycoside-containing plants sequester iridoid glycosides in higher concentrations. The adults of checkspot butterflies, *Euphydryas* spp., feeding on iridoid glycoside-containing plants contain as much as 9% dry weight of iridoid glycosides and are unpalatable to birds, and the larvae and pupae are warning colored and gregarious,⁷⁰ which are the typical characteristics of unpalatable insects. The molecular mechanism of sequestration has recently been studied in leaf beetles (Chrysomelidae) by feeding the larvae with structurally different artificial thioglucosides resembling natural iridoid glycosides (*O*-glucosides).⁷²

In spite of the above biological phenomena, the reason why iridoid glycosides show such effects are not explained at the molecular level. Several studies have shown that at least some defense activities of iridoid glycosides could be attributed to their unstable aglycones that have glutaraldehyde-like structures and have alkylating activity against nucleophilic residues in biomolecules, in particular, amino residues in amino acids, proteins, and nucleic acids (**Figure 12**).^{73,74} For example, two iridoid glycosides, aucubin and catalpol, in *Plantago lanceolata* showed growth inhibitory activity on specialist fungus *Diaporthe adunca* only in aglycone form and not in glycoside form.⁷⁴ Oleuropein, a secoiridoid glycoside with a diphenolic moiety, exists in high concentrations (3% of leaf dry weight) in *Ligustrum obtusifolium* and shows a strong growth inhibitory activity against herbivorous insects after being activated by an oleuropein-specific β -glucosidase and by a polyphenol oxidase (**Figure 12**).⁷³ Since the aglycone of oleuropein is a very strong electrophile due to its glutaraldehyde and α,β -unsaturated aldehyde structures, and covalently binds to amino residues of side chains of lysine, which is an essential amino acid, lysine becomes unavailable and privet leaves become nonnutritive to generalist insects (**Figure 12**).⁷³ The specialist caterpillars that feed on privet tree, such as *Brabmeae wallichii*, secrete high concentrations of free glycine, which also has amino residues, in the digestive juice as neutralizer to prevent the loss of lysine by competing with the amino residues in the side chain of lysine (**Figure 12**).^{75,76}

Figure 11 Iridoid glycosides.

Figure 12 Enzymatic activation of oleuropein in the defense system of the privet tree *Ligustrum obtusifolium* and adaptation of privet specialist insects.

4.08.1.2.2 Defense proteins

Recently, the importance of defense proteins in plant defenses against herbivores has become more recognized. Although there have been some well-known defense proteins such as proteinase inhibitors (PIs), the progress in studies on defense proteins had been slow until recently compared with those on secondary metabolites involved in defense, probably because methodologies used in protein sciences and organic chemistry are very different. For example, before performing bioassays of defense substances, the chemist often performs leaf extraction with an organic solvent such as methanol; however, the same extraction may denature and inactivate defense proteins irreversibly. Recently, DNA technology and gene expression analyses showed that many genes are induced when plants are damaged by herbivores, and that quite a few of these genes code defense proteins.

4.08.1.2.2(i) Proteinase inhibitors Among defense proteins, the best known and widely distributed are PIs, which are also called protease inhibitors.^{55,77,78} Serine proteinase inhibitors inhibit serine proteinases such as trypsin and chymotrypsin, the major digestive proteinases in lepidopteran and a part of coleopteran insects, and many of which are classified into Bowman–Birk inhibitors and Kunitz inhibitors according to the structures, but some, such as potato inhibitors, are not included in these classes.⁷⁷ Serine proteinase inhibitors are most abundant in storage tissues such as seeds and tubers, and their concentrations reach a few percent of the total protein. Legume seeds are a rich source of various serine proteinase inhibitors.

In foliage, the concentrations of serine proteinase inhibitors are lower than that in seeds; however, the induction of serine proteinase inhibitors has been reported in many plants including tobacco and tomato, and the inhibitors have been proven to function as a plant defense system.^{79–81} Leaf damage by the tobacco hornworm *Manduca sexta* was much bigger in tomato plant line deficient in proteinase inhibitor induction than in those that can induce proteinase inhibitor on herbivory.⁸²

Some lepidopteran larvae such as *Helicoverpa zea* and *P. xylostella* adapt themselves to plant PIs by changing digestive midgut proteinases from PI-sensitive ones to PI-insensitive ones when they are fed PI-containing diets.⁸³

4.08.1.2.2(ii) Amylase inhibitors Amylase inhibitors are found in the seeds of plants such as cereal grains (wheat, maize, rice, barley) and legumes (kidney beans, cowpea, adzuki beans). Amylase inhibitors inhibit amylases of insects in general and inhibit the growth of insects, and thus serve as defense proteins in both cereal grains and bean seeds.⁷⁷ Seed weevils not specialized in feeding on the kidney bean cannot grow on this bean because their amylase is inhibited by amylase inhibitor in kidney bean, but seed weevils specialized in feeding on kidney bean can feed on this bean because their digestive proteinase can digest and inactivate amylase inhibitor in kidney bean.⁸⁴ Seed weevils utilize cysteine proteinases (CPs) instead of serine proteinases as their digestive proteinase, which enables seed weevils to feed on beans rich in serine proteinase inhibitors.⁵⁵

4.08.1.2.2(iii) Proteinase Recently, proteinases have become recognized as plant defense proteins. In a maize line resistant to a lepidopteran pest, *Spodoptera frugiperda*, a unique 33-kDa cysteine proteinase, Mir1-CP, with chitin binding activity was found to accumulate in damaged foliage sites, move through vascular tissues, and confer defense on maize, *Zea mays*.^{85,86} In addition to Mir1-CP in maize, it has been directly demonstrated that CPs such as papain and ficin in the latex of papaya and fig latex, respectively, inhibit the growth and are responsible for the strong lethal toxicity and defense activities of these plants against herbivorous insects.⁸⁷

4.08.1.2.2(iv) Polyphenol oxidases and peroxidase Polyphenol oxidases (PPO), which occur in many plants together with phenolics, catalyze the oxidation of mono-, di-, and polyhydric phenols to quinones.⁵⁵ Quinones formed by these reactions are active and covalently bind to nucleophilic side chains (–SH, –NH₂, =NH) of proteins and, as a result, lysine, histidine, cysteine, and methionine are lost from dietary proteins and the nutritive value of the protein is significantly reduced (Figure 7).^{55,88} POD also catalyze the oxidation of phenolics and, as a result, oxidation products such as quinone and quinone methides are formed. These oxidation products also covalently bind to side chains of amino acids such as lysine and cysteine⁵² (Figure 7).⁵⁵

4.08.1.2.2(v) Lipoxxygenase Lipoxxygenases (LOX), which are widely distributed in plant species and abundant in legume seeds such as soybean, oxidize polyunsaturated fatty acid containing a *cis*, *cis*-1,4-pentadiene moiety into conjugated dienoid hydroperoxides, which are further oxidized into epoxides, C6 aldehydes, malondialdehyde, hydroxynonenal (an α,β -unsaturated aldehyde), and jasmonic acid (JA), an important elicitor of plant defense genes (see Section 4.08.1.3.3(ii)).^{55,81,89} These products bind covalently to amino acid side chains (–SH, –NH₂, =NH) and exert toxicity to insects including caterpillars and planthoppers.^{55,89–92}

4.08.1.2.2(vi) Lectins Lectins are proteins that bind to specific sugars and are found in a wide variety of plant species, but are abundant in legume seeds.^{93–96} Typical lectins have multiple binding sites in each molecule, and thus they have cell agglutinating property, but some lectins have single binding site. Lectins from kidney bean seeds (Phaseolus hemagglutinin (PHA)),⁹⁷ lectin from wheat germ (wheat germ agglutinin (WGA)),⁹⁸

and lectin from snowdrop, *Galanthus nivalis* (snowdrop lectin, GNA),⁹² are the three lectins whose chemical characters and toxicity against insects have been intensively studied.^{93,95,96} PHAs specifically bind to α -GalNAc and GNA specifically binds to mannose. WGA specifically binds to GlcNAc β (1,4) GlcNAc and chitin.^{93,96} Expression of GNA in a transgenic tobacco plant resulted in added protection against aphids, which suggested that plant lectins could be useful in the development of crops tolerant to pests.⁹⁹ PHAs and GNA bind to midgut epithelia and the binding is supposed to be the cause of toxicity of lectin against insects; however, the detailed mechanisms of toxicities are not clear.^{93,95,96} WGA is supposed to exert its toxicity by binding to or by preventing the formation of peritrophic membrane in midgut lumen rich in chitin;¹⁰⁰ however, the detailed mechanism of toxicity is unknown.

4.08.1.2.2(vii) Jasmonic acid-inducible proteins: Arginase, threonine deaminase, and vegetative storage proteins Recently, several proteins induced by JA, a plant hormone that plays important roles in the induction of many plant defense responses and defense genes⁸¹ (see Section 4.08.1.3.3(ii)), namely arginase, threonine deaminase,¹⁰¹ and vegetative storage protein (VSP),¹⁰² were proven to be plant defense proteins against insect herbivores. In tomato, *Solanum lycopersicum*, JA-inducible arginase and threonine deaminase degraded arginine and threonine, both of which are necessary for insect growth, and the tomato plant that overexpresses arginase was more resistant to *M. sexta* larvae¹⁰¹ (Figure 13). VSPs, which are widely distributed in plants such as soybean and *A. thaliana*, and are shown to be induced by JA, are found to have an acidic phosphatase activity and have toxic effects to insects such as bruchid beetles and dipteran insects that have acid gut lumen.¹⁰²

4.08.1.2.3 Temporally and spatially restricted distribution of defense substances

When we attempt to extract defense substances, sometimes we assume that the substances are ubiquitously present in plant tissues. However, recent studies show that quite often this is not the case. Without being aware of this fact, we might fail to detect and extract defense substances. Here, three types of such defense systems are introduced.

4.08.1.2.3(i) Enzymatic activation of precursors, compartmentation, and synergism of defense factors Some defense substances are kept in plants as precursors that do not have biological activities (or defense activities). The defense substances that are even hazardous and toxic to plants themselves are often kept in plant tissues as stable and safe precursors. The enzymes that convert or activate the precursors are

Figure 13 Degradation of amino acids by arginase and threonine deaminase, defense proteins induced in tomato leaves after insect herbivory.

kept in compartments separate from precursors. When plant tissues are damaged and the compartments are broken, activating enzymes come in contact with the precursors and activate them to bioactive forms.

Such compartmentation and enzymatic activation of precursors are commonly observed in several different groups of secondary metabolites,¹⁰³ which includes the activation of cyanide glycosides by β -glucosidase and hydroxynitrile lyase that produce toxic hydrogen cyanide in many plants such as sorghum, clover, and almond^{34,35,37–40} (Figure 4), the activation of glucosinolates by thioglucosidase that produce toxic isothiocyanates or nitriles in plants belonging to the order Brassicales^{43–45} (Figure 5), the activation of *O*-coumaric acid by β -glucosidase that produces coumarin in white melilot (*Melilotus alba*),¹⁰³ the activation of phenolics by PPO that produce highly reactive quinones in many plants⁵⁵ (Figure 7), and the activation of an iridoid glycoside, oleuropein, by β -glucosidase⁷³ (Figure 12).

Not only defense secondary metabolites but also defense proteins may exist as precursors. For example, papain, a cysteine proteinase that exists in papaya latex and recently found to have strong defense activity, is kept in laticifer as an inactive precursor and within 2 min after wounding, it becomes active.^{104,105}

To obtain precursors in stable form, preheating leaves in steam before extraction is essential to inactivate the activating enzyme; however, in order to detect or reproduce bioactivity, activation of precursor by activating enzyme is needed.^{73,106}

In compartmentation systems (or precursor activation systems), precursors and activating enzymes work synergistically to exert plant defense; however, other kinds of synergism were observed. For example, chemicals that enhance the toxicity of furanocoumarin by inhibiting detoxifying enzyme of insects (i.e., cytochrome P-450 monooxygenase) coexist with furanocoumarin in plants.⁵³

4.08.1.2.3(ii) Plant tissues specialized in defense and localization of defensive substances Defense substances are not always distributed evenly throughout plant tissues. In practice, there are some tissues specialized in defense against insects in which the defense substances are very abundant, whereas in other tissues the defense substances are almost absent. Glandular trichomes and latex (laticifer) are examples of such defense-specialized tissues.

Glandular trichomes are typically hair-shaped glands (some are peltate) on plant surface that exude solution containing defense substances.^{103,107,108} Some glandular trichomes constantly exude solution, whereas others contain solution and rupture when insects contact. The glandular trichomes are common in families such as Solanaceae, Labiatae, and Asteraceae. The exudates from trichomes on tobacco leaves contain alkaloids such as nicotine and anabasine, and are toxic to aphids and tobacco hornworm *M. sexta*.^{108,109} The resistance of wild tomato, *Lycopersicon hirsutum*, to lepidopteran larvae is due to 2-tridecanone and 2-undecanone, both of which are contact toxins to lepidopteran larvae and which are contained in the exudates of glandular trichomes.^{107,108,110,111} Similarly, the exudates of peltate glandular trichomes in mint, *Mentha × piperita*, contain essential oil consisting primarily of *p*-menthane-rich monoterpenes such as menthol, which are toxic to insects.^{45,112} In both tomato and mint cases, the localization, characterization, and/or genes of synthetic enzymes in trichomes have been studied in detail.^{111,112}

Plant latex is a sap that is exuded from damaged vein. It is kept in a tissue specialized in exuding latex called laticifer and is kept inside laticifer cells. It works as an efficient defense system against herbivorous insects.^{113–115} Resin, which is kept outside the cells, and exudate, which is transparent, and the contents of oil duct exudates are similar to latex in appearance and biological functions. Latex, exudates, and resin are found in more than 35 000 species of plants.¹¹⁶ Latex has been shown to contain a wide variety of bioactive substances, both secondary metabolites and proteins, in condensed form.^{113,116} Some of the latex substances have been shown to be toxic to insects, such as cardenolides in milkweed latex,⁶⁶ but the toxicities of latex ingredients against insects have not been tested in most latex-exuding plants. However, recently, more and more latex ingredients have been proven to have toxicity against insects and serve as defense against insect herbivores. The ingredients of plant latex shown to have defense activities are as follows.^{113,116} Cardenolides or cardiac glycosides, a group of glycosylated steroids that are inhibitors of $\text{Na}^+ - \text{K}^+$ ATPase of animals, exist in high concentrations in the latex of Asclepidaceae and Apocynaceae^{66,67} and are highly toxic to animals including insects and serve as plant defense. Morphine and other alkaloids in the latex of poppy (*Papaver somniferum*) and plants of poppy families, whose synthetic mechanism and localization have been recently elucidated in detail,¹¹⁷ are toxic to animals. Furanocoumarins such as xanthotoxin abundant in oil duct exudates of wild

parsnip *Pastinaca sativa*¹¹⁸ are toxic to insects in general. Recently, high titer of CPs such as papain and ficin in papaya and fig latex, respectively, are experimentally proven to be defense proteins that are highly toxic to herbivorous insects.⁸⁷ As CP and other types of proteinases are often found in plant latex such as latex of Asclepidaceae species,¹¹⁹ those proteinases could also function as plant defense. Latex that exudes from mulberry leaves, which have been used as a suitable host plant of the silkworm *Bombyx mori*, is recently found to function as effective defense to nonadapted insects because of latex ingredients toxic to nonadapted insects, namely high concentrations of sugar-mimic alkaloids such as 1,4-dideoxy-1,4-imino-D-arabinitol (D-AB1) and DNJ (Figure 1), which altogether make up 2.5% of wet latex and 18% of dried latex, and a novel unidentified defense protein.²² Latex-borne defense is unique in that even though the total amount of toxin that a latex-exuding plant individual contains is small, and thus the average concentration of toxin is low, for herbivorous insects small in size, a large amount of condensed toxin that moved through the laticifer rushes out at the very point of insect damage immediately after the damage. This means that the amount of toxin that the insects encounter and the defense effect of toxins in latex on insects would be much greater than those estimated from average concentration. For example, the average concentrations of sugar-mimic alkaloids in mulberry leaves are as low as 0.1–0.01% of leaf dry weight; however, herbivores feeding on mulberry latex would encounter latex containing 2.5% wet weight and 18% dry weight of sugar-mimic alkaloids.²² Similarly, the average titer of papain in papaya leaves is much lower than the lethal dose, and the titer of papain in latex that insects encounter is much higher than the lethal dose.⁸⁷

It is clear from the above discussion that if we do not know about the localization of defense substances in specialized tissues like latex and glandular trichomes, adopt extraction methods disregarding the localization, and rely on average concentration, we may underestimate or may even overlook the biological effects and the existence of defense substances in defense tissues. On the contrary, the plant substances found in such defense-specialized tissues are very likely to have roles as defense substances.

4.08.1.2.3(iii) Induction of defense substance The induction of defense substance (i.e., increase in the amount of defense substance in response to herbivory) was first reported in 1970s, where PIs were found to be induced in plant leaves after wounding and herbivory.⁷⁹ At present, induction of defense substances has been reported in more than 100 plant species belonging to 34 families,⁸¹ and the substances reported to be induced include defense proteins such as PIs,⁷⁹ cysteine proteinases (Mir1-CP),^{85,86} lipoxygenase,^{91,120} and secondary metabolites involved in defense such as tannins,¹²¹ phenolics,¹²¹ tobacco alkaloids (nicotines),^{122,123} glucosinolates,¹²⁴ and phytoecdysteroids.⁶⁵ Now, induction of defense substances is considered a common phenomenon. The extent of induction differs among each plant species and even within the same species. For example, nicotine in tobacco leaves increases after herbivory only twofold the original concentration before herbivory¹²³ and PIs are practically absent from undamaged tobacco leaves and increase to high titer after induction.⁸²

It is generally believed that in condition where herbivory is regularly expected, constitutive defense is more adaptive, whereas in condition where herbivory is expected only once in a while, it is better for plants to produce defense substances when plants are damaged by herbivory, and when there are no herbivores, it is better to invest resource to plant growth rather than defense. This idea is supported by field experiments using plant disabled to induce defense substance by gene modification techniques, and comparing seed production between plant deficient in induction and the control type.¹²³

Induced defense reaction is also triggered by oviposition. When rice hoppers lay eggs on rice blades, exudates containing benzyl benzoate exude around the eggs and kill them.¹²⁵ Induction of defense substances triggered by insect herbivory is partly reproduced by mechanical damage¹²⁶ or application of insect saliva to the leaves.¹²⁷ Even though a single artificial damage using razor blade or pattern wheel may induce defense substance, it was recently found that continuous multiple damages better mimic the damage of insects and cause responses that are very close to real herbivory, and such continuous multiple damages and other types of damage, different in magnitude, speed, and patterns, can be made using a mechanical caterpillar, MecWorm that has been designed in a computer-programmed manner to very closely resemble the herbivore in damages caused to leaves.¹²⁸ In many plant species, insect damages induce a plant hormone, JA, that mediate the defense responses in plants, including induction of many defense substances (see Section 4.08.1.3.3(ii)).^{81,126} Spraying JA on plants can induce defense substances that are induced by insect feeding.^{65,81,126}

Recently, using model plants such as *A. thaliana*, tobacco, tomato, and maize, the profiles of gene induction after treatment such as insect feeding, JA application, and mechanical damage were intensively analyzed using gene technologies such as microarray techniques.^{129–131} Some of the induced gene products (proteins) may be involved in signal transduction, others in the synthesis of secondary metabolites involved in defense, and the rest may work as defense proteins. In practice, gene products of two genes that had been known to be induced by JA in tomato, namely arginase and threonine deaminase, were recently proven to function as defense proteins by degrading amino acids in diets and by decreasing the nutritive value¹⁰¹ (Figure 13). Similarly, it is likely in future that more novel defense proteins or proteins involved in the synthesis of secondary metabolites involved in defense would be found among the products of such genes induced by herbivory, mechanical damages, saliva treatment, or JA treatment.

4.08.1.3 Indirect Defense of Plants against Herbivores

Indirect defense of plants against herbivores is another type of defense that involves all the ways by which plants enhance the effectiveness of natural enemies of the herbivores. There are three types of indirect defense of plants against herbivores: (1) offering shelters to carnivores, (2) offering alternative foods to carnivores, and (3) attracting carnivores by emitting the so-called 'herbivore-induced plant volatiles (HIPVs)'. Because the focus of this chapter is on the role of chemicals in plant defense, the second and the third type of indirect defense will be discussed here.

4.08.1.3.1 Offering food to carnivores

Secretion of extrafloral nectar (EFN) is shown to act as an indirect defense against herbivores. EFN is used by carnivorous natural enemies such as ants. Some plant species, including lima bean plants, increase EFN in response to herbivory.^{132–135} Further, Heil¹³⁵ reported that the production of EFN by lima bean plants increased after the exogenous application of JA, which is an important plant hormone regulating a defense signaling pathway against herbivores and pathogens. The components of EFN have been reported in several plant species. For example, Choh *et al.*¹³⁶ reported EFN of lima bean plants contained fructose, glucose, and sucrose as major sugars. Interestingly, *T. urticae*-infested lima bean plants contained smaller amounts of sucrose and larger amounts of fructose in EFN than uninfested plants. Whereas EFN provides fuel for survival and search, plants can also provide predatory arthropods with alternative food containing nutrients (amino acids and lipids) required for development and reproduction. Central American Acacia trees stand out as a landmark example.¹³⁷ Apart from providing nesting sites (in enlarged, hollow stipular thorns) to certain ants and nectar from large foliar nectaries, they also produce protein- and lipid-rich organs called Beltian bodies on the leaf pinnules. These food bodies are eagerly harvested by foraging ants and fed to their larvae. The ants in turn kill insect herbivores, repel mammalian herbivores, and destroy plants interfering with the Acacia tree. A major question is how plants benefit from producing such nutritive foods, even when they cannot prevent organisms harmful to the plant from using it. For example, protein-rich pollen of several plants can be utilized by many predatory mites (e.g., *Iphiseius degenerans*), but also by the herbivorous arthropods they prey on (e.g., Western flower thrips, *Frankliniella occidentalis*). Despite the danger of herbivores reaping the benefits, plants still profit because any surplus prey boosts predator populations and because pollen are usually provided locally, which allows predators to occupy the site and repel herbivores. Thus, by the distribution of pollen over the plant, plants influence the degree to which predatory mites can monopolize pollen as an alternative food source at the expense of the herbivore. It is an entirely open question how plant-provided foods affect the motivation of predators to capture herbivores. Nutritive deficiencies in the alternative foods could stimulate the predators to maintain a predatory lifestyle.

4.08.1.3.2 Attracting carnivorous natural enemies by using herbivore-induced plant volatiles

Plants respond to herbivory by producing volatiles that in turn attract carnivorous natural enemies of the herbivores. These volatiles are produced by the plants as a specific response to herbivore damage or mainly as a result of mechanical damage. These so-called HIPVs attract carnivores, which in turn reduce the damage caused by herbivorous arthropods. These chemical alarm calls thus represent an example of induced indirect

defense of the plant against herbivores. HIPVs may vary quantitatively and/or qualitatively depending on herbivore species, plant species, and growth conditions of plants and attacking herbivores,^{1,2,138} suggesting that HIPVs may convey information on the status of damage of plants.

4.08.1.3.2(i) Tritrophic interactions of plant–spider mite–carnivorous natural enemy Spider mites (Tetranychidae) are polyphagous herbivores that have a severe impact on plants due to their explosive population dynamics in the absence of their carnivorous natural enemies such as predatory mites and predatory insects. In many plant–spider mite–carnivorous natural enemy interactions, infested plants attract natural enemies by producing a blend of volatiles – several terpenoids, phenolics, green leaf volatiles – that differ from the volatiles emitted from intact plants. A well-studied example is a tritrophic system consisting of lima bean plants (*Phaseolus lunatus*), herbivorous two-spotted spider mites (*Tetranychus urticae*), and predatory mites (*Phytoseiulus persimilis*).¹³⁹ In this system, infested lima bean leaves emitted more than 100 *T. urticae*-induced volatiles. Among them, linalool, (*E*)- β -ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and methyl salicylate attract the specialist predatory mite *P. persimilis*¹⁴⁰ (Figure 14). The generalist predatory mite *Neoseiulus californicus* also preferred volatiles from lima bean leaves infested with *T. urticae* than from intact lima bean leaves, but this predator was attracted by other volatiles such as linalool, methyl salicylate, (*Z*)-3-hexen-1-ol, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate¹⁴¹ (Figure 14). The predatory mite *N. womersleyi* is attracted only to previously experienced plant volatiles and not to volatiles they have not experienced.¹⁴² The predatory mites reared on *Tetranychus kanzawai*-infested tea leaves showed significant preference for a mixture of three synthetic compounds (mimics of the *T. kanzawai*-induced tea leaves volatiles: (*E*)- β -ocimene, DMNT, and (*E,E*)- α -farnesene)¹⁴² (Figure 14). However, mixtures lacking any of these compounds did not attract the predatory mites. Likewise, *N. womersleyi* reared on *T. urticae*-infested kidney bean plants showed a significant preference for a mixture of four synthetic compounds (mimic of the *T. urticae*-induced kidney bean plant volatiles: DMNT, methyl salicylate, β -caryophyllene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene)¹⁴² (Figure 14). The lack of any of the four compounds resulted in no attraction.

Figure 14 Volatile compounds that attract carnivorous natural enemies of herbivores.

4.08.1.3.2(ii) Tritrophic interaction of plant–herbivorous insects–parasitoid The volatiles emitted from plants infested with caterpillars and aphids can also attract parasitic wasps of the herbivore. For example, maize plants infested with the noctuid larvae *Mythimna separata* emit volatiles that attract a specialist parasitic wasp *Cotesia kariyai* when volatiles from intact plants or artificially damaged plants are the alternative. These attractive volatiles include terpenoids, green leaf volatiles, indole, oxime, and nitriles.¹⁴³ The composition of the volatiles is specific for the larval stage damaging the plant. Among them, a blend of four chemicals, geranyl acetate, β -caryophyllene, (*E*)- α -farnesene, and indole, elicited a response in naive *C. kariyai*, but making them to learn to associate these volatiles with a reward did not enhance this response.¹⁴⁴ A blend of five chemicals, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, β -myrcene, and linalool, which are known to be released not only from plants infested with the host larvae, but also from artificially damaged plants or undamaged ones, elicited little response in naive wasps, but making them to learn to associate these volatiles and a reward significantly enhanced the wasp's response.¹⁴⁴ Both the host-induced and nonspecific volatile compounds appear to be important for *C. kariyai* females in host location. A tritrophic system of the parasitoids *Cotesia marginiventris*, host larvae *Spodoptera* spp. and maize plants is also a well-studied system (see D'Alessandro *et al.*¹⁴⁵ and citation therein).

Cabbage plants infested with cabbage white butterfly larvae *P. rapae* emit volatiles that attract a parasitic wasp *Cotesia glomerata*.¹⁴⁶ In this system, the attraction is not specific; the wasp is also attracted to either artificially damaged cabbage plants or cabbage plants infested with nonhost larvae (*P. xylostella*).¹⁴⁶ In fact, *Cotesia glomerata* is attracted to products of the lipoxygenase pathway, that is, to green leaf volatiles such as (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, and (*Z*)-3-hexenyl acetate (Figure 14), that are induced in response to mechanical damage of leaf tissue.¹⁴⁷

Psylla-infested pear trees attract anthocorid predators by release of at least two volatiles, (*E,E*)- α -farnesene and methyl salicylate.¹⁴⁸ A similar example exists for whiteflies (*Trialeurodes vaporariorum*). They induce four *de novo* emitted volatiles in beans and three of these elicit oriented flight and landing of *Encarsia formosa* when offered in pure form, most effective being a mixture of (*Z*)-3-hexen-1-ol and 3-octanone.¹⁴⁹ Plants infested with aphids also emit induced volatiles that attract parasitic wasps. For example, *Aphidius ervi* females were attracted to volatiles emitted from broad bean plants infested with host aphid *Acyrtosiphon pisum*, but not to those from plants infested with nonhost aphid *Aphis fabae*.¹⁵⁰ 6-Methyl-5-hepten-2-one, linalool, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenol, (*E*)- β -ocimene, and (*E*)- β -farnesene separately attract the wasps¹⁵¹ (Figure 14). 6-Methyl-5-hepten-2-one is most attractive and is found in the headspace of host-infested plants but not in that of nonhost-infested plants.¹⁵¹ An aphid parasitoid *Diaeretiella parae* female is attracted to 3-benzyl isothiocyanate of host plant origin.¹⁵² Some parasitic flies are also attracted to plant volatiles. Borneol attracts a tachinid fly *Cyzenis albicans* in the field¹⁵³ (Figure 14). A parasitic fly *Exorista japonica* is attracted to maize plants infested with common armyworms (*Mythimna separata*),¹⁵⁴ but the chemical nature of the attractants is now under investigation.

4.08.1.3.2(iii) HIPVs affect neighboring plant's indirect defense In response to volatiles emitted from herbivore-infested plants (HIPVs), neighboring intact plants enhance either their direct defense (i.e., becoming a less suitable resource for herbivores^{155–158}) or their indirect defense (i.e., attracting carnivorous natural enemies of herbivores^{159–162}). Such responses of plants prior to biotic stress are called priming. A well-studied example of the latter case is a tritrophic system consisting of lima bean plants (*Phaseolus lunatus*), herbivorous two-spotted spider mites (*Tetranychus urticae*), and predatory mites (*Phytoseiulus persimilis*). Lima bean plants infested with *T. urticae* emit HIPVs that in turn attract *P. persimilis*.¹⁵⁹ When intact lima bean plants are exposed to volatiles from conspecific plants infested with *T. urticae*, they become more attractive to *P. persimilis*.¹⁵⁹ This increased attractiveness was explained by the fact that uninfested lima bean leaves that were exposed to volatiles from infested conspecific leaves could adsorb the volatiles and re-emit them.¹⁶¹ Choh *et al.*¹³⁶ further reported that HIPV-exposed intact plants produced more EFN compared to control plants (plants exposed to volatiles from intact plants). It remains unanswered how the mechanisms involved in the production of components of EFN differ between infested plants and HIPV-exposed plants.

Bate and Rothstein¹⁶³ showed that (*E*)-2-hexenal induced several defense-related genes such as *chalcone synthase*, *AOS*, and *lipoxygenase 2 (LOX2)* in *A. thaliana*. Arimura *et al.*¹⁶⁴ also showed that (*E*)-2-hexenal or

(Z)-3-hexenol induced genes encoding basic pathogenesis-related proteins (PRs), LOX, or phenylalanine ammonia lyase in lima beans. In addition, it has been reported that (*E*)-2-hexenal induced phytoalexin accumulation in cotton plants (*Gossypium hirsutum* L.). Recently, Gomi *et al.*¹⁶⁵ reported that (*E*)-2-hexenal induced genes encoding AOS, LOX, and HPL, and (*E*)-2-hexenal enhanced resistance of citrus tree (*Citrus jambhiri*) against *Alternaria alternata*. Vancanneyt *et al.*¹⁶⁶ also reported that antisense-mediated deletion of HPL in transgenic potato (*Solanum tuberosum* cv. Desiree) led to an increase in aphid performance. These findings suggest that C6-aldehydes function as signals to initiate defense responses of undamaged plants.

4.08.1.3.2(iv) Do entomopathogens and non-arthropod insectivores use HIPVs as a signal? Predatory arthropods (phytoseiid mites and heteropteran bugs) and parasitoid wasps have been best explored for their responses to HIPVs, whereas other classes of natural enemies have received little attention. Some major advances have been achieved recently with respect to entomopathogens. Van Tol *et al.*¹⁶⁷ were the first to show that indirect plant defenses also operate underground. They found that the roots of a coniferous plant (*Thuja occidentalis*) release chemicals upon attack by vine weevil larvae (*Otiorhynchus sulcatus*) and that these chemicals attract nematodes that parasitize weevils (*Heterorhabditis megidis*). Hountondji *et al.*¹⁶⁸ were the first to show that volatiles emanating from cassava plants infested with green mites (*Mononychellus tanajoa*) trigger production of conidia, the infectious stage, in different isolates of a mite-pathogenic fungus (*Neozygites tanajoae*), whereas volatiles from clean plants suppress conidiation. These opposing effects make sense in that the entomopathogenic fungus tunes the release of conidia, the stage most vulnerable to environmental conditions, to herbivore-induced plant chemicals that signal the presence of hosts.

4.08.1.3.3 Mechanisms involved in the production of herbivore-induced plant volatiles

There are several lines of evidence for an active role of the plant in releasing HIPVs: (1) whereas many natural enemies are attracted to plants harboring herbivores as prey, herbivorous arthropods and some of the products they deposit on the plant (e.g., feces, silk) can be eliminated as the source of volatiles found in blends of HIPV;¹⁶⁹ (2) the compounds identified in blends of HIPV have been reported to occur in plants;^{140,148,151,170} (3) pathways for biosynthesis of compounds in blends of HIPV exist in plants and are shown to be inducible by herbivory;^{170–172} (4) blends of HIPV are not only emanated from the leaf under attack by the herbivore, but are also systemically induced by the plant;^{173–175} (5) application of the phytohormone JA to wild-type plants or defense-signaling mutants (e.g., JA) leads to induction of volatiles similar to HIPV;^{176–181} (6) herbivore-induced gene expression patterns in plants are similar to those mediated by jasmonates;^{181–184} (7) elicitors of HIPV synthesis in plants have been found in the regurgitate/saliva of herbivores (*N*-(17-hydroxylinolenoyl)-L-glutamine or volicitin) (Figure 15),^{185,186} β -glucosidase,^{127,177} and inceptin (⁺ICDINGVCVDA[−]).¹⁸⁷

4.08.1.3.3(i) Insect factors affecting the production of HIPVs One of the striking features of HIPV as a means to attract natural enemies is that there are usually several compounds involved and that these mixtures of volatiles contain a great deal of specific information: the plant provides predators with information that is sufficiently specific to allow discrimination by olfaction. This hypothesis was confirmed by several studies.^{146,174,188–190} For example, De Moraes *et al.*¹⁸⁹ carried out a study on olfactory responses of the parasitoid *Cardiobiles nigriceps* to three species of host plants (tobacco, maize, and cotton) that were attacked by two closely related herbivore species, the tobacco budworm (*Heliothis virescens*) and the corn earworm (*H. zea*). These authors found that tobacco, cotton, and maize plants each released distinct volatile blends in response to damage by the two herbivore species, and that the parasitoid exploits these differences in odor blends to distinguish infestation by its host *H. virescens* from that by the nonhost *H. zea*.

Figure 15 Volicitin from the oral secretion of *Spodoptera exigua* larvae.

How do plants manage to respond differently to different herbivore species? One of the possible cues is in the oral secretion of herbivores. In fact, several classes of elicitors of insect origin have been isolated. For example, volicitin (*N*-(17-hydroxylinolenoyl)-L-glutamine) (**Figure 15**) has been shown to induce the production of HIPVs in maize when applied to wounded parts.¹⁸⁵ Volicitin is, however, not generally active. Leaves of lima bean, for example, do not respond to volicitin with the induction of volatile emission.¹⁹⁰ Other fatty acid conjugates have been isolated from the regurgitate of several caterpillars.^{191,192} For example, *N*-linolenoyl-glutamine in the regurgitate of the tobacco hornworm *M. sexta* was characterized as a potential elicitor of volatile emission in tobacco plants.¹⁸⁹

The other elicitor of insect origin that induces the production of HIPVs is β -glucosidase present in the oral secretion of cabbage white butterfly larvae *Pieris brassicae*.¹²⁷ Cabbage plants infested with *P. brassicae* larvae attract a parasitic wasp *C. glomerata*. Although this plant emits the same volatiles as those emitted when damaged mechanically, it does so in larger quantities and for a longer time. In this system, major volatiles involved are fatty acid derivatives. The elicitor β -glucosidase also induces the production of homoterpenes ((*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene) in lima bean plants and maize.¹⁷⁶ Recently, a disulfide-bridged peptide called inceptin (⁺ICDINGVCVDA⁻) was isolated from the oral secretion of *Spodoptera flugiperda* larvae.¹⁸⁷ Inceptin promotes ethylene production by cowpea and triggers an increase in the defense-related phytohormone salicylic acid and JA.

Although elicitors from insect oral secretions have received special attention, the quality and quantity of HIPVs may also be affected by insect feeding behavior such as continuous or interrupted leaf chewing, phloem sucking, and even egg deposition.^{193,194} Recently, it was shown that continuous mechanical wounding was sufficient to induce local as well as systemic emission of volatiles that are emitted as HIPVs.¹⁹⁵ Thus, the role of insect elicitors in HIPV production still remains largely an open question.

4.08.1.3.3(ii) Signaling pathways involved in the production of HIPVs in plants The oxylipin pathway in plants (**Figure 16**) is known to be involved in the production of HIPVs. In the oxylipin pathway, an LOX generates either 9- or 13-hydroperoxide from unsaturated fatty acids such as linoleic and linolenic acids.¹⁹⁶ It serves as a substrate for two main pathways in the oxylipin metabolism. One is the allene oxide synthase (AOS) pathway, which leads to the formation of JA and methyl jasmonate (MeJA) (**Figure 16**). JA and MeJA play essential roles both in the responses against biotic/abiotic stresses and in plant development.¹⁹⁷ Further, JA and MeJA are involved in the production of compounds regarded as HIPVs. Hopke *et al.*¹⁷⁶ reported that most of *T. urticae*-induced volatiles could be triggered by treatment of lima bean plants with solutions of JA. Dicke *et al.*¹⁹⁸ also reported that damage by *T. urticae* and application of JA had similar, although not identical, effects on the composition of induced volatiles in lima bean leaves and the attraction of a carnivore (*Phytoseiulus persimilis*). Ozawa *et al.*¹⁷⁷ reported that in lima bean leaves, the JA-related signaling pathway is involved in the production of volatiles induced by armyworms (*M. separata* and *S. exigua*). Furthermore, in corn plants (*Z. mays*), JA triggers the emission of all volatiles that are known to be emitted in response to damage by *S. exigua*.¹⁷⁶

JA and ethylene act synergistically in response to wounding.¹⁹⁹ Arimura *et al.*²⁰⁰ found that lima bean plants infested with two-spotted spider mites (*T. urticae*) showed emission of ethylene. Synergistic effects of ethylene on JA-induced volatile production in lima bean leaves are reported.²⁰¹

Another pathway is the fatty acid hydroperoxidase lyase (HPL) pathway. This pathway produces C6-aldehydes and C12-oxo acids.²⁰² C6-volatiles, including (*E*)-2-hexenal, (*Z*)-3-hexenal, hexanal, as well as their corresponding alcohols or esters, are produced from mechanically wounded plant tissue.^{197,203} C6-aldehydes are also formed during hypersensitive response to infection by bacterial pathogens, after insect feeding, and after exogenous application of JA.^{204,205} As already shown in this chapter, some of C6-volatiles are known to be attractants of parasitoids and predators.

Salicylic acid (SA) is an endogenous signal implicated in eliciting plant resistance. SA is known to act as a signal for systemic acquired resistance (SAR) in pathogen-infected plants.²⁰⁶ SA levels in resistant tobacco increased in both the tobacco mosaic virus (TMV)-inoculated and uninoculated leaves of the same plant; the rise in SA levels preceded the induction of PR (Pathogenesis Related) genes in the uninoculated leaves. Exogenously supplied SA also induces the same genes as those that are activated systemically upon TMV infection.²⁰⁷ Ozawa *et al.*¹⁷⁷ showed that gaseous treatment of MeSA induces some of HIPVs in lima bean leaves. They suggested that both SA signaling pathway and JA signaling pathway are involved in the production of *T. urticae*-induced volatiles.

Figure 16 Phytooxylipin pathway.

4.08.1.3.3(iii) Cross talks Ozawa *et al.*¹⁷⁷ observed that the *T. urticae*-specific blend of HIPV from lima bean plants is different from that induced by exogenous JA. They discussed the possibility that this specificity arises from the interaction between JA and SA signaling pathways in *T. urticae*-infested leaves. In general, the upregulation of SA biosynthesis suppresses the production of JA, which is required for the general stimulation of induced volatile biosynthesis. Thus, the intensity of upregulation of SA biosynthesis might cause the blend of HIPVs in *T. urticae*-infested lima bean leaves to be unique. The cross talk between JA biosynthesis and SA biosynthesis caused by herbivory could be one of the important mechanisms for the production of herbivore-specific blend of HIPV. Specificity of HIPV blends arises especially from cross talk and feedback between different signaling pathways.

4.08.1.4 Coordination of Direct and Indirect Defense Responses

Effective plant defense requires coordination of direct and indirect defenses such that they do not interfere or even act synergistically. A relevant example comes from studies by Baldwin,²⁰⁸ who wondered why the nicotine defense of wild tobacco is downregulated by an ethylene burst following attack by young tobacco hornworm larvae. He formulated three – as yet untested – hypotheses to explain this.

First, the hornworm larvae may suppress the plant's nicotine response or they may reduce their food intake and therefore run less risk to alert the plant's nicotine response.

Second, the tobacco plant may improve the impact of its indirect plant defenses by reducing possibilities for hornworm larvae to sequester nicotine as a defense against predators and parasitoids, and by slowing down hornworm growth (through production of digestion inhibitors), thereby causing prolonged exposure of hornworm larvae to their predators. In this way, the plant saves fitness costs from investment in nicotine defense and gains more protection from indirect defense.²⁰⁹

Third, the plant may suppress the nicotine response until the hornworm larvae reach a size (i.e., fourth or fifth instar) where they impose a serious death risk to the tobacco plant and then switch to produce nicotine to make these large larvae move to their neighboring competitors.²¹⁰

These three hypotheses – herbivore stealth, indirect defense optimization, and herbivores as allies in plant competition – do not necessarily exclude each other. If the plant first chooses to optimize indirect defense against young hornworm larvae, it may switch to direct defense by the time the hornworm larvae are large enough, thereby deterring the larvae and imposing immediate and present danger to neighboring (=competing) plants. This defense scenario is not likely to be general because the best scenario depends on the efficacy of alternative defenses. There are examples of plants switching on direct defenses in response to feeding by caterpillars even though this reduces the suitability of the caterpillars as hosts for parasitic wasps.^{211,212}

4.08.2 Antimicrobial Chemical Defense

4.08.2.1 Introduction

Higher plants produce a remarkably diverse array of secondary metabolites that are involved in defense against pathogens,⁴ although antimicrobial proteins such as PRs²¹³ are also involved in defense. Antimicrobial secondary metabolites can be preformed in the plant, the so-called 'constitutive antimicrobial compounds', or they are induced after infection through *de novo* enzyme synthesis, the 'phytoalexins'.²¹⁴ It is often difficult to determine whether compounds are phytoalexins or constitutive antimicrobial compounds, as the distinction between them is not always clear. In addition, some compounds are phytoalexins in one organ and constitutive in another organ of the same plant species. Some of these secondary metabolites are implicated in defense against not only microbial attack, but also herbivore/animal predation. Several books and reviews on constitutive antimicrobial compounds and/or phytoalexins have been published.^{4,214–219} This section will focus mainly on the chemical structures and distribution of constitutive antimicrobial compounds and phytoalexins in higher plants. In addition, recent findings from biosynthetic studies of antimicrobial compounds will be summarized.

4.08.2.2 Constitutive Chemical Defense

The first chemical barrier to microbial infection is the constitutive presence of antimicrobial substances that occur in sufficient concentrations to represent a barrier to infection. The constitutive antimicrobial compounds are largely divided into two groups, preinfection plant metabolites, which are normally present in concentrations high enough to inhibit the growth of pathogens, and antimicrobial metabolites produced by plants in response to infection, but whose formation does not involve *de novo* biosynthetic enzyme synthesis. The latter metabolites are normally present in plants in an inactive, bound form, but are converted into the active form after infection by means of a short and simple biochemical reaction such as enzymatic hydrolysis. VanEtten *et al.*²²⁰ suggested that all these constitutive antimicrobial compounds be called 'phytoanticipins', which can be defined as low-molecular-weight antimicrobial compounds that are present in plants before challenge by microorganisms or are produced after infection solely from pre-existing constituents. The structurally diverse array of constitutive antimicrobial compounds can be classified into several groups such as terpenoids, nitrogen-containing compounds, and aromatics.

Terpenoids: In *Nicotiana* spp. (Solanaceae), the diterpenoids sclareol, episclareol, and 2-ketoepimanool (Figure 17) on the leaf surface have been identified as constitutive antimicrobial compounds.²²¹ Another series of diterpenoids, α - and β -4,8,13-duvatriene-1,3-diol (Figure 17), have been identified from *N. tabacum* leaves, where they appear to play a role in resistance to blue mold.^{214,222} Diterpenoid resin acids such as 7-ketodehydroabietic acid and 7-hydroxyabietic acid have been identified from the needles of *Pinus radiata* (Pinaceae) (Figure 17). These compounds inhibit both spore germination and mycelial growth of a pine pathogen.²¹⁴ The diterpenoid momilactones A and B accumulate in rice seeds,²²³ although they are induced in rice leaves as phytoalexins,²²⁴ as described in Section 4.08.2.3. In rice, the antimicrobial diterpenoid oryzalide A (Figure 17) and related compounds have been identified in healthy rice leaves, but were found to further increase after inoculation with the pathogen *Xanthomonas campestris* pv. *oryzae*.²²⁵ The constitutive antimicrobial triterpenoids cucurbitacin I (Figure 17) and meliacins have been identified in *Ecballium elaterium* (Cucurbitaceae) and *Chisocheton paniculatus* (Meliaceae), respectively.²¹⁴

Figure 17 Structures of some constitutive antimicrobial terpenoids.

Saponins are an important source of constitutive antimicrobial triterpenoids. The antimicrobial activity is generally correlated with the sugar moiety glycosylated to the 3-hydroxyl of the triterpenoids. The ivy *Hedera helix* (Araliaceae) produces two related saponins, hederasaponins B and C, which are stored in the cell vacuoles. When ivy leaves are damaged by pathogen infection, these two saponins undergo partial hydrolysis, with loss of the sugars attached to the 26-carboxylic acid group, to yield α - and β -hederins, which are highly toxic to pathogens. Further loss of sugar at the three positions of α - and β -hederin results in sapogenins, which are completely inactive (**Figure 18**).²¹⁴ In addition to the ivy saponins, triterpenoid saponins have been identified from many species, including oat (*Avena sativa*), *Dolichos kilimandscharicus* (Leguminosae), *Rapanea melanophloeos* (Myrsinaceae), *Primula sieboldii* (Primulaceae), and *Camellia japonica* (Theaceae).²¹⁴

Nitrogen-containing compounds. Many alkaloids showing activity against human pathogens have been identified. Although it remains almost unknown whether these alkaloids play a role in defense of the host plant against pathogens, some show activity against plant pathogens: for example, the indole alkaloid gramine in barley leaves (*Hordeum vulgare*) (**Figure 19**) and quinolizidine alkaloids in Leguminosae species.²¹⁴

Figure 18 Release of sugars by hydrolysis from the hederasaponins B and C.

Figure 19 Structures of some *N*-containing, constitutive antimicrobial compounds.

The cyclic hydroxamic acid 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and its precursor 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) (Figure 19) form part of the defense against microbial pathogens and insects in the Gramineae. DIBOA is the main hydroxamic acid in rye (*Secale cereale*), whereas DIMBOA is the predominant form in wheat (*Triticum aestivum*) and maize (*Z. mays*).²²⁶ The hydroxamic acids, while not present in the seeds of cereals, appear upon germination in maize, wheat, and rye. Their levels increase with age to reach a maximum a few days after germination in maize and wheat, and are higher in the stem than in the leaf tissue.²²⁷ Five genes, *Bx1*–*Bx5*, that are involved in DIBOA biosynthesis are localized to a 6-cM region on the short arm of chromosome 4 in maize (Figure 20).²²⁶

Glucosinolates (Figure 19) are nitrogen- and sulfur-containing anionic natural products that have been reported almost exclusively from the order Brassicales, containing Brassicaceae. They produce toxic products such as isothiocyanates, thiocyanates, and nitriles upon hydrolysis by endogenous myrosinases, as described in Section 4.08.1.2. In some cases, resistance to pathogens is associated with glucosinolate content, but some pathogens do not cause enough cell damage to activate the glucosinolate–myrosinase system.²²⁸ Glucosinolates are biosynthesized from aliphatic or aromatic amino acids. In *A. thaliana*, indole glucosinolates are biosynthesized from tryptophan via indole-3-acetaldoxime, which is also a biosynthetic intermediate of the phytoalexin camalexin.²²⁹

Cyanogenic glycosides, which are widely distributed in higher plants, are a bound form of toxic hydrogen cyanide, which is released from the glucoside following enzyme hydrolysis.²¹⁴

Aromatics: Several flavonoids, including pinocembrin in *Populus deltoides*, sakuranetin in black currant bushes (*Ribes nigrum*, Grossulariaceae),²³⁰ and 6-isopentenylnaringenin in the hop plant (*Humulus lupulus*), are present on the leaf surface and exhibit antimicrobial activity (Figure 21). The flavanone sakuranetin occurs constitutively in the leaves of black currant, but is induced as a phytoalexin in rice leaves (*Oryza sativa*, Gramineae), as described in Section 4.08.2.3. Flavan-4-ols occur in grains and leaves of *Sorghum*, and a positive correlation has been found between flavan-4-ols and disease resistance.²¹⁴ The isopentenyl isoflavonoids luteone and wightone (Figure 21) occur on the leaf surface of *Lupinus* species (Leguminosae).²¹⁴ Stilbene glucosides such as astringin (5,3',4'-trihydroxystilbene-3 β -D-glucoside) and rhaponticin (5,3'-dihydroxy-4'-methoxystilbene-3 β -D-glucoside) occur in the bark of sitka spruce (*Picea sitchensis*, Pinaceae) and are antimicrobial themselves,

Figure 20 Biosynthetic pathway of DIMBOA in *Zea mays*.

Figure 21 Structures of some constitutive antimicrobial flavonoids.

while their aglycones have much higher activity.²³¹ In the Rosaceae, the majority of plants are devoid of phytoalexins; instead, many contain catechin-like phenolic compounds with antimicrobial activity.²¹⁴

Others: Alkadienals and a number of epoxy and hydroxyl linoleic and linolenic acids have been identified from wheat and rice leaves, respectively.²¹⁴

Acetylenic compounds such as safynol are constitutive in some species of Compositae and Umbelliferae, and are phytoalexins in other species (see Section 4.08.2.3). Antimicrobial long-chain alcohols, some of which are acetylenic, have been identified in the skin of immature avocado fruits (*Persea americana*, Lauraceae).²¹⁴

4.08.2.3 Phytoalexins

The term 'phytoalexin' was originally coined by K. O. Muller for plant-formed antibiotics that are synthesized *de novo* after the plant tissue is exposed to microbial infection.²³² A more recent consensus definition has gained general acceptance: phytoalexins are low-molecular-weight antimicrobial compounds that are synthesized by and accumulated in plants after exposure to microorganisms.^{220,233} Production of phytoalexins is also induced by abiotic elicitors, including heavy metals and biosurfactants, biotic elicitors such as pathogen-derived constituents (the so-called pathogen-associated molecular patterns), and ultraviolet (UV) light.

The pterocarpan pisatin was first isolated and characterized as a phytoalexin in *Pisum sativum* in the early 1960s.^{234,235} Since then, structurally diverse phytoalexins have been reported from more than 30 families of higher plants. Much progress is also being made in studies on phytoalexin biosynthesis based on genomic information.

Terpenoids: The sesquiterpenoid capsidiol has been identified as a major phytoalexin from several Solanaceae species, including *Nicotiana tabacum* and *Capsicum annuum*^{236,237} (Figure 22). The sesquiterpene hydrocarbon 5-*epi*-aristolophene, a biosynthetic precursor of capsidiol, is biosynthesized from farnesyl diphosphate by the action of 5-*epi*-aristolophene synthase (EAS) and is sequentially hydroxylated at C1 and C3 to form capsidiol.²³⁸ Two EAS genes (*EAS1* and *EAS2*) and a P-450 gene encoding 5-*epi*-aristolophene-1,3-dihydroxylase (*CYP71D*) were functionally identified from cDNA libraries prepared from elicitor-induced tobacco cells, with both *EAS* and *CYP71D* being elicitor-inducible.^{237,239} In potato (*S. tuberosum*), production of sesquiterpenoid phytoalexins, including rishitin, is induced in response to elicitor treatments and pathogen infection.²³⁶ In pathogen-infected sweet potato (*Ipomoea batatas*) root tissue, various furanosesquiterpene phytoalexins, including ipomeamarone, have been identified²⁴⁰ (Figure 22). The sesquiterpenoid phytoalexin lettuceenin A was reported from lettuce, *Lactuca sativa* (Compositae) (Figure 22).²¹⁴

Figure 22 Structures of some sesquiterpenoid phytoalexins.

In *Gossypium* spp. (Malvaceae), sesquiterpenoid phytoalexins identified so far are cadinene derivatives that are biosynthesized from δ -cadinene.²⁴¹ CAD1-A and CAD1-C were functionally identified as sesquiterpene cyclases that catalyze the conversion of farnesyl diphosphate into δ -cadinene^{242,243} (Figure 23). GaWRKY1 is a transcription factor involved in elicitor-inducible *CAD1-A* expression.²⁴¹ Gossypol, a major phytoalexin in *G. arboreum*, is likely to be biosynthesized via 8-hydroxy- δ -cadinene (Figure 23).²⁴⁴ CYP706B1 was functionally identified as a cytochrome P-450 enzyme that catalyzes the conversion of δ -cadinene into 8-hydroxy- δ -cadinene, with *CYP706B1* being highly elicitor-inducible in suspension-cultured cotton cells.²⁴⁴

In rice (*Oryza sativa*), 15 compounds have been identified as phytoalexins in leaves infected with the blast fungus *Magnaporthe grisea* or on UV irradiation. Except for the flavanone sakuranetin (Figure 21), they are all diterpenoids. The rice diterpenoid phytoalexins are classified into four groups based on their basic carbon frameworks: phytocassanes A–E,^{245–247} oryzalexins A–F,^{248–250} momilactones A and B,^{223,224} and oryzalexin S (Figure 24).²⁵¹ By analogy with known biosynthetic pathways of polycyclic diterpenes such as

Figure 23 Putative biosynthetic pathway of a sesquiterpenoid phytoalexin, gossypol.

Figure 24 Structures of rice diterpenoid phytoalexins.

gibberellins,²⁵² the common precursor of these molecules, geranylgeranyl diphosphate (GGDP), is postulated to be sequentially cyclized via *ent*-copalyl diphosphate (*ent*-CDP) to *ent*-cassa-12,15-diene and *ent*-sandaracopimaradiene, leading to phytocassanes A–E and oryzalexins A–F. GGDP is also cyclized via *syn*-CDP to 9 β H-pimara-7, 15-diene and stemar-13-ene, leading to momilactones A and B and oryzalexin S. By utilizing the information from the rice genome database that was recently opened to the public, six diterpene cyclases have been demonstrated to be involved in the conversion of GGDP into the four diterpene hydrocarbon precursors via *ent*- or *syn*-CDP. OsCPS2 (OsCyc2) and OsCPS4 (OsCyc1) catalyze the conversion of GGDP into *ent*-CDP and *syn*-CDP,^{253,254} and OsKSL7 (OsDTC1), OsKSL10 (OsKS10), OsKSL4 (OsKS4), and OsKSL8 (OsDTC2) catalyze the conversion of *ent*-CDP or *syn*-CDP into the four diterpene hydrocarbons *ent*-cassa-12,15-diene, *ent*-sandaracopimaradiene, 9 β H-pimara-7,15-diene, and stemar-13-ene, respectively (Figure 25).^{255–258} These diterpene cyclase genes have also been shown to be induced by irradiation of rice leaves with UV light and by treatment of suspension-cultured rice cells with a chitin oligosaccharide elicitor, with the accumulation of all of their mRNAs peaking at 4–8 h after elicitor treatment.^{255,257,259} It should be noted that *OsCPS1* encodes an *ent*-CDP synthase involved in the biosynthesis of GAs, and that expression of the *OsCPS1* transcript was not induced either by UV light in the rice leaves²⁵³ or by a chitin oligosaccharide elicitor in suspension-cultured rice cells.²⁵⁴

It has been suggested that cytochrome P-450s are involved in the downstream oxidation of diterpene hydrocarbons, leading to the bioactive phytoalexins, by analogy to known biosynthetic pathways for the diterpenoid gibberellins.²⁵⁸ On chromosome 2 of the rice genome, *OsCPS2* and *OsKSL7* are closely located. Similarly, on chromosome 4, *OsKSL4* is located near *OsCPS4*. In addition, several chitin oligosaccharide-inducible P-450s have been found near the cyclase genes on chromosomes 2 and 4.²⁵⁶ In fact, it was shown that a 168-kb gene cluster on chromosome 4 encodes, in addition to *OsCPS4* and *OsKSL4*, momilactone A synthase (a dehydrogenase named *OsMAS*) and two cytochrome P-450s, either or both of which are involved in momilactone biosynthesis.²⁶⁰ These results suggest that phytocassane and momilactone biosynthesis genes are clustered on chromosomes 2 and 4, respectively. Phytocassanes and momilactones are major representatives of four distinct types of diterpenoid phytoalexins in rice. Although the biological significance of gene clusters in the synthesis of secondary metabolites is not clear, such gene clusters might contribute to efficient coordinated expression of the genes after elicitation, followed by production of high levels of diterpenoid phytoalexins.

Figure 25 Biosynthetic pathways of gibberellins and diterpenoid phytoalexins in rice.

Figure 26 Structures of some *N*-containing phytoalexins.

Nitrogen-containing compounds: Anthranilate derivatives, such as avenanthramides, and avenalmin I, II, and III have been reported in *A. sativa* (Gramineae) (Figure 26).^{261,262}

In the Brassicaceae, sulfur-containing indole phytoalexins such as brassinin, camalexin, and their derivatives have been identified (Figure 26).²¹⁴ Within this family, *A. thaliana* is a useful plant to investigate biological roles and regulatory mechanisms of phytoalexins, because the plant appears to produce only camalexin. In addition, the *Arabidopsis* genome database is open to the public, and useful genetic tools such as mutant lines and gene chips are readily available for *A. thaliana*.

Camalexin was originally isolated from the leaves of the crucifer *Camelina sativa* infected with *Alternaria brassicae*.²⁶³ Infection of *A. thaliana* leaves with both biotrophic and necrotrophic pathogens induces camalexin production.²²⁹ Camalexin originates from tryptophan via indole-3-acetaldoxime (IAOx) and (*S*)-dihydrocamalexic acid (Figure 27). IAOx is also an intermediate of the indole glucosinolates that are constitutively formed in *A. thaliana* and the auxin indole-3-acetic acid (IAA). Synthesis of IAOx from tryptophan is catalyzed by P-450 enzymes CYP79B2 and CYP79B3.²⁶⁴ Expression of the CYP79B2 transcript was elicitor (silver nitrate)-inducible, but that of the CYP79B3 transcript was not elicitor-inducible. A *cyp79B2/cyp79B3* double knockout mutant was shown to be devoid of both camalexin and indole glucosinolates, and could only synthesize reduced levels of IAA.²⁶⁵ The camalexin-deficient mutants *pad1-pad5* were isolated^{266,267}

Figure 27 Biosynthetic pathway of camalexin.

and *PAD3* was shown to encode the P-450 enzyme CYP71B15, which catalyzes the last step in camalexin biosynthesis, the conversion of (*S*)-dihydrocamalexic acid into camalexin.²⁶⁸ *PAD2* was shown to encode γ -glutamylcysteine synthetase 1 involved in glutathione biosynthesis,²⁶⁹ suggesting the involvement of glutathione in camalexin biosynthesis, either as a regulatory component or as a biosynthetic precursor of the thiazole ring moiety of camalexin. *PAD4* encodes a lipase-like protein²⁷⁰ that acts upstream from SA to affect expression of the pathogenesis-related protein gene *PR-1* and camalexin synthesis.²⁷¹ The cytochrome P-450 genes *CYP79B2* and *CYP79B15* (*PAD3*) involved in camalexin biosynthesis and the tryptophan biosynthetic gene *ASA1* (anthranilate synthase) are transcriptionally induced by *P. syringae* infection. Camalexin synthesis and the induction of *CYP79B2*, *PAD3*, and *ASA1* were strictly colocalized with the infection site of *Alternaria alternata*, a potent camalexin inducer.²⁷²

Aromatics: In the Leguminosae, most phytoalexins are isoflavonoids. These phytoalexins have been identified mainly in the leaves of herbaceous members of the Leguminosae, and include pisatin, kievitone, and glyceollin I in *Pisum sativum*, *Phaseolus vulgaris*, and *Glycine max*, respectively (**Figure 28**). Pisatin is biosynthesized from 6a-hydroxymaackian by 6a-hydroxymaackian 3-*O*-methyltransferase (HMM). Wu *et al.*²⁷³ reported isolation and characterization of two HMM cDNA clones (pHMM1 and pHMM2) created from RNA obtained from pathogen-infected pea tissue; the deduced amino acid sequences of HMM1 and HMM2 were highly homologous to each other.

Gramineae species also produce flavonoid phytoalexins. The flavanone sakuranetin (**Figure 21**) is a major phytoalexin in rice leaves,²⁷⁴ although this compound is constitutive in black currant leaves, as described in Section 4.08.2.2. *S. bicolor* produces 3-deoxyanthocyanidins such as luteolinidin and apigeninidin (**Figure 29**) in response to fungal infection.²⁷⁵

In carrot, *Daucus carota* (Umbelliferae), 6-methoxymellein was identified as a phytoalexin (**Figure 29**). It is biosynthesized by 6-hydroxymellein-*O*-methyltransferase from 6-hydroxymellein, which is biosynthesized from 1 mol of acetyl coenzyme A (CoA) and 4 mol of malonyl-CoA by a polyketide biosynthetic enzyme, 6-hydroxymellein hydroxylase.^{276,277}

Hydroxystilbenes such as resveratrol (**Figure 29**) have been identified in several unrelated families, including the grapevine *Vitis vinifera* (Vitaceae), peanut *Arachis hypogaea* (Leguminosae), *Veratrum grandifolia*

Figure 28 Structures of some isoflavonoid phytoalexins.

Figure 29 Structures of some typical aromatic phytoalexins.

(Liliaceae), and *Festuca versuta* (Gramineae). Constitutive overexpression of a grapevine stilbene synthase gene in tobacco and alfalfa resulted in increased resistance to pathogens.^{219,278}

In the banana *Musa acuminata* (Musaceae), a series of novel phenalenone-type compounds, including irenolone and 2-(4'-hydroxyphenyl)-1,8-naphthalenedicarboxylic anhydride, have been identified (Figure 29).^{279,280} In the Compositae, acetophenone derivatives in yacon (*Polymnia sonchifolia*)²¹⁴ and the coumarins scopoletin and ayapin in sunflower (*Helianthus annuus*)²⁸¹ have been reported (Figure 29).

Others: In the Compositae, acetylenic compounds such as safynol and dehydrosafynol in safflower (*Carthamus tinctorius*)²⁸² and (*E*)- and (*Z*)-mycosinol in corn marigold (*Coleostephus myconis*)²⁸³ have been reported (Figure 30).

4.08.2.4 Chemical Defenses of Plants

Antimicrobial plant metabolites that function as chemical barriers against pathogens are divided into constitutive compounds and phytoalexins. These two types of chemical barriers are likely to cooperatively function as defense systems against pathogens, together with other defenses. Although induction of phytoalexin production under stressed conditions, including pathogen infection, is general within the flowering plants, and a variety of compounds have been identified as phytoalexins from over 30 families, there are also many plants in which phytoalexin production is not induced. For example, a survey of Rosaceae leaf tissue indicated a relatively low frequency of phytoalexins, with no more than 15% of species forming phytoalexins. Constitutive barriers might be well developed in such leaves.²¹⁴ In the Cucurbitaceae, phytoalexins have not been reported, but these plants appear to respond to pathogen infection by SAR, in which the resistance is provided by antimicrobial proteins, including PRs,²¹⁶ although SAR is also a general defense mechanism throughout the higher plants.

Figure 30 Structures of some acetylenic phytoalexins.

Several model plants, including *O. sativa*, *A. thaliana*, *Z. mays*, *G. max*, and *Medicago truncatula*, for which genome information is currently available,²¹⁹ are a rich source of antimicrobial metabolites. Genetic and reverse genetic approaches are providing evidence for the biological importance of antimicrobial compounds in host defense mechanisms.

4.08.3 Phytotoxins

4.08.3.1 Introduction

Microbial pathogenesis in plants is an intricate developmental process requiring biological components found in most microorganisms, as well as factors that are unique to microbial species that participate in particular microorganism–plant interactions. Toxic substances isolated from plant pathogens are often called phytotoxins. In many cases, it is known that phytotoxins play an important role in disease development causing chlorosis, necrosis, or wilting.^{4,284} Because of agricultural importance, crop–phytopathogenic microbe interaction is studied extensively and is one of the well-studied examples of plant–microbe relationship. In some cases, phytotoxins also participate in particular microorganism–plant interactions. This type of phytotoxin is called host-specific or host-selective toxin (HST)²⁸⁵ to distinguish from host nonselective toxin. HST reproduces the symptom of disease and the ability to produce phytotoxins is strongly related to virulence. The phytotoxins are usually isolated from fermentation media with the guidance of appropriate bioassay using host plants. In the last decade, significant progress has been made in elucidating phytotoxin biosynthetic genes, which are usually clustered on chromosomes. The study on biosynthetic gene cluster of phytotoxins provides information on transcriptional regulation of gene expression, transport of pathway products, and self-resistance mechanism.

Similar to other natural products, phytotoxins are classified on the basis of their structural types considering their biosynthetic pathways. Among various phytotoxin families, polyketides including aromatic and reduced polyketides, peptides including diketopiperazines, and terpenes are most frequently described in the literature. In this section, representative phytotoxins that are well-characterized biosynthetically and physiologically will be introduced.

4.08.3.2 Polyketides

Fungal polyketide synthase (PKS)²⁸⁶ is a large protein and consists of a single set of module containing a keto synthase (KS), an acyltransferase (AT), a thioesterase/Claisen cyclase (TE/CYC), and two acyl carrier protein (ACP) domains. This enzyme catalyzes the condensation of malonyl CoA and enzyme-bound acyl group to afford complex fatty acid possessing methyl substituent and various functional groups. Interestingly, fungal type-I PKS produces both aromatic and reduced polyketides contrary to bacterial PKSs, which are divided into type-I and type-II for reduced and aromatic ones.

Cochliobolus heterostrophus (*Helminthosporium maydis*) race T, the causal agent of southern corn leaf blight, produces polyketol of a long carbon chain polyketide (C41) T-toxin (or HMT-toxin).²⁸⁷ High virulence of this fungus on T-cytoplasm maize is responsible for the production of host-selective toxin T-toxin. Production of T-toxin requires PKS1 and a decarboxylase DEC1.²⁸⁸ Recently, the second PKS gene (PKS2) has been identified. It is speculated that rather long backbone of T-toxin is constructed by two separate PKSs using one of the products as a starter unit of the second PKS (Figure 31).

Cercosporin²⁸⁹ is a light-activated, nonhost-selective toxin produced by many *Cercospora* fungal species. The dimeric perylenequinone structure indicates that cercosporin is a typical aromatic polyketide biosynthesized by iterative type-I PKS.²⁹⁰ This toxin acts as a photosensitizer that activates molecular oxygen in the presence of light,²⁸⁹ and the resultant reactive oxygen species causes oxidative damage to cells. Studies on toxin-deficient mutants and on the involvement of light in symptom development have demonstrated the importance of this toxin in diseases by *Cercospora* pathogen. Interestingly, light is the primary signal to trigger cercosporin biosynthesis.

Another fungal pathogen *Alternaria* produces a variety of nonhost-selective phytotoxins²⁹¹ such as aromatic polyketides (alternariol, zinniol) and reduced polyketides (alternaric acid, solanapyrones). Alternaric acid²⁹² isolated from *Alternaria solani* shows unique physiological effects on the hypersensitive cell death of potato

Figure 31 Structures of polyketide

cells.²⁹³ The causal fungi of potato early blight *Alternaria solani* produce solanapyrones,²⁹⁴ which are also known as specific inhibitors of DNA polymerase β .²⁹⁵ Solanapyrone is the first demonstrated example biosynthesized via enzymatic Diels–Alder reaction.²⁹⁶ Recently, a biosynthetic gene cluster of solanapyrones has been identified²⁹⁷ (Figure 32).

In addition to those host nonselective toxins, *Alternaria* pathogens produce a number of host-selective toxins such as AK-toxin, AF-toxin, and ACT-toxin.²⁹⁸ 9,10-Epoxy-8-hydroxy-9-methyldecatrienoic acid, the common backbone of these toxins, was biosynthesized via the polyketide pathway. Genetic approach has allowed the identification of a gene cluster for AF-toxin biosynthesis.²⁹⁹

Figure 32 Structures of polyketide phytotoxins (continued).

Host-selective sphinganine-like phytotoxin AAL-toxin²⁹⁸ produced by *Alternaria alternata* f. sp. *lycopersici*, a causal fungus of tomato stem canker disease, reproduces symptoms similar to those of the disease for susceptible genotype of tomato leaf in concentrations less than 10 ng ml⁻¹.³⁰⁰ Interestingly, AAL-toxin and its structural analogue fumonisin, which is known as a mycotoxin, are phytotoxic to tomato and are cytotoxic to cultured mammalian cells due to apoptosis induced by inhibition of ceramide synthase.³⁰¹ Similar overlapping toxicity between plants and animals has been reported in the case of phytotoxins (and also mycotoxin) such as tricothecenes³⁰² and cytochalasins.³⁰³

Nonhost-selective toxin coronatine was originally isolated as a chlorosis-inducing factor from Italian ryegrass infected with *Pseudomonas syringae* pv. *atropurpurea*³⁰⁴ and contributes to virulence in several host-pathogen interactions.³⁰⁵ Later, it was found that coronatine showed very similar, but stronger, activities to that of the phytohormone JA.³⁰⁶ Its potency of biological activities is attributed to fixing the *cis* configuration of cyclopentane ring, which is easy to isomerize *trans* in the case of JA.

The biosynthetic pathway of coronatine has been extensively studied. The coronafacic acid moiety is proposed to be constructed by bacterial modular type-I PKS via 5-oxocyclopentene carboxylic acid. A recent study clarified the detailed mechanism of the formation of highly unusual cyclopropane amino acid via D-3-chloroalloisoleucine (enzyme-bound form).³⁰⁷

4.08.3.3 Nonribosomal Peptides

Fungi and bacteria produce various phytotoxic cyclic peptides using nonribosomal peptide synthetase (NRPS).³⁰⁸ Similar to modular organization of PKS, NRPS consists of several modules containing condensation (C), adenylation (A), and thiolation (T) domains.

Alternaria alternata apple pathotype (previously described as *A. mali* Roberts) causes *Alternaria* blotch of susceptible apple cultivars through production of a cyclic peptide host-selective toxin, AM-toxin, whose complete structure has been determined for the first time among host-specific toxins.²⁹⁸ Disruption of AM-toxin synthetase (AMT) gene resulted in toxin-minus mutants, which were also unable to cause disease symptoms in susceptible apple cultivars, indicating that AMT is a primary determinant of virulence and specificity in the *A. alternata* apple pathotype³⁰⁹ (Figure 33).

Figure 33 Structures of nonribosomal peptide phytotoxins.

Maize *Helminthosporium* leaf spot is caused by *Cochliobolus carbonum* race 1, a fungal pathogen whose infection is dependent on the production of a cyclic depsipeptide HC-toxin. HC-toxin consists of a family of four related compounds the most abundant of which is a cyclic depsitetrapeptide. In the backbone biosynthesis of HC-toxin, NRPS (HTS1), encoding a large peptide synthetase of about 574 kDa, and PKS (TOXC) are required, which are responsible for depsipeptide formation and biosynthesis of unusual amino acid, respectively.³¹⁰ Nontoxic mutants that are avirulent for maize lines susceptible to the wild type are obtained only if both copies are disrupted.³¹¹

Pseudomonas syringae parasitizes a wide variety of plant species and is divided into more than 50 different pathovars (pv.) based on host preference.³¹² In many *P. syringae* pv., nonhost-selective phytotoxins are produced, which induce chlorotic and necrotic symptoms in various host plants. For example, strain B301D (a pathogen of pear) of *P. syringae* pv. *syringae* produced cyclic depsipeptide syringomycin,³¹³ whereas strain SY12 (lilac blights) produced structurally related analogue syringostatin.³¹⁴ Their roles in disease development are extensively studied. Halogenation at the aliphatic carbon is a rare transformation in the biosynthesis of natural products. The detailed mechanism of halogenation with *syrB1* and *syrB2* in the biosynthetic gene cluster of syringomycin E has been elucidated.³¹⁵

Streptomyces pathogens are quite rare in plant diseases. Thaxtomin A from *Streptomyces scabies* causes scab disease of potato, which is characterized by conspicuous corky lesions on tubers. Molecular genetic investigation has revealed that thaxtomins are biosynthesized by nonribosomal peptide synthetases (*txtAB*) that condense modified L-phenylalanyl and L-4-nitrotryptophanyl units to form a 2,5-dioxopiperazine skeleton.³¹⁶ Disruption of *txtA* results in the formation of nonpathogenic strain. This toxin is shown to affect the movement of calcium ions and protons across the plasma membrane and also inhibit cellulose biosynthesis.³¹⁷

4.08.3.4 Terpenoids

Terpenoid phytotoxins, including diverse fungal diterpenoids and sesquiterpenoids, are produced by many phytopathogenic fungi. In general, the biosynthesis of terpenes starts with the formation of the molecular skeleton with terpene cyclase to afford cyclic hydrocarbon, which is usually hydroxylated and subsequently modified by alkylation, acylation, and glycosylation. Some of these phytotoxins show useful biological activity, and are used as plant growth regulators in agriculture and as biochemical agents for plant and cell physiology (Figure 34).

Diterpene glucoside fusicoccin is produced by *Phomopsis* (*Fusicoccum*) *amygdali* as a principal toxin implicated in the wilting disease of almond and peach in Italy, and a fusicoccin-producing *P. amygdali* Niigata 2 is newly found in a peach *Fusicoccum* canker fungus in Japan. Fusicoccin A shows potent phytohormone-like activities and is used as a biochemical agent for plant physiology. It permanently activates plasma membrane H⁺-ATPase in all higher plants and its mode of action is investigated by X-ray crystallographic analysis of the ternary complex of a plant 14-3-3 adapter protein, fusicoccin, and a synthetic phosphopeptide of the C-terminus of H⁺-ATPase.³¹⁸ Current biosynthetic studies of fusicoccin aglycone indicate the presence of the genuine biosynthetic tricyclic hydrocarbon intermediate (+)-fusicocca-2,10(14)-diene in the mycelia of

Figure 34 Structures of terpenoid phytotoxins.

P. amygdali Niigata 2.³¹⁹ Cloning of fusicoccadiene synthase gene and its expression allowed elucidation of the highly unusual multistep conversion of C₅ isoprene units into fusicoccadiene, showing that fusicoccadiene synthase possesses both prenyltransferase and terpene cyclase activities.³²⁰ Fusicoccin biosynthetic gene cluster has been identified by chromosomal walking. In connection with structure and bioactivities of fusicoccin, its sole congener cotylenin A, a plant growth regulator isolated from a fungus *Cladosporium* sp. 501-7W, is originally characterized as a potent differentiation-inducing substance in mammalian cells and an antitumor agent against human lung carcinoma cells.³²¹

The plant hormones gibberellins GA_{1/3} and GA₄ are produced by *Gibberella fujikuroi*, a Bakanae disease fungus of rice, and *Sphaceloma maniboticola*, a super-elongation disease fungus of cassava, respectively. Fungal gibberellins GA₃ and GA₄ are used as plant growth regulators for horticultural production. The biosynthesis of gibberellins in *G. fujikuroi* is determined at the molecular level; biosynthetic enzymes responsible for GA₁ formation include only *ent*-kaurene synthase and four cytochrome P-450 enzymes.³²² This pathway is totally different from the corresponding plant counterpart. Another gibberellin-producing fungus *Phaeosphaeria* sp. L487, known as one of the phytopathogenic fungi, produces GA₁ in plant-like gibberellin biosynthetic pathway through GA₉ and GA_{4/20}. Its *ent*-kaurene synthase in the GA₁ biosynthesis catalyzes the formation of *ent*-kaurene from GGDP through *ent*-copalyl diphosphate³²³ (Figure 35).

Aphidicolin, a well-known biochemical agent that functions as a specific inhibitor of DNA polymerase α , is produced by *Phoma betae*, a fungal pathogen of beet. Its biosynthetic precursors, including aphidicolan-16 β -ol, were elucidated by treatment of *P. betae* with cytochrome P-450 inhibitors.³²⁴ From this fungus, a cDNA encoding aphidicolan-16 β -ol synthase was cloned, and its recombinant fusion protein was found to catalyze the direct formation of 16 β -ol from GGDP through *syn*-copalyl diphosphate.³²⁵ Furthermore, chromosomal walking adjacent to the aphidicolol synthase gene allowed to identify the aphidicolin biosynthetic gene cluster.

Diterpene phytotoxins sphaeropsidins A–F, tri- and tetracyclic unrearranged pimarane skeleton, are isolated from *Sphaeropsis sapinea*, a fungus that causes a canker disease of Italian cypress. Sphaeropsidin A is the major toxic substance showing nonhost-selective phytotoxic activity³²⁶ (Figure 36).

Trichothecene phytotoxins such as deoxynivaenol are produced by some phytopathogenic species of *Fusarium*. Biosynthetic studies on this phytotoxin show that a bicyclic hydrocarbon intermediate trichodiene, formed from FDP, and the subsequent oxidations with a series of cytochrome P-450s such as Tri4 give isotrichodiol, isotrichotriol, and deoxynivaenol.³⁰² The gene cluster responsible for trichothecene biosynthesis was found in *Fusarium* and *Myrothecium* fungi.

Another phytohormone abscisic acid is produced by phytopathogenic fungi *Cercospora cruenta*, *C. cruenta*, and *Botrytis cinerea*. These fungi biosynthesize abscisic acid by oxidation of ionylideneethane with molecular oxygen following cyclization of allofarnesene.³²⁷ This direct pathway via ionylideneethane and subsequent ionylideneethanol is common among abscisic acid-producing fungi.

Sorokinianin, an unusual sesquiterpenoid, is isolated from *Bipolaris sorokiniana*, a fungal phytopathogen that causes spot blotch or foot and root rot diseases in wheat, barley, and oat. It is biosynthesized from phytotoxic prehelminthosporol and C₃ unit derived from oxaloacetic acid.³²⁸ Prehelminthosporol itself was isolated as a phytotoxin of *Helminthosporium sativum*. Sorokinianin is more phytotoxic than prehelminthosporol in inhibiting the germination of barley seeds.

Figure 35 Structures of terpenoid phytotoxins (continued).

Figure 36 Structures of terpenoid phytotoxins (continued).

4.08.3.5 Macromolecular Phytotoxins

Various phytopathogenic microorganisms produce glycoproteins and polysaccharides that have been implicated as phytotoxic agents. For example, *Pseudomonas syringae* pv. *glycinea* produces exopolysaccharide alginate, a copolymer of D-mannuronic acid and L-glucuronic acid, which is strongly correlated with virulence in host plants.³²⁹ In the case of *Verticillium* wilt, it is suggested that protein–lipopolysaccharide and glycoprotein are involved in the production of disease symptoms in susceptible host plants.³³⁰

Stereum purpureum, the causal fungus of apple silver leaf disease, produces three endopolygalacturonases (EndoPG Ia, Ib, and Ic), which are fully characterized. EndoPGs, present at a concentration of about 50 ng in an infected leaf, degrade the pectin in leaves and cause the silver leaf symptoms. EndoPG Ia contains 379 amino acid residues in the amino acid sequence excluding the signal peptide and has two N-binding sugar chains with Man₅GlacNac₂. Its glycosylated asparagines are Asn92 and 161, and three disulfide bridges are at Cys3–Cys17, Cys175–Cys191, and Cys300–Cys303.³³¹ Three crystal structures of EndoPG Ia (the unliganded EndoPG Ia and the binary and ternary complexes of EndoPG Ia with galacturonate) are determined in native and two galacturonic acid complex states by X-ray crystallography and, consequently, the catalytic mechanism of EndoPG Ia is elucidated.³³²

4.08.3.6 Perspectives

Completion of genomic analysis of several plant pathogens *Magnaporthe grisea* (rice blast), *Gibberella zeae* (head blight in wheat and barley), and *Streptomyces scabies*, causing potato scab, allowed us to identify genes for phytotoxin biosynthesis and to study disease development in detail at molecular level. It has been found that rice blast fungus produces some polyketide–nonribosomal peptide hybrid molecules during infection. The dynamic action and role of phytotoxins will be elucidated by transcriptome and metabolome analysis. Comparative genetic study in usual and pathogenic strains will provide information on how pathogenicity is acquired and developed. These studies on various types of diseases give a general idea on the strategy of microorganisms in disease development, and also the solution to prevent their infection.

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