An Introduction to the Biosynthesis of Chemicals Used in Plant-Microbe Communication

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ABSTRACT

Plants accumulate a diverse array of natural products, which can serve either to defend the plant against various microbes in its environment or to attract various microbes, both beneficial and pathogenic. Plants must also attract pollinators, repel or poison herbivores, compete with other plant species, and protect themselves from environmental dangers such as high light intensities. Some compounds have been implicated in playing a role in multiple interactions. Although the structures vary immensely in size and complexity, most are derived from a limited number of core biosynthetic pathways. This review briefly summarizes the biosynthetic origins of phenylpropanoid (including simple phenolics, flavonoids, anthocyanins and isoflavonoids), polyacetate, terpenoid, and alkaloid classes of metabolites. Compounds reported to be important in plant-microbe, plant-animal, and plant-plant interactions will be given as examples of each of these classes. Other aspects of biosynthesis also will be discussed, including the timing or location of biosynthesis, the potential for genetic manipulation of these pathways, and various questions regarding the biosynthesis of these compounds.

Key words: Biosynthesis; Secondary metabolite; Natural products; Phytoalexin; Phenylpropanoid; Terpenoid; Alkaloid; Polyacetate

INTRODUCTION

Plants accumulate a diverse array of natural products, which are thought to be involved in their interactions with the environment. These chemicals function in interactions with microbes, animals, and even other plants, as well as protecting the plant from ultraviolet radiation and oxidants. Some compounds may attract beneficial insects or microbes, whereas others kill or repel predators. Many of these compounds have been referred to as "secondary metabolites" to distinguish them from the "primary metabolites" required for the growth of all plants. These secondary metabolites, however, are likely to be es-

sential for the successful competition or reproduction of a given plant species in its natural environment. Several of these same compounds, such as codeine or taxol, have important medicinal activities and have sparked a resurgence in the studies of their biosynthetic origins.

Natural plant products that are important in plant-microbe interactions range in size from the volatile two-carbon molecule ethylene to insoluble polymeric compounds such as rubber or lignin. The structures characterized to date number well over 10,000. However, each structure is built from simple building blocks derived from products of primary metabolism. Most natural products can be classified into one of three or four basic structural classes, but some are hybrids of two or more classes. Many

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structures are widespread among diverse plant species, whereas some have only been isolated from a single species or a small group of highly related species.

This review will discuss briefly the biosynthetic origins of the major classes of natural products, focusing on the core reactions leading from primary metabolism to the standard building blocks of each class. Molecules that are important in plant-microbe or plant-animal interactions will be used as examples of each structural class. In this review, the names of illustrated compounds are in bold text. Several biochemistry texts and in-depth reviews are available for those interested in learning more about the enzyme mechanisms or details of the biosynthesis of a specific molecule (Bailey and Mansfield 1982; Goodwin and Mercer 1983; Luckner 1990; Nelson and Cox 2000; Stafford and Ibrahim 1992; Stryer 1995; Towers and Stafford 1990). Mechanisms that the plant uses to protect itself from the potential negative effects of these compounds will also be reviewed. Some recent examples of genetically altering the levels or structures of secondary metabolites will be described, along with the practical utility of such modifications. Nevertheless, despite amazing progress in gene cloning and how much is now known about the origins of these compounds, several significant questions still remain to be answered.

PHENYLPROPANOIDS: SIMPLE PHENOLICS, FLAVONOIDS, AND ISOFLAVONOIDS

The shikimate pathway gives rise to the aromatic amino acids, phenylalanine, tyrosine, and tryptophan, as well as numerous essential vitamins and co-factors. In plants, phenylalanine ammonia lyase (**PAL**) converts phenylalanine to cinnamate, which is the precursor of thousands of phenolic compounds, including simple phenolics, phenylpropanoids, and flavonoids (Figure 1).

Hydroxylation of **cinnamate** by means of cinnamic acid 4-hydroxylase (**CA4H**) produces **para-coumaric acid,** which can be further hydroxylated and methylated to form **caffeic, ferulic,** and **sinapic** acids. These simple phenylpropanoids are the building blocks of the complex polymers, suberin and lignin, as well as other polyphenolic barriers formed after wounding or pathogen infection. For example, **chlorogenic acid** (a quinic acid ester of caffeic acid) is quickly polymerized by means of enzymatic and oxidative (nonenzymatic) mechanisms activated on crushing of cells. Both the free acids and their coenzyme A (CoA) thioesters appear to be involved in
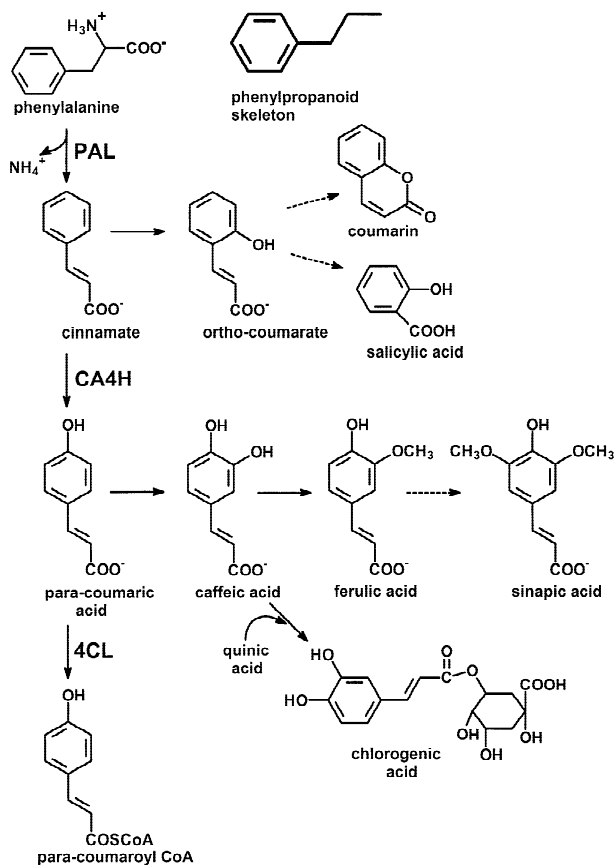
Figure 1.   Core phenylpropanoid pathway reactions and examples of simple phenylpropanoids. PAL, phenylalanine ammonia lyase; CA4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate:CoA ligase.

lignin biosynthesis, but the exact form of the monomers that are polymerized and how the structure is regulated are still unclear. The monomers must be exported outside the cell membrane before being polymerized into the cell wall.

Ortho-hydroxylation of cinnamate produces **ortho-coumaric acid** (coumarate), which can serve as the precursor of the coumarins. Coumarins are antimicrobial and can also be converted to the powerful anticoagulant dicoumarol. Such compounds may serve as a defense against herbivores. Ortho-coumaric acid also undergoes chain shortening reactions to form **salicylic acid,** which is antimicrobial and plays an important role in plant signal transduction during pathogen attack (Friedrich and others 1995).

Cinnamate and benzoate derivatives are somewhat antimicrobial and phytotoxic and are thought to be involved in allelopathic interactions between plants, especially when released from decaying crop residue. **Acetosyringone** and **methylsinapate** (Fig-
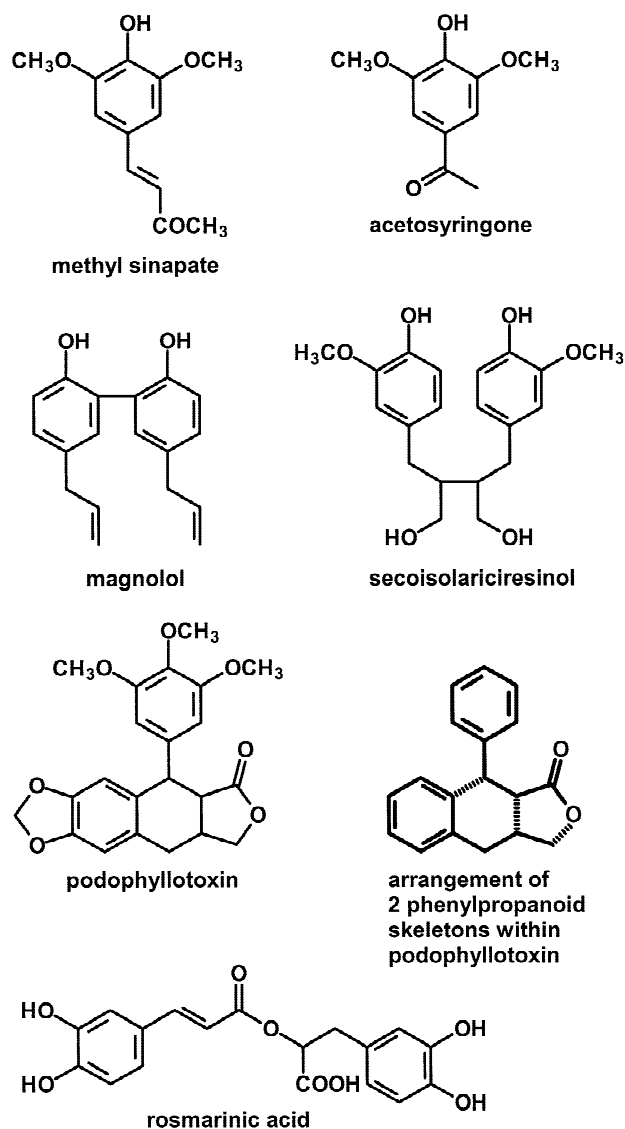
Figure 2. Examples of phenylpropanoids, lignans, and rosmarinic acid, a phenolic ester.

Figure 3. Reactions carried out by chalcone synthase (CHS), resveratrol synthase (RS), chalcone isomerase (CHI), and chalcone reductase (CHR) leading to common chalcones, stilbenes, and flavonoids.

members of the mint family, is an ester formed from two very similar phenylpropanoids. However, one is derived from phenylalanine, whereas the other comes from tyrosine, with the two phenylpropanoid units being synthesized by very different enzyme mechanisms (Petersen and others 1995).

Phenylpropanoids can serve as starter units in various polyketide synthase reactions. The most widely distributed reaction is that catalyzed by chalcone synthase **(CHS)** to produce chalcones and flavonoids by way of the condensation of para-coumaroyl-CoA and malonyl-CoA (Figure 3). Every vascular land plant is believed to contain CHS because the products of this enzyme are effective UV light protectants and can act as a "sunscreen" to protect cells from the harmful effects of the sun's rays. For example, *Arabidopsis* mutants lacking CHS-derived flavonoids or other UV-absorbing phenolics are more sensitive to high light intensity (Landry and others 1995; Li and others 1993). The tetrahydroxychalcone produced by CHS can be isomerized by chalcone isomerase **(CHI)** to produce the common trihydroxyflavanone, **naringenin.** Chalcone reductase **(CHR)** is present in legumes and can act on the intermediate polyketide chain to produce a

ure 2) are two simple phenolics that induce *vir* gene expression in *Agrobacterium tumefaciens;* acetosyringone is often added during the cocultivation step of plant transformation. Simple phenylpropanoids can be reduced and dimerized to form lignans, including the antifungal **magnolol, secoisolariciresinol** (an anticancer agent found in grains), and **podophyllotoxin** (an extremely toxic component of mayapple rhizomes). The stereochemistry of the lignans varies among plants, and it has been shown that the stereochemistry of the radical coupling reaction is tightly controlled by a "dirigent protein"—one that does not initiate the reaction but helps guide the orientation of the monomers (Davin and others 1997). **Rosmarinic acid,** a common constituent of
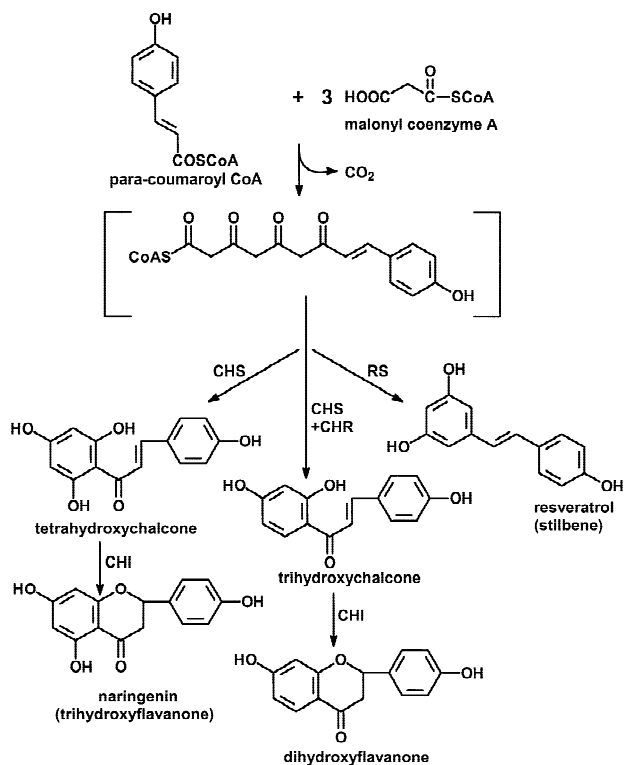
trihydroxychalcone that can be cyclized to dihydroxyflavanone, which in turn gives rise to numerous 5-deoxyflavonoids and isoflavonoids. Resveratrol synthase **(RS)** (also known as stilbene synthase) produces the same polyketide intermediate as CHS but cyclizes the chain in a different manner, losing an additional molecule of CO_2 to form 3,5,4′-trihydroxystilbene, or **resveratrol.**

Modifications in these basic structures are produced by variations in the starter unit (for example, cinnamoyl-CoA in place of coumaroyl-CoA) and by further hydroxylations, methylations, glycosylation, reductions, oxidations, acetylations, prenylation, and so on, resulting in hundreds of other flavonoids and stilbenes. **Naringenin** accumulates in several plants, but it is also the precursor of several important classes of flavonoids. Hydroxylation followed by oxidation to introduce a double bond produces **kaempferol** and **quercetin,** two common flavonols (Figure 4). Reduction of the carbonyl group, followed by introduction of a positive charge into the oxygen-containing ring, produces the anthocyanidins **pelargonidin** and **cyanidin** (containing mono- and dihydroxylated phenyl rings, respectively). Subsequent 3-glycosylation converts these anthocyanidins to the corresponding **anthocyanins** such as **callestephin (pelargonidin-3-glucoside),** a red component of strawberries. Additional sugars may be added, as in the case of **cyanin (cyanidin-3, 5-diglucoside),** and additional hydroxylation, methylation, and esterification reactions further add to the diversity of the anthocyanins. These compounds and their derivatives are the red, purple, and blue pigments of many plant parts, such as flower petals, fruits, leaves, and roots.

The carbon skeletons of both trihydroxy- and dihydroxyflavanoids can undergo an aryl migration and dehydration, resulting in a rearrangement to form **isoflavonoids.** The three carbons that were originally derived from the linear side chain of phenylalanine become a branched carbon chain. This class of compounds is widespread in the legumes but has also occasionally been isolated from other species, such as beet and iris. Isoflavonoid glucoside malonates accumulate to high levels in many legume seeds and roots. Simple isoflavonoids also can be converted by reductions and dehydrations to tetracyclic **pterocarpans,** one of the largest subclasses of flavonoids, which is found in diverse species of legumes. **Medicarpin** and **glyceollins** are important pterocarpan antifungal compounds in alfalfa and soybean, respectively.

Flavonoids are important in many different plant-
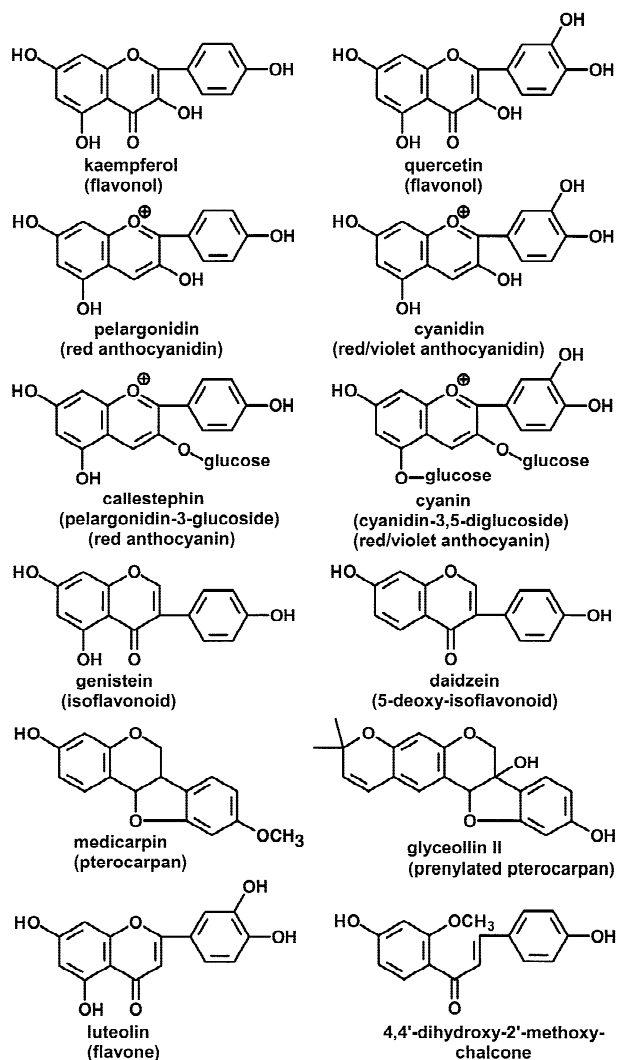
Figure 4. Biologically active examples of common classes of flavonoids, including flavonols, anthocyanidins, anthocyanins, isoflavonoids, pterocarpans, a flavone, and a chalcone.

microbe interactions and are therefore highly studied in plants. Several types of flavonoids influence the early stages of the establishment of biologic nitrogen fixation in root nodules of legumes. **Luteolin** (a flavone) was one of the first flavonoids identified that induce *nod* genes in *Rhizobium meliloti* (recently renamed *Sinorhizobium),* the nitrogen-fixing symbiont that nodulates roots of alfalfa (Peters and others 1986). Subsequently, it was shown that the luteolin is actually released from alfalfa seed coats, whereas **4,4′-dihydroxy-2′-methoxychalcone** is released from the roots at very low levels but is 100 to 1000 times more potent as a *nod*-gene inducer (Maxwell and others 1989). The simple flavonoid

naringenin and isoflavonoids **daidzein** and **genistein** act as *nod*-gene inducers in other species. In addition to possible roles as coloring agents, anthocyanins from bean seed coats are active as *nod*-gene inducers of *Bradyrhizobium,* the bean symbiont.

In addition to attracting and stimulating nodulating bacteria, flavonoids have numerous other activities. Whereas free (nonglycosylated) **naringenin** induces the *nod* genes of certain rhizobia, naringin (the glucose conjugate of naringenin) is bitter and may help deter herbivores. **Naringenin** and the very widespread flavonols **quercetin, kaempferol,** and rutin (quercetin-3-rutinoside) are somewhat antimicrobial. Various isoflavonoid phytoalexins (antimicrobial small molecules that accumulate after pathogen attack) are much more toxic (Bailey and Mansfield 1982). For example, the pterocarpans **glyceollin** (from soybean) and **medicarpin** (from alfalfa and clover) are very toxic to several fungal pathogens of these plants. Both are only biosynthesized in foliage after induction of the pathways after pathogen infection or suitable abiotic stresses. The anthocyanidin peonidin (3′-*O*-methyl-cyanidin) promotes haustoria formation in the root parasitic plant *Triphysaria versicolor* (Albrecht and others 1999), suggesting the compounds released from the host's seed coat may induce the parasite to attack. Flavonoids induced during the initial stages of rhizobial colonization may both stimulate further invasion and modify the growth of plant cells, in that flavonoids have been proposed to alter the transport of auxins (Faulkner and Rubery 1992; Mathesius and others 1998).

POLYACETATES

Several natural products are formed by the condensation of "activated" acetate units supplied either as malonyl CoA or acetyl CoA. Several are believed to be derived from common fatty acids. For example, linoleic acid can be repeatedly oxidized to introduce additional carbon–carbon double bonds, and these are oxidized to form carbon–carbon triple bonds. In combination with chain-shortening reactions (beta-oxidations), a series of polyacetylenic compounds are generated (Figure 5). **Wyerone,** an antifungal and antibacterial phytoalexin from fava beans, and the **thiophenes** and **thiarubrines,** antimicrobial and insecticidal compounds common in the Asteraceae, probably arise from the interaction of the acetylenes with hydroxyl and thio donor molecules.

Polyacetate molecules may also be synthesized by specific "polyketide synthases" and modifying en-
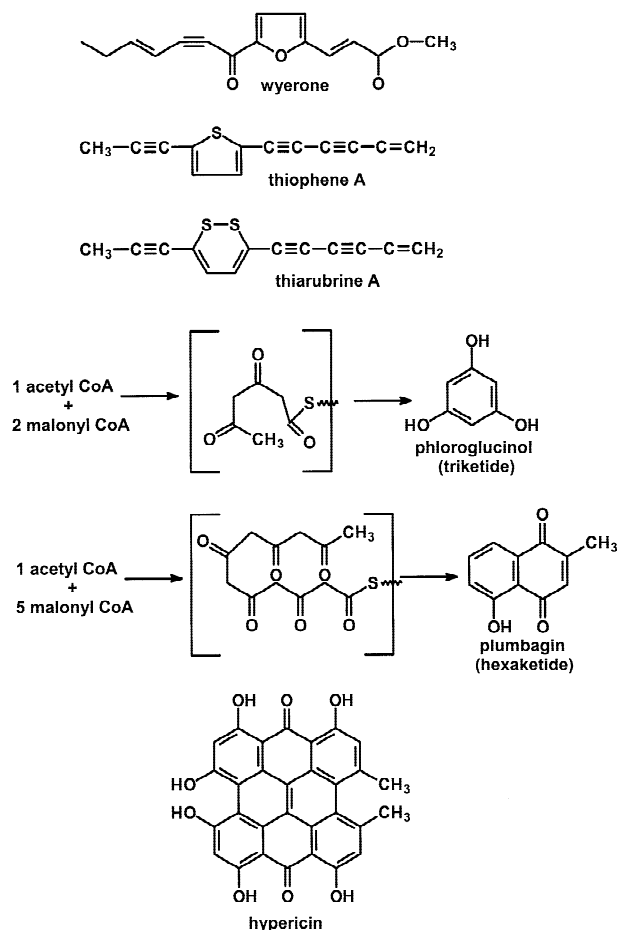

Figure 5. Polyacetate molecules derived from either modification of fatty acids or condensation of acetyl and malonyl CoA by polyketide synthases.

zymes. In most cases, acetyl-CoA serves as the starter unit, and malonyl CoA units are condensed onto the growing chain, using reactions very similar to those involved in fatty acid chain elongation. Linear polyketides (hydrocarbon chains with carbonyl or "keto" groups at alternating carbons) may be formed by such reactions, or the unreduced intermediates may cyclize, generating aromatic rings. The smallest aromatic polyketide in plants is the triketide **phloroglucinol** (Figure 5). (A similar mechanism is used by CHS and RS mentioned earlier to form the nonshikimate aromatic ring in the flavonoids and stilbenes.) **Plumbagin** is a bicyclic hexaketide from *Plumbago* spp., whereas **hypericin** is a complex polyketide from St. John's wort. Both are antimicrobial, and hypericin acts as an insect antifeedant and as an antidepressant in humans. Acetates also can be condensed to form branched chains, such as in the biosynthesis of mevalonate

(discussed later), or they can be combined with other organic acid derivatives (discussed previously).

TERPENOIDS

Another diverse family of metabolites is classified as terpenoids or isoprenoids. Terpenoids are derived from the condensation of branched five-carbon units, either isopentenyl pyrophosphate **(IPP)** or its isomer, dimethylallyl pyrophosphate **(DMAPP).** These same precursors are incorporated into many primary metabolites essential to plant life, such as carotenoids, ubiquinones, sterols, and the phytyl side chain of chlorophyll.

Two routes to IPP have been demonstrated in plants (Figure 6). One is referred to as the **mevalonate** pathway, using the same enzymatic steps early in the synthesis of cholesterol in humans. Mevalonate is formed by the reduction of 3S-3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), a product of the condensation of three acetyl CoA units. This reduction is catalyzed by HMG-CoA reductase **(HMGR),** which is often a key regulatory enzyme in the synthesis of certain groups of terpenoids. Mevalonate is then phosphorylated twice and decarboxylated to form IPP. A second mevalonate-independent route was first discovered in bacteria and recently shown to be present in plants (Eisenreich and others 1996; Lange and others 1998). Pyruvate and glyceraldehyde-3-phosphate are condensed to form **1-deoxy-D-xylulose-5-phosphate** (Figure 6), which is isomerized and otherwise modified by as yet uncharacterized steps to form IPP. The mevalonate pathway appears to function in the cytoplasm, whereas enzymes of the nonmevalonate pathway appear to be targeted to plastids, although studies are still in progress to localize the enzymes involved conclusively. A portion of the IPP synthesized by either pathway is converted to DMAPP by means of IPP isomerase. IPP and DMAPP either serve directly as prenyl donors or polymerize to form terpenoid precursors of varying chain lengths (Figure 7).

Terpenoids vary greatly in molecular weight, ranging from **isoprene,** a highly volatile five-carbon hemiterpene involved in allelopathy of desert plants, to **rubber,** a polyterpene with a molecular weight up to 100,000, which may serve as a polymeric barrier to microbes and herbivores (Figure 8). IPP can condense with one, two, or three molecules of DMAPP to form **geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP),** or **geranylgeranyl pyrophosphate (GGPP)** (Figure 7), which are then
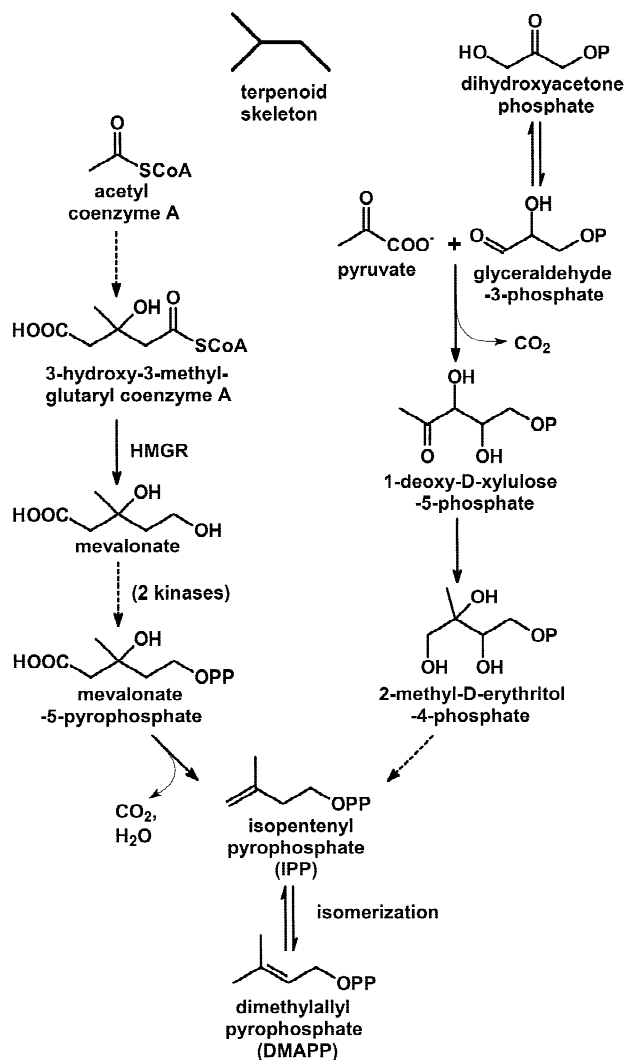

Figure 6. The mevalonate and 1-deoxy-D-xylulose-5-phosphate pathways leading to isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), the precursors of terpenoids. HMGR = 3-hydroxy-3-methyl-glutaryl coenzyme A reductase.

rearranged, cyclized, hydroxylated, and otherwise modified by highly specific enzymes. GPP is the 10-carbon precursor of monoterpenes, which include many volatile flavor and aroma components, and compounds involved in plant-insect interactions, including pollinator attractants. Representative monoterpenes include the mildly antimicrobial **menthol,** aroma components **citral** and geraniol, insect repellant **citronellal,** parasitoid wasp attractant **linalool,** and allelopathic **camphor** (Figure 8). FPP can be cyclized and rearranged to form a variety of 15-carbon sesquiterpenes. Sesquiterpenes are among the most heavily studied class of phytoalexins. Microbial infection induces the accumulation of
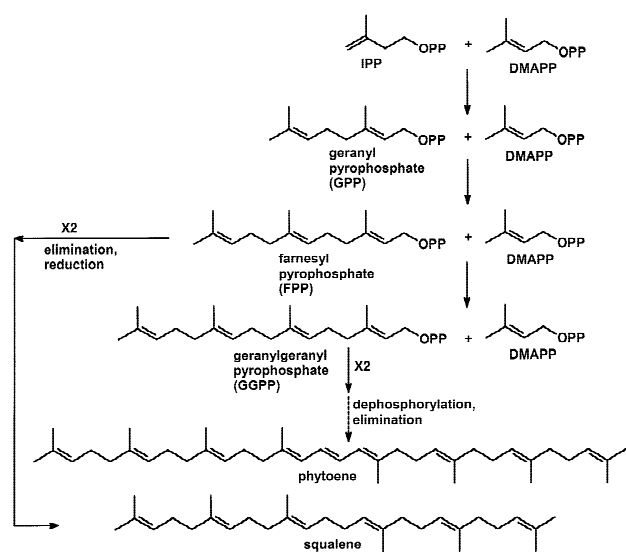
Figure 7. The series of condensations of IPP and DMAPP leading to the terpenoid intermediates geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP), phytoene, and squalene.

Figure 8. Representative terpenes, including monoterpenes and sesquiterpenes.

rishitin and lubimin in potato, capsidiol in peppers and tobacco, and 2,7-dihydroxycadalene in cotton. Caryophyllene and turmerone are better known as flavor components of the spices cloves and tumeric, but are also antimicrobial. FPP is also the precursor of squalene, which gives rise to sterols and steroidal glycoalkaloids such as tomatine and solanine from tomato and potato—bitter, toxic components of the leaves and tuber skins—and the triterpenoid cucurbitacins, which are antinematode compounds in cucumber roots (Figure 9). GGPP is the precursor of hundreds of diterpenes, including the growth hormone gibberellic acid and C20 diterpenes in pine rosin, such as abietic acid. Casbene is a powerful antifungal agent from the castor bean plant. Taxol is famous for use as a human anticancer agent but is toxic to all eukaryotic cells through its interaction with tubulin and therefore would serve as protection against pathogens and herbivores. GGPP can also be dimerized to produce phytoene, which is rearranged to carotenoids including lycopene and β-carotene, two colorful nutrients beneficial for human health. IPP or DMAPP can also serve as the source of the prenyl side of a number of natural products, including cytokinin, a growth regulator in plants. Prenylated flavonoids and isoflavonoids are found in many plant species. The prenylation of dihydroxypterocarpan to form the glyceollins in soybean and specific isoflavonoids in lupine greatly increase their antifungal activity.
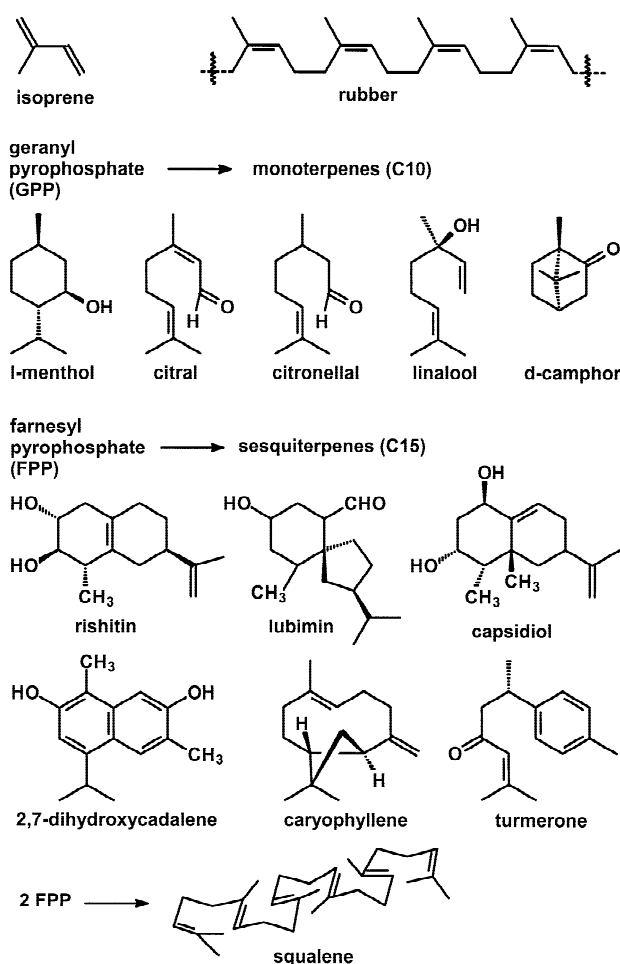
By means of incorporation of ^{13}C-labeled pres-

cursors, taxol and carotenoids have been shown to be synthesized by way of the 1-deoxy-D-xylulose-5-phosphate pathway, whereas steroidal compounds appear to be derived from the mevalonate pathway (Eisenreich and others 1996; Lange and others 1998). The source of the IPP for many other metabolites has not yet been determined.

ALKALOIDS

Alkaloids are loosely defined as compounds with a nitrogen-containing heterocyclic ring. The rings vary in size and oxidation state, ranging from aromatic to completely reduced. Thousands of alkaloids have been identified and are classified according to the structure of the ring system. Alkaloids are generally synthesized from amino acids.

Nicotinic acid (derived from aspartic acid and glyceraldehyde-3-phosphate) is a precursor of several pyridine alkaloids (Figure 10). Methylation of
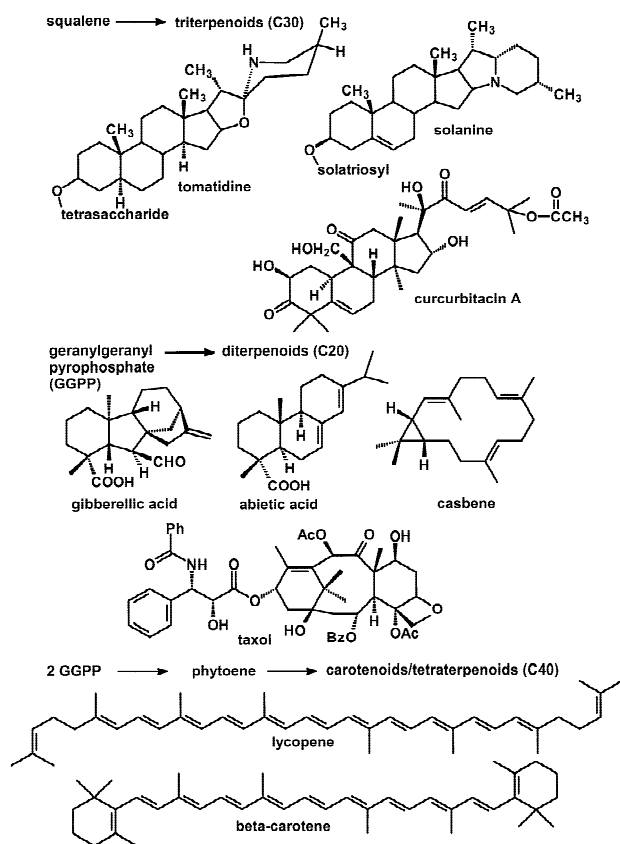
Figure 9. Representative terpenes, including triterpenoids, diterpenoids, and carotenoids.

Figure 10. Representative alkaloids, including pyridine, tropane, and isoquinoline alkaloid classes.

the nitrogen produces **trigonelline,** a compound found in many seeds including leguminous beans, wherein it has been shown to produce significant induction of nodulation-related genes in rhizobia. **Nicotine,** the infamous alkaloid component of tobacco, is produced by the fusion of nicotinic acid and another nitrogen-containing ring. Nicotine is insecticidal and inhibits feeding of herbivores.

Tropinone, a precursor of the tropane alkaloids containing a bicyclic ring system, is derived from ornithine, which is cyclized and modified to form *N*-methyl-pyrrolinium cation, and malonyl CoA. **Scopolamine,** a powerful sedative, is formed from the ester of tropine (reduced tropinone) and an aromatic residue; **cocaine** is similarly formed. Lysine, its precursors and derivatives, can be converted to the six-membered rings, pyridine or piperidine rings, in several alkaloids.

Reticuline is a precursor of hundreds of isoquinoline alkaloids (Figure 10). It is derived from two molecules of tyrosine, by way of **dopamine** and **4-hydroxyphenyl acetaldehyde.** One branch pathway leads to the tetracyclic benzophenanthridine al-
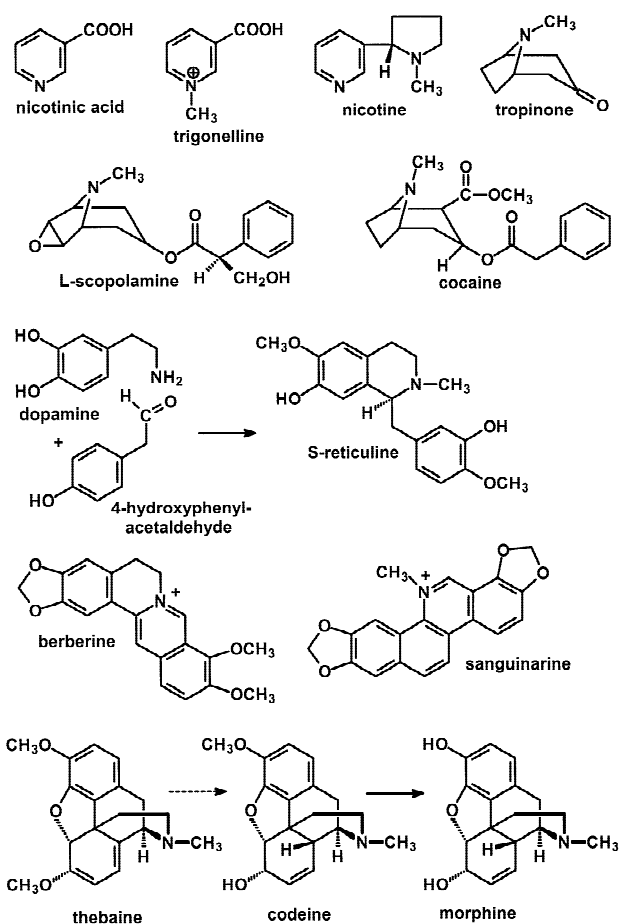
kaloids **berberine** and **sanguinarine,** alkaloids with high antibacterial, antifungal, and antiviral activity. Their structure allows intercalation into DNA strands, which will interfere with many cellular processes. Reticuline is also converted to the morphinan ("opium") alkaloids, including **thebaine, codeine, morphine.** Unusual aryl demethylation reactions occur during the interconversions of these alkaloids. Although valuable for centuries in human medicine, these bitter alkaloids may serve as antifeedants in nature.

Indole alkaloids are formed from strictosidine, a condensation product of **tryptophan** and **geraniol** derivatives (Figure 11). Strictosidine is then modified to form several types of monomeric indole alkaloids such as **ajmalicine, vindoline,** and **camptothecin.** Monomers can be further dimerized in the medicinal plant *Catharanthus roseus* (rosey periwinkle) to form powerful anticancer drugs such as vinblastine and vincristine. These and other alkaloids in the plants are highly toxic to herbivores.
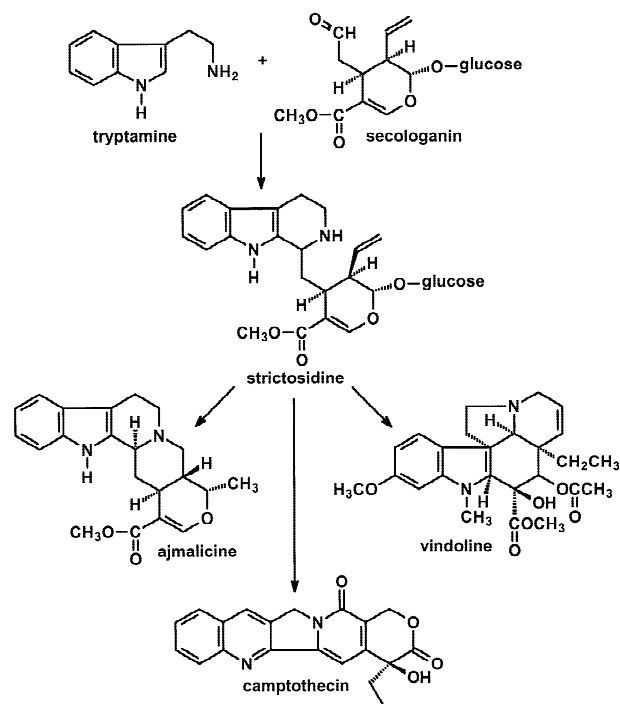
Figure 11. Representative indole alkaloids.

Nonplant Sources of Secondary Metabolites in Plants

Several secondary metabolites are isolated from plant tissues or the plant rhizosphere but are not synthesized by the plant itself. Some are beneficial to the plant, but others are toxic to the plant or other organisms. These compounds can greatly affect the plant's interactions with other organisms.

Ergot alkaloids accumulate in both rye and fescue, whereas fescue also accumulates pyrrolizidine **(loline)** and pyrrolopyrazine (peramine) alkaloids (Figure 12). Ergot alkaloids **(ergosine)** are used in medicine but are extremely toxic to mammals. The loline and peramine alkaloids confer insect resistance to fescue without causing much mammalian toxicity. In rye, the ergot alkaloids are synthesized by the endophytic fungus *Claviceps purpurea,* whereas in fescue, the primary endophyte is *Neotyphodium coenophialum.* Neither fungus causes damage to the vegetative portions of the plant and are not detectable without microscopic examination.

Several mycotoxins and aflatoxins are produced by fungi during fruit ripening, food storage, or food processing. In some cases the fungi are highly visible, whereas in others they are growing within the plant structures. The highly carcinogenic **aflatoxin B1,** found in stored grains and peanuts, is produced
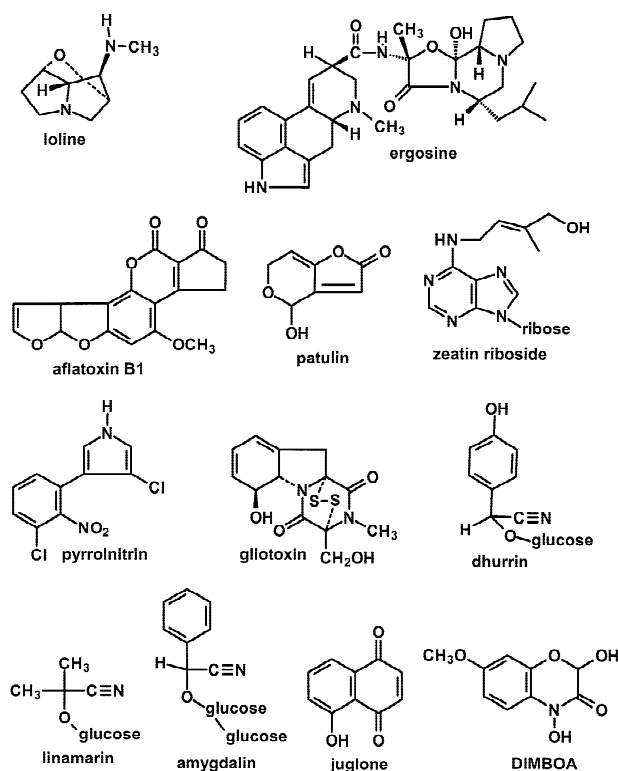

Figure 12. Compounds accumulating in plants or in the rhizosphere but not synthesized by plants (*upper portion*), three cyanogenic glucosides from plants, and two allelopathic compounds released from plant leaves.

by *Aspergillus parasiticus* and other *Aspergillus* sp. The carcinogenic and hepatotoxic **patulin,** found in apples, apple juice, and malted beer, is produced by *Penicillium urticae.* Both are polyketides, although aflatoxin B1 contains a coumarin moiety; both it and patulin are polyketides derived solely from acetyl and malonyl CoA.

Plant pathogens often synthesize plant hormone analogs or phytotoxins as part of their manipulations of the host plant's metabolism. The isopentenyl transferase (*ipt*) gene introduced into plant cells by *Agrobacterium tumefaciens* causes the accumulation of abnormally high levels of the cytokinin **zeatin riboside,** resulting in a corresponding increase in cell division (McKenzie and others 1994). Strains of the fungus *Gibberella fujikuroi* (anamorph, *Fusarium moniliforme*) produce high levels of the mycotoxin and phytotoxin fumonisin B1 but also produce **gibberellic acid** (Figure 9), which causes hyperelongation of rice seedlings.

Many species of fungi and bacteria are being investigated for their use as biocontrol agents. They are introduced into the rhizosphere of crop plants,

where they produce compounds toxic to plant pathogens. Two well-known examples are strains of *Pseudomonas fluorescens,* which produce **pyrrolnitrin** (toxic to pathogens such as *Rhizoctonia solani*), and *Gliocladium virens,* which produces **gliotoxin** (toxic to pathogens such as *Pythium* spp.)

SPATIAL AND TEMPORAL REGULATION OF BIOSYNTHESIS

Many of the secondary metabolites discussed earlier only accumulate in certain parts of the plant, or at certain times, and the mechanisms that the plant uses to control the accumulation vary. The plant may control where the compound is accumulated or synthesized, when it is synthesized, or both.

In the case of the phytoalexins and other toxins, the plant product is not only toxic to the microbial pathogen, but also potentially toxic to the plant cells. Many of the phytoalexin pathway genes are not expressed until the plant senses the presence of the pathogen, when complex signal transduction mechanisms activate transcription of each of the biosynthetic steps. For example, in the case of phenylpropanoid phytoalexins, there is a huge increase in the mRNA and enzyme activities of all of the core phenylpropanoid enzymes, especially the branch-point enzyme PAL, as well as the phytoalexin-specific enzymes, such as those late in the biosynthesis of medicarpin and glyceollin (Stafford and Ibrahim 1992). Similar pathway induction has been observed for terpenoids (requiring induction of the branch-point enzyme HMGR) (Towers and Stafford 1990) and various alkaloids (requiring induction of branch-point amino acid decarboxylases) (Lopez-Meyer and Nessler 1997). The cells immediately around the infection site will synthesize high levels of phytoalexins and may die, whereas cells at a distance from the lesion may accumulate no phytoalexins.

Many plant toxins are accumulated constitutively but are accumulated in specialized compartments. The monoterpene components of various mint oils are accumulated in glandular cells on the surface of the leaves. The early stages of biosynthesis occur in supporting cells, and the product is exported to accumulate to high levels in the glands. Similarly, the thiophenes and thiarubine often accumulate in specialized veins in the roots. There they are kept away from metabolically active cells and protected from photoactivation by sunlight. Rubber polymer is only synthesized in lacticifers, which form a network throughout the plant. Antifungal deoxyanthocyanidins and several alkaloids are synthesized in vesicles inside the producing cells and transported to the side of the cell closest to the fungal pathogen. Some toxins are also synthesized as glycosides or other "conjugates" and often are stored in the producing-cell's vacuole. There they are harmless until the cell is damaged or stressed, at which time an enzyme in the plant or pathogen activates the compound. For example, glucosinolates accumulate in canola and other members of the mustard family. On wounding or crushing of the plant or seeds, myrosinase (beta-thioglucoside glucohydrolase) is released from plant cells and hydrolyzes the glucosinolates to toxic isothiocyanate derivatives. **Dhurrin** (sorghum), **linamarin** (cassava), and **amygdalin** (*Prunus* spp.) (Figure 12) are three cyanogenic glucosides, that release HCN on hydrolysis. Each of the producing plants contain enzymes highly active on their respective component but separated spatially until the tissue is crushed. In each of the preceding cases, enzymes from microbes or in the digestive systems of herbivores also catalyze the same conversion.

In many cases, a natural product is only synthesized in a specific plant part. For example, flower color and scent components are produced at highly specific developmental stages, correlating with the optimal times to attract pollinators. However, not all metabolites are synthesized in the part of the plant where they accumulate. For example, 4,4'-dihydroxy-2'-methoxychalcone (Figure 4), a powerful inducer of rhizobial nodulation genes, is synthesized and released from root tips, but two other nodulation inducers, luteolin (Figure 4) and trigonelline (Figure 10), are actually synthesized in the seed and released during imbibition and germination. Much of the toxic linamarin in cassava roots is apparently synthesized in the leaves and transported to the roots for storage, whereas the opium alkaloids are at least in part synthesized in the roots and transported to the pod latex vesicles in waves with a diurnal rhythm. Many allelopathic compounds are synthesized in leaves, then are washed onto the surrounding ground during rain or are deposited when the leaves drop, providing a toxic barrier to competitors. Two famous examples are **juglone** from walnut and **DIMBOA** from rye (derived from isochorismic acid and anthranilic acid, respectively, from the shikimate biosynthetic pathway) (Figure 12).

GENETIC MODIFICATIONS OF SECONDARY METABOLITES

For centuries, humans have been modifying the secondary metabolite profiles of plants, often without

realizing it. In selecting fruits or flowers with modified colors, people selected for altered anthocyanin content, whereas in selecting for scents, they selected for plants with a modified profile of volatile phenolics or terpenoids. In some classic plant-breeding programs, increased pest resistance was selected for, resulting in less palatable food. For example, insect-resistant celery contained irritating psoralens, and insect-resistant potatoes contained unacceptable levels of bitter, toxic steroidal glycoalkaloids.

In recent years, more directed approaches have been undertaken, using chemical screening for increased or decreased compound levels. Breeders selected rapeseed varieties for lines low in erucic acid (a polyunsaturated fatty acid) and glucosinolates in the seeds to create canola. Researchers working with medicinal plants have often screened individual plant lines or cultures grown under various conditions to find ways to maximize the content of the active ingredient(s).

Alterations of secondary metabolites in plants by genetic engineering using molecular techniques (as opposed to traditional breeding and selection) have become more prominent in recent years. Much of the early work was driven by funding from the floriculture industry and focused on the easily observed flower pigments. Overexpression of a chalcone synthase in petunia could convert a pink or red flower into a very dark red flower, indicating that more substrate was being directed into the flavonoid/anthocyanin pathway. During some of this early work, it was also found that in the presence of very high levels of foreign mRNA ("co-suppression"; van Blokland and others 1994), or when the complementary strand was highly transcribed instead of the sense strand ("antisense mRNA"; van der Krol and others 1990), the transcript for the endogenous message was greatly decreased, resulting in a lack of expression of the gene. For example, many white-flowered lines were created either by overexpressing CHS mRNA at high levels or by inserting the CHS coding region in the "antisense" orientation downstream of a very strong promoter. As a consequence, the flowers accumulated little or no CHS enzyme and therefore could not produce anthocyanins.

Many recent studies have focused on either (1) using overexpression of an endogenous branch-point or putative rate-limiting enzyme to increase flux into a pathway, or (2) using antisense and cosuppression strategies to eliminate or reduce flux toward an undesirable metabolite. Several laboratories have used antisense or cosuppression to alter the level of enzymes involved in lignin biosynthesis.

Consequently, alterations in both lignin quality and quantity have been reported (Baucher and others 1999; Lee and others 1997; Ni and others 1994; Tsai and others 1998; Zhong and others 1998). Other approaches have taken genes from various plants, fungi, bacteria, or animals to introduce new pathways into plants. Trichodiene synthase, a sesquiterpene synthase from the fungus *Fusarium sporotrichioides,* was used to transform tobacco and successfully converted endogenous FPP into trichodiene, a precursor of fungal toxins (Zook and others 1996). In the author's laboratory, a resveratrol synthase coding region from peanut was expressed constitutively in alfalfa, allowing the accumulation of a resveratrol glucoside, a stilbene phytoalexin not normally found in alfalfa (Hipskind and Paiva 2000). Bacterial enzymes involved in the synthesis of polyhydroxybutyrate polymers have been introduced into various plants to evaluate the feasibility of using plants to produce economical levels of biodegradable plastics (Nawrath and others 1994). Foreign enzymes can also be used to destroy metabolites. The salicylate hydroxylase (*nahG*) gene from bacteria has been introduced into *Arabidopsis* and tobacco (Friedrich and others 1995), where it successfully converts salicylic acid to catechol. Such plants were important in illustrating the importance of salicylic acid in defense responses. In the author's laboratory, we are also attempting to use a fungal enzyme specific for phytoalexin detoxification to hydroxylate medicarpin, and then test the modified plants for increased pathogen susceptibility.

Many problems with plant transformation and tissue-specific promoters have been overcome, but understanding the biochemistry of the target metabolites and cloning the necessary genes are now often the rate-limiting steps in modifying secondary metabolite profiles. In some of the early work on lignin manipulation, researchers were surprised that drastic reductions in certain enzymes did not cause corresponding decreases in lignin content. Such results led to the realization that plants had more than one route to lignin biosynthesis and that a block in one route was compensated for by an increased flux through another (Zhong and others 1998). In the case of the abovementioned introduction of resveratrol into alfalfa, previous studies using a similar approach had always reported the accumulation of free resveratrol, whereas alfalfa only accumulated the glucoside. The compound is still toxic to fungal pathogens but could be missed in assays specific to resveratrol (Hipskind and Paiva 2000). Efforts to increase tryptamine and related metabolites by overexpressing tryptophan decarboxylase were successful in potato but reduced the level of antimicrobial

phenylpropanoids by creating an increased demand for shikimate pathway intermediates (Yao and others 1995).

Many products also play multiple roles in plants or in the creatures that interact with them, so alteration of the levels could have unintended effects. For example, medicarpin functions as a phytoalexin and a medicarpin conjugate as a phytoanticipin (preformed antimicrobial compound) in roots in alfalfa because of antifungal activity. However, medicarpin also inhibits the germination of alfalfa seeds and thus has been implicated in allelopathy in established alfalfa fields (Dornbos and others 1990). Antisensing CHS not only reduced the colored anthocyanins in flower petals but also reduced flavonol glycosides in the pollen. In Solanaceous plants, flavonols are required for pollination, so the antisense plants were made sterile. Several acyclic terpenoids, for example, alpha-farnesene and cyclic terpenoids such as linalool (Figure 8), myrcene, and beta-ocimene, and volatile compounds, are released from damaged leaves in response to feeding by caterpillars. Although some compounds were known to be antimicrobial and are not directly toxic to caterpillars, it was recently shown that they act indirectly to attract parasitic wasps, which then prey on the feeding larvae (de Moraes and others 1998). For centuries, whole plants or their extracts have been used as medicines, leading to many early phytochemical studies. Several natural products such as flavonoids, resveratrol (Jang and others 1997), lignans, and isoflavonoids (Jung and others 2000) are currently under investigation as "nutraceuticals," plant constituents that are somehow beneficial to animal health, although not absolutely required (Briskin 2000). Several of these compounds are antioxidants and have other properties that are purported to decrease heart disease and cancer rates in humans. Genetic engineering studies allowing the accumulation of resveratrol (Hipskind and Paiva 2000) and genistein (Jung and others 2000) in crop plants that do not normally accumulate these compounds have already been successful and may allow increased human consumption of these beneficial metabolites in the future.

Recent advances in molecular techniques, expressed sequence tags (EST) and genome sequencing programs, and bioinformatics technology are accelerating the identification of biosynthetic genes, particularly those encoding enzymes difficult to purify using standard biochemical techniques. However, there are still many unanswered questions about the biosynthetic origins of compounds, the regulation of their accumulation, and the roles they play in plants. With new compounds constantly being identified, the list of questions may grow as fast as the questions are answered.

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