Chapter 6

THE ROLE OF PHENOLS IN PLANT DEFENSE

1. PREFORMED ANTIMICROBIAL AND INSECTICIDAL METABOLITES

When considering substances produced by plants that act as agents that protect the plant from pathogens and insect pests, we must first consider whether the compounds are present prior to the time of infection or whether they are synthesized in response to infection. When compounds are present prior to attempted infection they are known as *preformed antimicrobial metabolites*. Such preformed compounds are part of a *passive* resistance mechanism. In general, such preformed metabolites are toxic to a broad spectrum of fungi and bacteria, but the compounds have a relatively low level of toxicity. Thus, preformed compounds are present in all plant species and help plants to ward off pathogens that are not considered as highly aggressive organisms. They are also referred to as *phytoanticipins* (Van Etten et al., 1994).

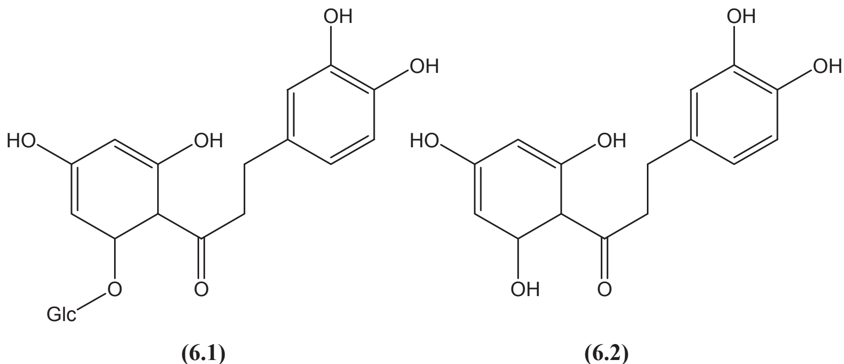
When one considers resistance expression in plants, it is necessary to consider whether resistance expression is part of a passive or active response system. There are several situations that could arise:

1. Compound "A" is present in the plant and is toxic to the potential pathogen. The compound is present in cells or tissues that the pathogen must come into contact with, at some time during the attempted infection. The compound is not further metabolized, but may or may not be changed by the pathogen, and the compound as such then accounts

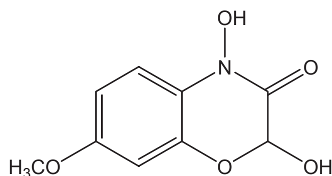
for toxicity to the pathogen. This example represents a form of *passive* resistance.
2. A preformed substance is degraded or metabolized to a different compound by the host in direct response to the pathogen and it is this compound that accounts in part for toxicity to the pathogen. Because the host changes the compound, this would be considered a mechanism of *active* resistance.

There are several criteria that must be satisfied before it can be decided whether a particular compound plays a significant role in the resistance to a pathogen. These are as follows:

1. The compound must be present in those parts of the plant where the pathogen will come in contact with it. For example, apple leaves contain phloridzin (**6.1**) and its aglycone phloretin (**6.2**), but these compounds are not present in the fruit.
2. The compound must be present at concentrations high enough to affect the pathogen. For example, maize contains the preformed cyclic hydroxamic acid DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; **6.3**). The concentration of DIMBOA, however, decreases over time and is initially not high enough for it to serve as a fungi-toxic agent.
3. Directly related to the criterion above is the requirement that the compound be "available" in the host in a form or place where it can express its toxicity. This may ultimately be a question of whether the compound is changed when it is extracted. Is the compound compartmentalized in a cell and possibly inaccessible to the pathogen? Is it in only one tissue?
4. Another criterion that must be met deals with the time of appearance of the compound. Thus, even if preformed, the compound must be at a sufficient *in vivo* concentration by the time resistance is being expressed.



(6.1) **(6.2)**



(6.3)

Phenolic substances are often thought of when referring to preformed rsistance compounds. The following classes of metabolites, however, should also be included: alkaloids, carbohydrates that can bind bacteria, proteins that act as lectins, amino acids, terpenoids, and polyacetylenes. Some of these compounds will be included in the discussion below.

1.1 Chlorogenic acid

Chlorogenic acid (**6.4**) is an example of a preformed compound that has a relatively low level of toxicity to most microorganisms. It is effective against microorganisms considered as weak pathogens of potato. In potato tubers, chlorogenic acid is present in the periderm (Kojima et al., 1985) and is toxic to the organism that causes potato scab, *Streptomyces scabies*. In general, there is more of the compound in tubers of cultivars that are resistant to the pathogen than in tubers of cultivars that are susceptible. Furthermore, more of the compound is present during the time of tuber expansion. Chlorogenic acid also affects the growth of the vascular pathogen *Verticillium albo-atrum* and is present in the vascular tissue of the potato (Dao and Friedman, 1994). The mechanism by which *ortho*-dihydroxy phenolic compounds such as chlorogenic acid provide defense against insect pests was studied by Felton et al. (1989). They investigated the fate of chlorogenic acid in tomato (*Lycopersicon esculentum*) on the feeding behavior of the tomato fruit worm (*Heliothis zea*) and beet army worm (*Spodoptera exigua*). Upon feeding by the insects, polyphenol oxidases that are compartmentally separated from chlorogenic acid in the plant, come in contact with their substrate and convert chlorogenic acid to the toxic chlorogenoquinone (**6.5;** Figure 6-1). This quinone is a highly reactive electrophile and will react with nucleophilic –SH and $-NH_2$ moieties in proteins (as indicated by structure **6.6;** Matheis and Whitaker, 1984). This results in the cross-linking of proteins with chlorogenic acid,, which reduces the availability of free amino acids and proteins to the insect.

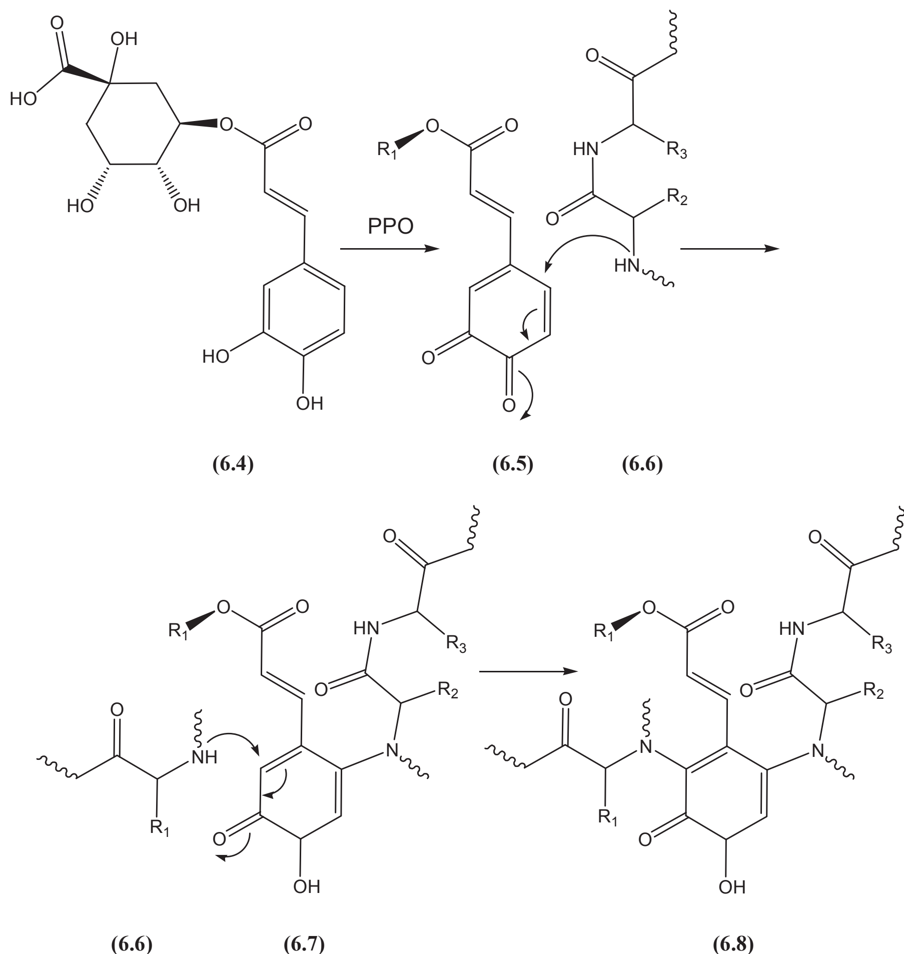


Figure 6-1. Oxidation of chlorogenic acid (**6.4**) by polyphenoloxidase (PPO), resulting in chlorogenoquinone (**6.5**), which can react with nucleophilic groups in proteins (**6.6**) to give the cross-linked compound **6.7**, which can react with another protein molecule to yield **6.8**. The quinate residue in structures **6.5**, **6.7** and **6.8** is represented by R, whereas R_1 and R_2 indicate different amino acid residues.

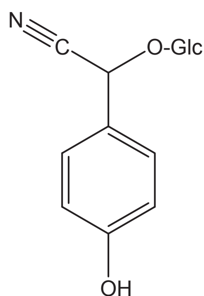
1.2 Phloridzin and phloretin

In apple, the glycoside phloridzin (**1.27**) and its aglycone phloretin are thought to inhibit the apple scab fungus *Venturia inaequalis*. Phloridzin is also an *ortho*-dihydroxyphenolic compound, and, like chlorogenic acid, can also be easily converted to a reactive quinone upon attack by a pathogen. Raa (1968) demonstrated that oxidation products of phloridzin inhibit fungal

pectinases. Fungal pectinases hydrolyze pectin, a cell wall compound that is abundant in the middle lamella and plays a role in cell adhesion. Thus, by inhbiting pectinases, the ability of the fungus to hydrolyze and invade the plant cell wall would be compromised. Although phloridzin and phloretin are toxic at high concentrations, based on the poor correlation between resistance to scab and the concentration of phenolic compounds such as phloridzin, they are probably not the factors that account for the actual resistance of apple cultivars to this fungus (Nicholson and Rahe, 2004). This was further substantiated by Leser and Treutter (2005), who investigated the effect of nitrogen supply on the scab susceptibility of the susceptible apple cultivar 'Golden Delicious' and the resistant cultivar 'Rewena'. Increased nitrogen supply was hypothesized to stimulate growth and decrease the levels of phenolic compounds. This was shown to indeed be the case. Consistent with this hypothesis, the susceptible cultivar became more susceptible under high nitrogen supplements. The resistant cultivar, however, did not become susceptible, even though the levels of phenolic compounds, including phloridzin, decreased.

1.3 Cyanogenic glycosides

Many plants contain cyanogenic glycosides. Toxicity of the cyanogenic glycoside results when the compound is enzymatically cleaved to release hydrogen cyanide (HCN) that is toxic to the pathogen. Sorghum contains the cyanogenic glycoside dhurrin (**6.9**). This compound is of interest to both pathologists and entomologists as an example of a preformed resistance compound and acts as an insect feeding deterrent and as a fungicidal agent (Starr et al., 1984; Adewusi, 1990).



(6.9)

1.4 Tuliposides

Tulips contain preformed compounds known as tuliposides, (tuliposide A, **6.10**, and tuliposide B, **6.11**). Hydrolysis of the tuliposides results in formation of aglycones (**6.12**) and (**6.13**) which will form butyrolactones (**6.14**) and (**6.15**). These lactone forms are inhibitory to fungi.

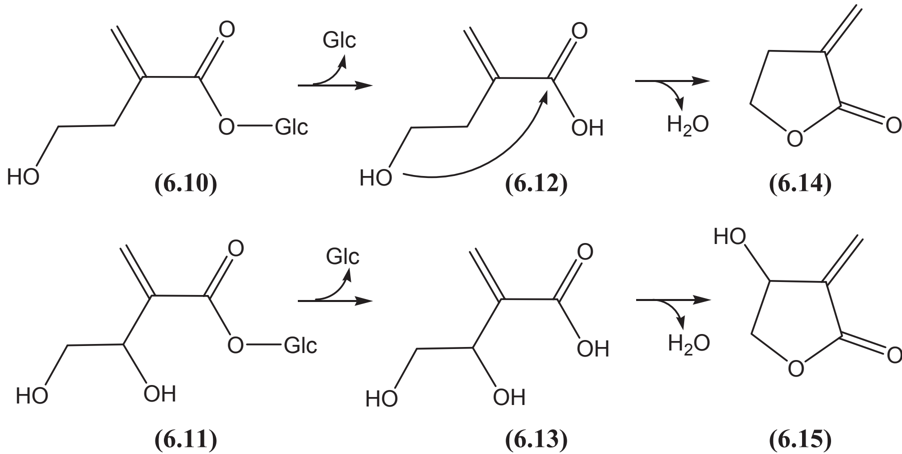
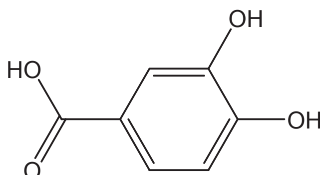


Figure 6-2. Formation of antifungal butyrolactones from tuliposides *via* an internal esterification reaction.

Some fungi are able to metabolize the lactone form to a butyric acid form that is not inhibitory to a variety of organisms, including the tulip pathogen *Fusarium oxysporum*. Two important diseases of tulip are bulb rot and grey mold. *Botrytis tulipae* infects all parts of the tulip, including the pistils, macerating the tissue. In contrast, *Botrytis cinerea* does not develop on tulips in the field, but eventually infects various parts of the plant when kept in a high humidity chamber. *B. cinerea* never infects the flower pistils because they contain exceptionally high concentrations of tuliposides. Tuliposides are stored in cell vacuoles. Importantly, growth of the pathogen is at first intercellular. Under these conditions the pathogen does not encounter the tuliposides. It is only when the tuliposides are released from the vacuoles that the effects of their toxicity can be expressed. *B. cinerea* converts tuliposides into inhibitory lactones. *B. tulipae* converts tuliposides into hydroxycarboxylic acids which are non-toxic (Schönbeck and Schroeder, 1972).

1.5 Protocatechuic acid

Probably the most commonly referred to compound which accounts for a form of passive resistance of a chemical nature is protocatechuic acid (**6.16**) which is found in yellow and red skinned onions and prevents the germination of spores of the onion smudge fungus, *Colletotrichum circinans*. Thus, protocatechuic acid serves as a barrier to infection prior to penetration. Once penetration occurs the compound is ineffective.



(6.16)

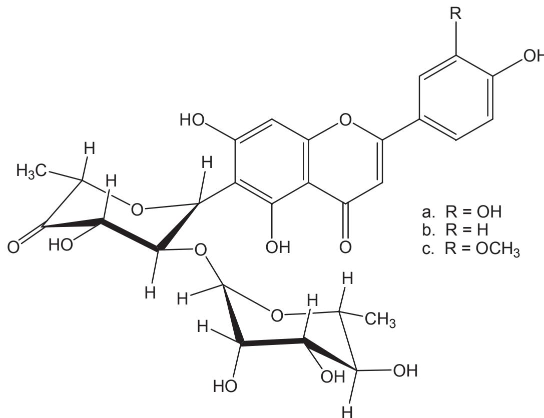
1.6 Lignin

The cell wall polymer lignin (see Chapter 1, Section 3.12, and Chapter 3, Section 12) has been implicated in defense against pests and pathogens, as a preformed, passive defense compound. It is a suitable defense compound in that respect, because it hardens the cell wall and thus creates a physical barrier against invasion. In addition, the chemical structure of lignin is very complex because of the different lignin subunits, and the many different types of chemical bonds that exist between subunits. Hydrolysis would, therefore, require a battery of enzymes, which is something most pests and pathogens do not have access to (Vance et al., 1980; Denton, 1998). Exceptions are white rot fungi, which can oxidize lignin in order to degrade it (Chen and Chang, 1985). This is how fallen tree logs eventually disintegrate. There is evidence that lignin can also be synthesized *de novo*. This lignin is synthesized locally, and specifically in response to pathogenic attack. This mechanism resembles papilla formation (Cadenagomez and Nicholson, 1987) and will be discussed in the next section.

1.7 C-glycosyl flavones

Maysin (2´´-*O*-α-l-rhamnosyl-6-C-(6-deoxy*xylo*-hexos-4-ulosyl)-luteolin; **6.17a**), apimaysin (**6.17b**) and methoxymaysin (**6.17c**) are C-glycosyl flavones that confer resistance against the corn earworm (*Helicoverpa zea* (Boddie)), a major silk- and kernel-feeding insect pest in

the United States. These three compounds differ from each other in the substitution pattern of the B-ring: apimaysin has one hydroxyl group on the 4′ position of the B-ring, maysin has a 3′,4′-dihydroxy substitution pattern, and methoxymaysin has a 4′-hydroxy, 3′-methoxy substitution pattern. These compounds accumulate in the silks of maize (*i.e.* the styles attached to the ovules) and are thought to act in a manner similar to chlorogenic acid (see Figure 6-1) when insects damage the silks.



(6.17)

Maysin is generally the most abundant of these three compounds, and is typically present at concentrations of 0.3% fresh silk weight, which is very high for a single compound. As a consequence, the C-glycosyl flavones can be considered preformed defense compounds.

The concentration of C-glycosyl flavones varies considerably among different maize lines. Since the concentration of the C-glycosyl flavones does not have a discrete value, but rather varies along a continuum, it can be considered a quantitative trait (see Chapter 3, Section 3.4). In order to identify loci controlling maysin concentration in silks, Byrne et al. (1996) investigated the role of a number of structural and regulatory genes known to play a role in flavonoid biosynthesis. They generated an F2 population derived from the maize inbred lines GT119 and GT114, which had low (0.031%) and high (0.56%) maysin levels, respectively. They determined the genotype at a number of loci at or near flavonoid biosynthetic genes, and concluded that the *P1* (‘P-one’; see Chapter 3, Section 9.2) and a locus referred to as *recessive enhancer of maysin* (*rem1*) near the *Brown pericarp1* (*Bp1*) locus accounted for 58 and 11% of the variance, respectively. In addition, a QTL near the centromere of chromosome 1 was uncovered, but there were no obvious candidate genes at this locus.

The effects of the *P1* gene and the tightly linked homolog *P2* on maysin biosynthesis were further investigated by Zhang et al. (2003). These researchers used introduced the *P1* and *P2* cDNA's under control of the ubiquitin promoter in cultured Black Mexican Sweet maize cells using microprojectile bombardment. This is a method in which small gold or tungsten particles coated with an expression construct are introduced into target cells using a burst of pressure. Based on gene expression studies, P1 and P2 activate the same genes, including *phenylalanine ammonia lyase* (Chapter 3, Section 7), *chalcone synthase*, *chalcone isomerase*, and *dihydroflavonol 4-reductase* (Chapter 3, Section 9), but not genes involved in the biosynthesis of flavonols and anthocyanins. Increased levels of flavones were detected in extracts obtained from the transformed cells. Further evidence for a role of *both* genes in maysin biosynthesis came from the observation that maize plants in which both *P1* and *P2* were deleted did not produce maysin, whereas plants in which *P1* was deleted, but *P2* was still present, still produced maysin, albeit at reduced levels.

Maysin is two times more effective in its ability to inhibit growth of the corn earworm, which is attributed to the fact that two neighboring hydroxyl groups (such as on the on the 3′ and the 4′ positions in maysin) will result in the efficient formation of a toxic quinone, whereas the quinone formation from apimaysin and methoxymaysin is less efficient (Elliger et al., 1980; Snook et al., 1994). The genetic basis of the substitution reactions of the B-ring have been the subject of several studies. Using an F2 population derived from the maize inbred lines GT114 (moderately high levels of maysin, negligible levels of apimaysin) and NC7A (moderately high levels of apimaysin, maysin, and chlorogenic acid (**6.4**), Lee et al. (1998) determined that the *rem1* locus identified by Byrne et al. (1996) explained 55% of the variance for maysin, whereas a QTL that mapped near the *Pr1* gene, which is thought to encode flavonoid 3′ hydroxylase (F3′H), explained 65% of the variance for apimaysin. Furthermore, the levels of maysin and apimaysin were independent of each other, suggesting these two compounds are synthesized *via* different pathways. Surprisingly, a functional *Pr1* gene was not required for maysin production. Lee et al. (1998) speculated that the actual gene responsible for maysin biosynthesis may be near *Pr1*, but does not have to be *Pr1*, that a second *F3′H* gene is responsible for maysin production, or that the hydroxylation at C3′ occurs at the level of the hydroxycinnamoyl CoA ester rather than at the level of the flavone.

The genetic control of the substitution of the C-glycosyl flavones was investigated in further detail by Cortés-Cruz et al. (2003). Two F2 populations were generated from maize inbred lines that differed from each

other in the relative concentrations of maysin, apimaysin, and methoxymaysin. In both F2 populations the main QTL associated with levels of chlorogenic acid, maysin and methoxymaysin was located on the short arm of chromosome 4, whereas the main QTL associated with levels of apimaysin was located on the long arm of chromosome 5. Presence of a specific allele in the QTL on chromosome 4 resulted in higher levels of methoxymaysin and lower levels of maysin and chlorogenic acid. The fact that a single QTL affects the concentrations of three compounds (methoxymaysin, maysin and chlorogenic acid) suggests that there may be a regulatory gene underlying the QTL, or that there is a branched rather than a linear biosynthetic pathway leading to these different compounds. The QTL for apimaysin on chromosome 5 coincided with the *Pr1* locus, consistent with the data reported by Lee et al. (1998).

The biosynthetic pathway leading to maysin starts with flavanone (**6.18**), which is hydroxylated by flavone 3′ hydroxylase to yield di-hydroxyl flavanone (**6.19**) and is the reduced by flavone synthase to the flavone luteolin (**6.20**). The next steps were recently investigated in more detail by McMullen et al. (2004) using two *salmon silk* mutants, *sm1* (Anderson, 1921) and a newly discovered mutant *sm2*. These mutants have salmon colored silks instead of green silks as a result of pigment accumulation throughout the shaft of the silks, as opposed to only in the silk hairs, but do require a functional *P1* gene in order for the mutant phenotype to be apparent (see also Chapter 3, Section 9.2).

Detailed chemical analyses of flavone composition in the silks in wild-type, *sm1*, *sm2* and *sm1-sm2* plants revealed that isoorientin (**6.21**) is the only flavone accumulating in *sm1-sm2* double mutants, indicating the synthesis of this compound precedes the action of the gene products of the functional *Sm1* and *Sm2* genes. Isoorientin (**6.21**) is present at high levels in *sm2* but not *sm1* mutants, so that a functional *Sm2* gene is required for the formation of rhamnosyl-isoorientin (**6.22**) from isoorientin. The *Sm2* gene may encode a rhamnosyl transferase, or control the expression of a *rhamnosyl tranferase* gene. Rhamnosyl-isoorientin (**6.22**) accumulates in *sm1* mutants, suggesting that *Sm1* encodes a protein that catalyzes the formation of maysin (**6.17a**), or otherwise controls the expression of gene(s) encoding the necessary enzymes.

Taking all of the abovementioned data into consideration, the most likely biosynthetic pathway leading to maysin is as shown in *Figure 6-2*, although further research is needed to fully elucidate the pathway and the regulatory genes.

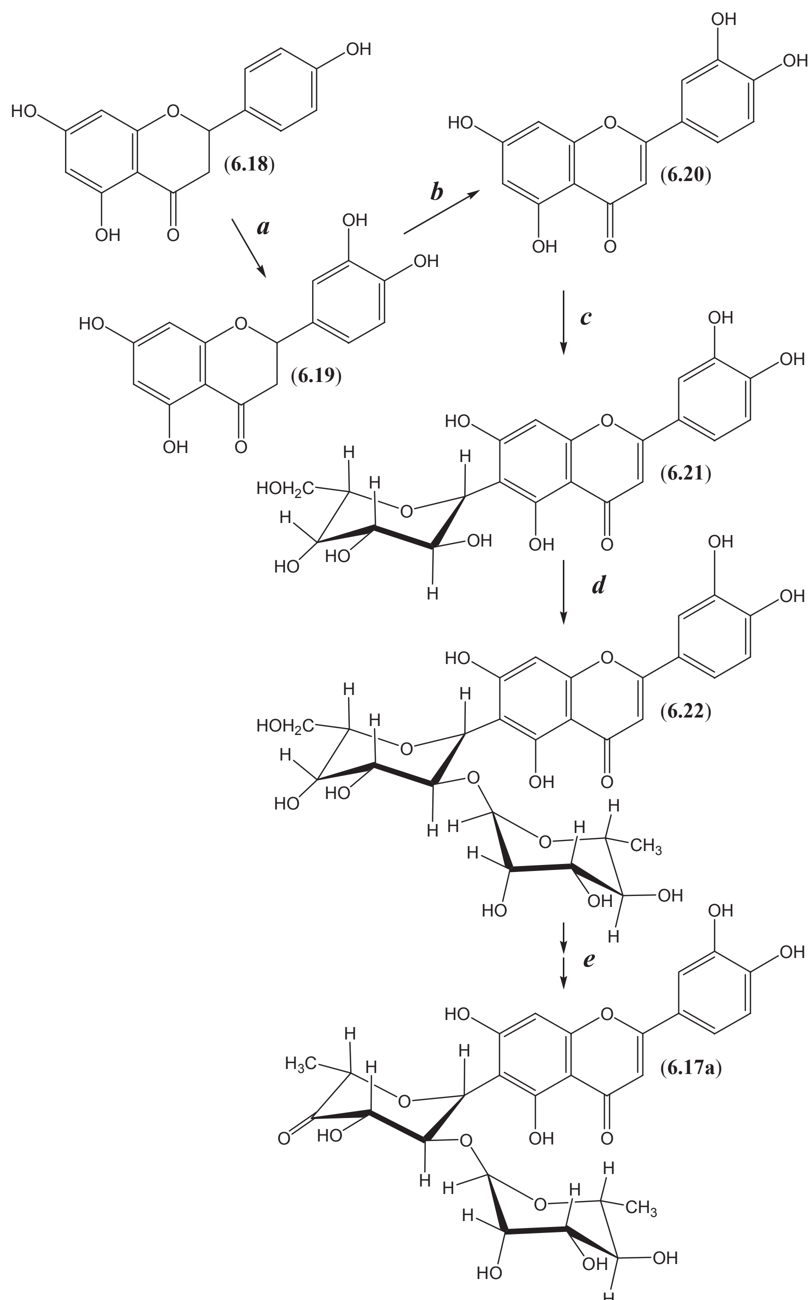


Figure 6-2. Biosynthesis of maysin proposed by McMullen et al. (2004) based on the analysis of flavones in the silks of maize *salmon silk* mutants. **a.** flavone 3´ hydroxylase (encoded by the maize *Pr1* gene), **b.** flavone synthase, **c.** *C*-glucosyltransferase, **d.** putative rhamnosyl transferase (encoded by the *Salmon silk2* gene), **e.** the step(s) controlled by the *Salmon silk1* gene.

2. COMPOUNDS FORMED IN RESPONSE TO PATHOGEN ATTACK

Compounds formed in response to stress may occur in at least two ways. In one response, the plant may form compounds throughout the tissue at a considerable distance from the infection site (Hammerschmidt, 1999). In another response, the plant may form compounds specifically at the infection site. This may include only a few cells and in rare cases, as few as one or two cells. (Snyder and Nicholson, 1990; Nicholson and Wood, 2001). In general, such compounds are referred to as either stress metabolites or more often as *phytoalexins*. By definition phytoalexins are formed in response to infection (Aguero et al., 2002; Lo et al., 2002; Hammerschmidt and Nicholson, 2001; Lo and Nicholson, 1998). Phytoalexins often exhibit toxicity to specific pathogens. In this case there is a genetic relationship between the expression of phytoalexin synthesis and the organism that induces that synthesis (Essenberg et al., 1985).

2.1 3-Deoxyanthocyanidins

The 3-deoxyanthocyanidins are a class of phytoalexins found in sorghum. These compounds are so fungi-toxic that they are effective at femtogram levels (Snyder and Nicholson, 1990; Nicholson and Wood, 2001). The synthesis of these compounds is initiated on the endoplasmic reticulum. Compounds are then trafficked in subcellular inclusions. The inclusions appear similar to vesicles, but there is no evidence that membranes surround the inclusions (Snyder and Nicholson, 1990). Nielsen et al. (2004) recently summarized this defense response. The cytological response commences when clear, colorless inclusions (less than 0.1. µm in diameter) accumulate in leaf cells under fungal attack. The inclusions eventually are seen as red bodies at the infection site. When the 3-deoxyanthocyanidins enter the apoplast, the host cell collapses. The phytoalexins then accumulate in the pathogen and cause its death. Excess phytoalexins are trapped in host cell walls at infection sites (Lo et al., 1998; 1999).

In the publication by Nielsen et al. (2004) images of the pigmented inclusions that contain the phytoalexins were prepared by confocal microscopy. This provided a three-dimensional perspective of inclusion body formation and visualization of the phytoalexins. A representation of deoxyanthocyanidin accumulation is shown in *Figure 6-3* where inclusions begin to form by 5 to 8 hours after a fungal appressorium was formed by a hypha.

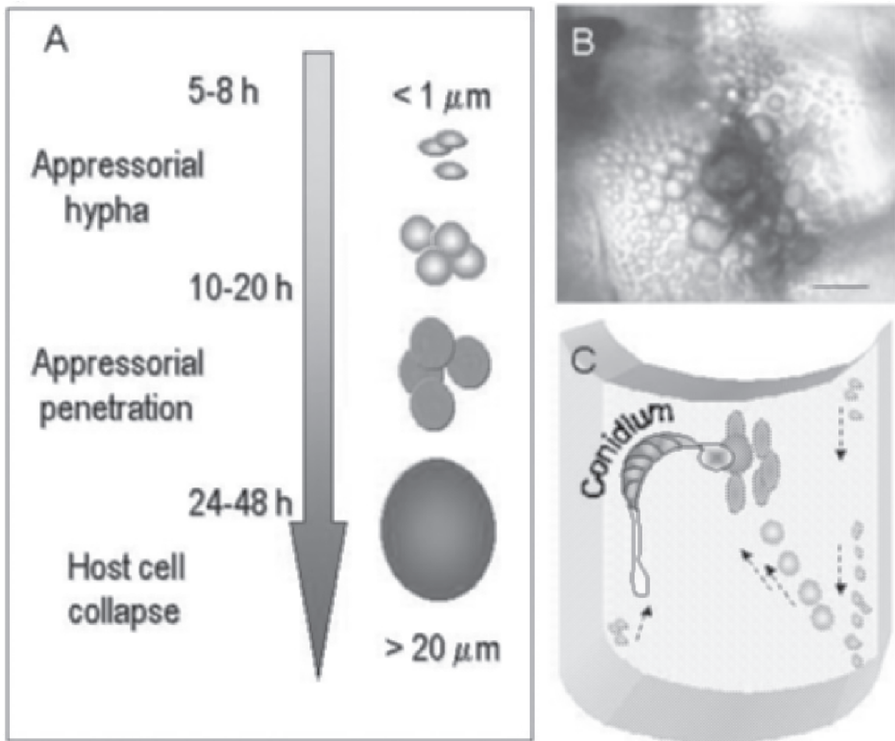


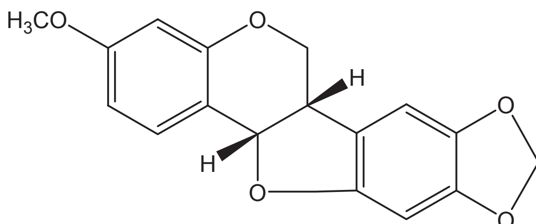
Figure 6-3. Cell-specific accumulation of 3-deoxyanthocyanidins in *Sorghum bicolor* in response to attempted fungal attack. (**A**) Illustration of changes in inclusion morphology in cells under fungal attack in response to formation of infectious structures 0–48 h after inoculation. (**B**) Site-specific accumulation of 3-deoxyanthocyanidins at site of incipient penetration, before host cell collapse 24 h after inoculation. (**C**) Illustration of site-specific trafficking (arrows) of inclusions in relation to position of fungal infectious structures. Reprinted from Phys. Mol. Plant Pathol., 65, Nielsen, K. A., Gottfredsen, C. H., Buch-Pedersen, M. J., Ammitzbøll, H., Mattsson, O., Duus, J. Ø., and Nicholson, R. L., Anti-microbial flavonoid 3-deoxyanthocyanidins in *Sorghum bicolor* self-organize into spherical structures, 187-196, Copyright 2004, with permission from Elsevier.

Note that the inclusions at this early time are not pigmented; rather they are colorless bodies that move through the cytoplasm toward the site of appressorial attack. Over time, the inclusions take on a yellow color and eventually become deep red in pigmentation. Inclusion size changes from less than 1 µm to 20 µm or even larger. Inclusions move to the penetration site and cluster in the area where the penetration peg has made physiological contact with the host cell. When the appressorium begins the process of penetration the inclusions burst, releasing their contents into the cytoplasm. The deoxyanthocyanidins kill the host cell itself *and* are taken up by the

pathogen. This is possible because these deoxyanthocyanidins are soluble in both water and organic solvents. In this manner the pathogen is also killed and prevented from causing extensive damage and cell death of the host.

2.2 Pisatin

Pisatin (**6.23**) is an isoflavonoid phytoalexin that is synthesized by pea (*Pisum sativum* L.) as a response to infection (Preisig et al., 1989). Subsequently, it was shown that pathogens capable of demethylating pisatin were tolerant of this phytoalexin. The enzyme responsible for demethylation is a specific cytochrome P450 mono-oxygenase released by the fungus *Nectria haematococca* (Delserone et al., 1999).

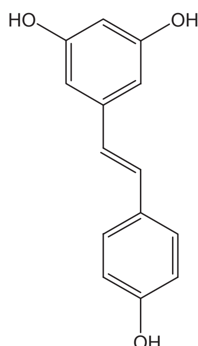


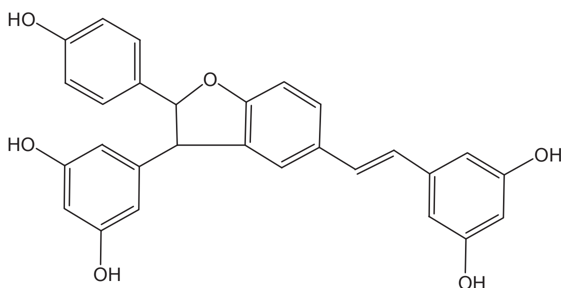
(6.23)

2.3 Stilbenes

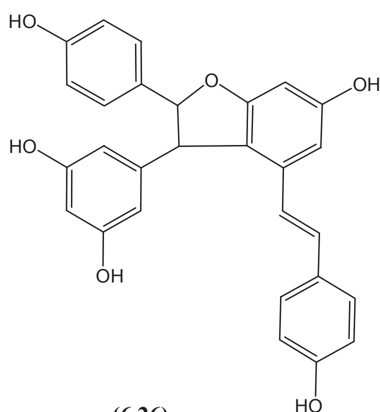
Aside from being UV-protectants, in a number of species certain stilbenes act as phytoalexins. Resveratrol (**6.24**; *trans*-3,5,4′–trihydroxystilbene), its *cis*-isomer, as well as their glucosides and dehydrodimer *trans*-ε-viniferin (**6.25**) are present in grape leaves and berries and play a role in the defense against gray rot caused by the fungal pathogen *Botrytis cinerea*.

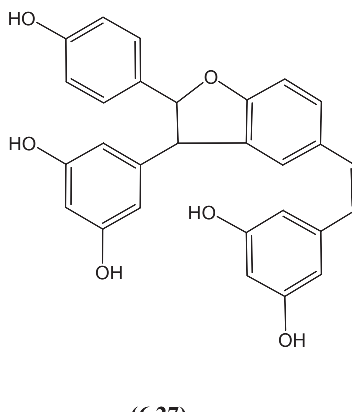
Viniferin is synthesized by a grape peroxidase (Morales et al., 1997). The fungus in return is able to inactivate resveratrol through the action of a laccase-like stilbene oxidase (Breuil et al., 1998). This results in the formation of resveratrol *trans*-dehydrodimer (**6.26**), as well as the corresponding *cis*-dehydrodimer (**6.27**), both of which structurally resemble viniferin.





(6.24) **(6.25)**





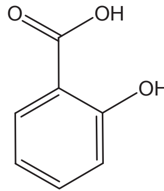
(6.26) **(6.27)**

2.4 Salicylic acid

When a specific part of a plant is attacked by a fungal pathogen, distant parts of the plant may display an enhanced state of resistance involving the accumulation of pathogenesis-related (PR) proteins, a class of plant proteins that are normally not present but that are induced upon pathogen attack (reviewed by Van Loon and Van Strien, 1999). In addition, salicylic acid (SA; **6.28**) and hydrogen peroxide accumulate at the wound site and in other parts of the plant. This response is referred to as *systemic acquired resistance* (SAR) and is thought to be mediated by one or more signaling molecules in the phloem.

SA was hypothesized to be one of those signaling molecules, because **1**) SA accumulation was shown to be correlated with SAR and resistance (Uknes et al., 1993), **2**) exogenous SA applied to an uninfected plant induced SAR and resistance in a manner similar to that of an infected plant (Ward

et al., 1991), and **3**) transgenic plants expression the *nahG* gene from *Pseudomonas putida*, which encodes a salicilate hydroxylase, were unable to display SAR (Gaffney et al., 1993).



(6.28)

SA was hypothesized to be one of those signaling molecules, because **1**) SA accumulation was shown to be correlated with SAR and resistance (Uknes et al., 1993), **2**) exogenous SA applied to an uninfected plant induced SAR and resistance in a manner similar to that of an infected plant (Ward et al., 1991), and **3**) transgenic plants expression the *nahG* gene from *Pseudomonas putida*, which encodes a salicilate hydroxylase, were unable to display SAR (Gaffney et al., 1993). While this latter study demonstrated the role of SA in initiating SAR, it did not address whether SA was the signaling molecule that transmitted the SAR signal through the phloem to other parts of the plant. Vernooij et al. (1994) performed a series of elegant experiments to investigate the role of SA in signaling. They grafted a scion from a transgenic tobacco plant expressing the *NahG* gene onto the root stock of an untransformed tobacco plant. In addition, an untransformed scion was grafted onto a transgenic rootstock. Ungrafted plants and plants where the scion was grafted back on the rootstock from which it came were used as controls. The root stocks were inoculated with the viral pathogen tobacco mosaic virus (TMV) to induce SAR. The degree of SAR was evaluated by challenging the scions of the inoculated plants 7 days later with TMV or the fungal pathogen *Cercospora nicotianae*. In the untransformed graft control, the lesions induced by TMV were 41% smaller compared to a mock-inoculated control. This indicated that the SAR signal was not hampered by the graft. When the scions of the transgenic grafted plants were inoculated, the lesion size was the same as in the corresponding mock-inoculated control. This indicated that the expression of the *NahG* gene prevented SAR, as had been shown by Gaffney et al. (1993). The TMV-inoculated transgenic scions on untransformed root stocks behaved similarly as the mock-inoculated controls, indicating that SA was required to induce SAR in the scions. When untransformed scions on transgenic root stocks were inoculated with TMV, however, they displayed SAR. This reveals that the *NahG*-expressing tissues were able to transmit the signal required for

SAR. Similar results were obtained when the SAR response was induced by *C. nicotianae*, suggesting that SAR is a response to a broad range of pathogens. These experiments thus demonstrated that SA is required for the induction of SAR, but that it is not the actual signaling molecule.

After an additional 10 years of research it is still not entirely clear what the signaling molecule is. Van Bel and Gaupels (2004) recently reviewed the possible signaling molecules that could induce SAR. The list includes jasmonic acid, lipid-derived molecules, reactive oxygen species (see Chapter 2, Section 1.9), oligosaccharides, mRNA molecules, calcium, and various peptides.

2.5 Lignin

There is evidence that lignin can be synthesized *de novo*. This lignin is synthesized locally, and specifically in response to pathogen attack. There is new evidence that this lignin requires different biosynthetic enzymes, which results in a different subunit composition than the lignin of the vascular tissue.

Wheat cultivars resistant to *Puccinia recondita* f. sp. *tritici*, a fungal pathogen causing leaf rust, were shown to accumulate more lignin than susceptible cultivars, based on histochemical stains and a quantitative assay to detect total phenolics (Southerton and Deverall, 1990). Similar results were reported for the response of wheat to infection with *Fusarium graminearum*, which causes Fusarium head blight. In this case immunogold labeling against lignin was used to evaluate the accumulation of lignin in inoculated and non-inoculated spikes of a resistant and susceptible cultivar. Labeling densities were significantly higher in inoculated spikes of the resistant cultivar, compared to either non-inoculated spikes and inoculated spikes of the susceptible cultivar (Kang and Buchenauer, 2000).

Several studies have focused on the role lignin biosynthetic enzymes play in response to pathogenic attack. Moerschbacher et al. (1990) used specific inhibitors of the enzymes phenylalanine ammonia lyase (PAL) and cinnamyl alcohol dehydrogenase (CAD). These inhibitors were applied to wheat cultivars highly resistant to stem rust (*Puccinia graminis* Pers f.sp. *tritici* Erics. & E. Henn.). Regardless of the inhibitor that was used, *de novo* lignification was decreased and fungal development increased. Thus, a strict correlation between resistance and lignification was demonstrated.

Lignification in response to infection has been found to be associated by an increase activity of several enzymes of the lignin biosynthetic pathway. When a wheat lines carrying the stem rust resistance gene *Sr5* was compared with a near-isogenicline without this resistance gene, different patterns of enzymatic activities were observed (Moerschbacher et al., 1988). Both lines had an early activation of the enzymes PAL, 4-coumarate:CoA ligase (4CL), and CAD, but only the resistant genotype exhibited a *second* significant increase at the time of the hypersensitive response. Similarly, Mitchell et al. (1994) measured *p*-coumaryl, coniferyl and sinapyl alcohol dehydrogenase activity in lignifying leaves of wheat. Lignification induced by wounding or elicitors was found to be specifically associated with an increase in sinapyl alcohol dehydrogenase activity, which is expected to result in a lignin rich in syringyl units. In a subsequent study Mitchell et al. (1999) investigated the role of CAD in the defense response of wheat, mimicked by wounding or the application of the elicitors chitosan and chitin. Three major forms of CAD were identified, but only one, CAD-C, was found to be induced during lignification at the wound margin. This particular form had a substrate preference for sinapyl alcohol. Thus, in agreement with the previous study, *de novo* lignin could contain a high level of syringyl units.

Deborah et al. (2001) studied PAL and peroxidase (PO) activity as well as accumulation of lignin in response to inoculation of rice leaf sheaths with a pathogen and a non-pathogen. Infection with the non-pathogen *Pestalotia palmarum* resulted in a stronger increase in PO and PAL activity and a higher accumulation of lignin than after inoculation with the pathogen *Rhizoctonia solani*.

Altogether, these results indicate that if lignin plays a role in the resistance against a pathogen, a coordinated activation of lignin biosynthetic enzymes is required.

Two recent studies, one in maize and one in Arabidopsis, provided evidence for the existence of multiple copies of the same gene and a divergence in their function. Pichon et al. (1998) cloned two different maize cDNA's encoding cinnamoyl-CoA reductase (CCR) and found that the corresponding genes are differentially expressed in different parts of the plant. A similar situation was observed in Arabidopsis (Lauvergeat et al., 2001). The expression of the two Arabidopisis genes was studied during plant development, and in response to infection with the pathogenic bacteria *Xanthomonas campestris pv. campestris*. The *AtCCR1* gene was mainly expressed in lignifying tissues during development. In contrast, the *AtCCR2* gene had a low expression level during development, but was induced when

the plant was challenged with the bacteria. With the availability of whole genome sequences, it is apparent that many of the lignin biosynthetic genes are respresented by multiple copies, some of which are likely to be involved in defense responses (Raes et al., 2003).

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