

Preformed Antimicrobial Compounds and Plant Defense against Fungal Attack

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INTRODUCTION

Plants produce a diverse array of secondary metabolites, many of which have antifungal activity. Some of these compounds are constitutive, existing in healthy plants in their biologically active forms. Others, such as cyanogenic glycosides and glucosinolates, occur as inactive precursors and are activated in response to tissue damage or pathogen attack. This activation often involves plant enzymes, which are released as a result of breakdown in cell integrity. Compounds belonging to the latter category are still regarded as constitutive because they are immediately derived from preexisting constituents (Mansfield, 1983). VanEtten et al. (1994) have proposed the term "phytoanticipin" to distinguish these preformed antimicrobial compounds from phytoalexins, which are synthesized from remote precursors in response to pathogen attack, probably as a result of *de novo* synthesis of enzymes. In recent years, studies of plant disease resistance mechanisms have tended to focus on phytoalexin biosynthesis and other active responses triggered after pathogen attack (Hammond-Kosack and Jones, 1996, in this issue). In contrast, preformed inhibitory compounds have received relatively little attention, despite the fact that these plant antibiotics are likely to represent one of the first chemical barriers to potential pathogens.

The distribution of preformed inhibitors within plants is often tissue specific (e.g., Price et al., 1987; Poulton, 1988; Davis, 1991; Fenwick et al., 1992; Bennett and Wallsgrave, 1994), and there is a tendency for these compounds to be concentrated in the outer cell layers of plant organs, suggesting that they may indeed act as deterrents to pathogens and pests. Some diffusible preformed inhibitors, such as catechol and protocatechuic acid (which are found in onion scales), may influence fungal growth at the plant surface. In general, however, preformed antifungal compounds are commonly sequestered in vacuoles or organelles in healthy plants. Therefore, the concentrations that are encountered by an invading fungus will depend on the extent to which that fungus causes tissue damage. Biotrophs may avoid the release of preformed inhibitors by minimizing damage to the host, whereas necrotrophs are likely to cause substantial release of these compounds. The nature and level of preformed inhibitors to which a potential pathogen is exposed will also vary, depending on factors such as host genotype, age, and environmental conditions (Price et al., 1987; Davis, 1991).

There have been numerous attempts to associate natural variation in levels of preformed inhibitors in plants with resis-

tance to particular pathogens, but often these attempts have failed to reveal any positive correlation. However, whereas preformed inhibitors may be effective against a broad spectrum of potential pathogens, successful pathogens are likely to be able to circumvent the effects of these antibiotics either by avoiding them altogether or by tolerating or detoxifying them (Schönbeck and Schlösser, 1976; Fry and Myers, 1981; Bennett and Wallsgrave, 1994; VanEtten et al., 1995; Osbourn, 1996). The isolation of plant mutants defective in the biosynthesis of preformed inhibitors would allow a direct genetic test of the importance of these compounds in plant defense. However, in most cases this approach is technically difficult because of the problems associated with screening for loss of the compounds. In the absence of plant mutants, a complementary approach involving the study of fungal mechanisms of resistance to preformed inhibitors, and of the contribution of this resistance to fungal pathogenicity to the relevant host plants, offers another route toward investigating the importance of these inhibitors in plant defense.

A large number of constitutive plant compounds have been reported to have antifungal activity. Well-known examples include phenols and phenolic glycosides, unsaturated lactones, sulphur compounds, saponins, cyanogenic glycosides, and glucosinolates (reviewed in Ingham, 1973; Schönbeck and Schlösser, 1976; Fry and Myers, 1981; Mansfield, 1983; Kuć, 1992; Bennett and Wallsgrave, 1994; Grayer and Harborne, 1994; Osbourn, 1996). More recently, 5-alkylated resorcinols and dienes have been associated with disease resistance, in this case, resistance of subtropical fruits to infection by *Colletotrichum gloeosporioides* (Prusky and Keen, 1993). However, only a few classes of preformed inhibitor have been studied in detail to determine their possible roles in plant defense against fungal pathogens. This review focuses on three of these classes—saponins, cyanogenic glycosides, and glucosinolates—and summarizes our current knowledge of the role of these preformed inhibitors in determining the outcome of encounters between plants and phytopathogenic fungi.

SAPONINS

Because many saponins exhibit potent antifungal activity and are often present in relatively high levels in healthy plants, these

molecules have been implicated as determinants of a plant's resistance to fungal attack (Osbourn, 1996). A number of other properties are also associated with these compounds, including piscicidal, insecticidal, and molluscicidal activity; allelopathic action; and antinutritional effects (Price et al., 1987; Fenwick et al., 1992; Hostettmann and Marston, 1995).

Saponins are glycosylated compounds that are widely distributed in the plant kingdom and can be divided into three major groups, depending on the structure of the aglycone, which may be a triterpenoid, a steroid, or a steroidal glycoalkaloid. Triterpenoid saponins are found primarily in dicotyledonous plants but also in some monocots, whereas steroid saponins occur mainly in monocots, such as the *Liliaceae*, *Dioscoreaceae*, and *Agavaceae*, and in certain dicots, such as foxglove, which contains the saponin digitonin (Hostettmann and Marston, 1995). Oats (genus *Avena*) are unusual because they contain both triterpenoid and steroid saponins (Price et al., 1987). Steroidal glycoalkaloids are found primarily in members of the family *Solanaceae*, which includes potato and tomato, but also in the *Liliaceae* (Hostettmann and Marston, 1995). The saponins produced by oats and tomato have been studied in the greatest detail in relation to their potential role in the defense of plants against phytopathogenic fungi (Osbourn, 1996). These saponins are considered in depth below.

Avenacins and Avenacosides

Oats contain two different families of saponins, the triterpenoid avenacins (A-1, B-1, A-2, and B-2) and the steroidal avenacosides (avenacosides A and B; Price et al., 1987). The chemical structures of an example of each family (avenacin A-1 and avenacoside A) are illustrated in Figure 1. The occurrence of avenacins and avenacosides is restricted primarily to the genus *Avena*, although avenacins also occur in the closely related species *Arrhenatherum elatius* (Turner, 1953; Crombie and Crombie, 1986). The distribution of the two types of saponin within the plant is mutually exclusive: the avenacins are located in the root (Goodwin and Pollock, 1954; Maizel et al., 1964), and the avenacosides are located in the leaves and shoots (Tschesche et al., 1969; Tschesche and Lauven, 1971). Within these tissues, the major avenacin, avenacin A-1, is restricted to the root epidermal cells (Osbourn et al., 1994), whereas the avenacosides are present at higher concentrations in the epidermis than in the rest of the leaf (Kesselmeier and Urban, 1983).

The avenacins are referred to as monodesmosidic because they have a single sugar chain attached at the C-3 position (Figure 1A). They exist in the plant in an active form. The avenacosides are bisdesmosidic and have an additional D-glucose molecule attached to C-26 (Figure 1B). Avenacosides are biologically inactive but are converted into the antifungal monodesmosidic saponins 26-desglucoavenacosides A and B in response to tissue damage by a specific plant glucosyl hydrolase (oat avenacosidase), which cleaves the

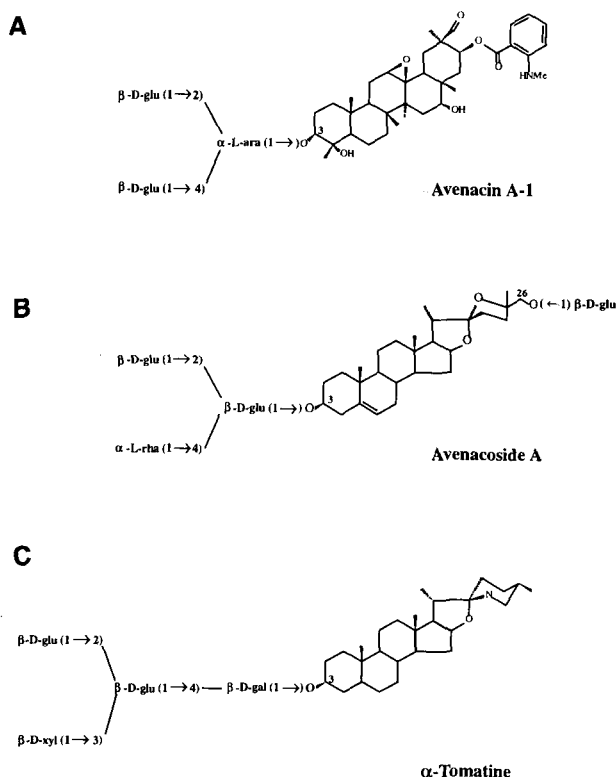


Figure 1. Structures of Oat and Tomato Saponins.

(A) The molecular structure of the major oat root saponin avenacin A-1 (a monodesmosidic triterpenoid). The structures of the other avenacins (not shown) are very similar: avenacin B-1, like avenacin A-1, is esterified with *N*-methyl anthranilic acid, whereas avenacins A-2 and B-2 are esterified with benzoic acid. The A-1 and A-2 compounds each contain one extra oxygen atom, relative to their B-1 and B-2 counterparts. All four avenacin compounds have the same trisaccharide group attached to C-3.

(B) The molecular structure of the bisdesmosidic foliar oat saponin avenacoside A. Avenacoside B (not shown) differs from avenacoside A only in that it has an additional β ,1-3-linked D-glucose molecule attached to the C-3 sugar chain.

(C) The monodesmosidic tomato steroidal glycoalkaloid, α -tomatine. These saponins all have a sugar chain attached to C-3 via oxygen. Avenacosides A and B each have an additional sugar moiety (D-glucose) attached via oxygen to C-26. Removal of this C-26 D-glucose by a specific oat glucosyl hydrolase (avenacosidase) converts the avenacosides to their biologically active monodesmosidic forms (26-desglucoavenacosides A and B).

D-glucose molecule at C-26 (Lüning and Schlösser, 1975; Nisius, 1988; Gus-Mayer et al., 1994a, 1994b).

There has been considerable interest in the importance of the triterpenoid avenacin saponins in determining the resistance of oats to the root-infecting fungus *Gaeumannomyces graminis* var *tritici*, which causes the disease known as "take-

all." Although *G. g. tritici* causes severe yield losses in wheat and barley, it is unable to infect oats, and unlike the oat-attacking variety of *G. graminis*, *G. graminis* var *avenae*, it is relatively sensitive to avenacins. Thus, the resistance of oats to *G. g. tritici* has been attributed to the presence of these saponins in oat roots (Turner, 1953).

Searches for natural variation in avenacin content have identified one diploid oat species (*A. longiglumis*) as lacking avenacin A-1 (Osbourn et al., 1994). Significantly, this species is susceptible to infection by *G. g. tritici*. Unfortunately, *A. longiglumis* does not hybridize readily with other diploid oat species that produce avenacins, making it difficult to test whether the ability to synthesize avenacins cosegregates with resistance to *G. g. tritici*. Recently, mutants of the diploid oat species *A. strigosa* lacking avenacin A-1 have been isolated after sodium azide mutagenesis (A.E. Osbourn, R.E. Melton, and M.J. Daniels, unpublished data). The mutants show increased susceptibility to isolates of both *G. g. avenae* and *G. g. tritici* and also to infection by other root-infecting fungi, such as the *Fusarium* species *F. avenaceum*, *F. culmorum*, and *F. graminearum*. These observations are consistent with a role for avenacin A-1 as a determinant of resistance of oats to a range of phytopathogenic fungi, although further genetic analysis is required to test whether the absence of this saponin and increased disease susceptibility are indeed causally related.

Natural variation in avenacoside content also exists in the genus *Avena*. *Avena* species belonging to the C-genome subgroup (*A. clauda*, *A. pilosa*, and *A. ventricosa*) are all known to lack both avenacosides A and B (Nisius, 1988), although there is no information in the literature to indicate whether the absence of these saponins is linked with increased susceptibility to disease.

The Tomato Saponin, α -Tomatine

The major saponin in tomato is the steroidal glycoalkaloid, α -tomatine. α -Tomatine is monodesmosidic and, like the avenacins, is present in healthy plants in its biologically active form. The sugar moiety attached to C-3 consists of two molecules of D-glucose and one each of D-galactose and D-xylose, and is known as β -lycotetraose (Figure 1C). The levels of this saponin are particularly high in the leaves, flowers, and green fruits of tomato (Roddick, 1974).

Despite the considerable variation in α -tomatine levels in the genus *Lycopersicon* (Courtney and Lambeth, 1977; Juvik and Stevens, 1982; Juvik et al., 1982; Rick et al., 1994), relationships between saponin content and disease resistance are not well documented. Attempts have been made to address the contribution of α -tomatine to specific resistance of near-isogenic tomato lines to the vascular wilt fungi *Fusarium oxysporum* f sp *lycopersici* (Smith and MacHardy, 1982) and *Verticillium albo-atrum* (Pegg and Woodward, 1986). No preferential accumulation of α -tomatine was seen in resistant

interactions when compared with compatible interactions, leading to the conclusion that α -tomatine does not appear to have a role in variety-specific resistance (although these experiments are complicated by the fact that both of these fungi are able to degrade α -tomatine enzymatically). However, it has been suggested that α -tomatine may play a secondary role in the variety-specific resistance of tomato to incompatible races of the biotroph *Cladosporium fulvum* and that release of the saponin from leaf cells as a consequence of an incompatible interaction may act to kill or contain the pathogen (Dow and Callow, 1978).

Mechanisms of Resistance of Fungi to Saponins

Fungi that invade saponin-containing plants must have strategies for protecting themselves from host saponins. For biotrophic fungi such as the tomato pathogen *C. fulvum*, which restricts its growth to the intercellular spaces of tomato leaves, this may be achieved simply by avoiding the release of α -tomatine. However, correlations have been made between the ability of various fungi to infect saponin-containing plants and the resistance of these fungi to the relevant saponins in vitro. These data suggest that for many fungi, saponin resistance may be a prerequisite for successful infection (Osbourn, 1996).

Membrane Composition

The toxic action of saponins to fungi is associated with the ability of these compounds to complex with membrane sterols and cause pore formation (Price et al., 1987; Fønwick et al., 1992). The membraneolytic action of α -tomatine is pH dependent (Roddick and Drysdale, 1984), and some tomato-infecting fungi, such as *Alternaria solani*, may counter the effects of α -tomatine by lowering the pH at the infection site to levels at which the saponin is ineffective as an antifungal agent (Schönbeck and Schlösser, 1976). However, a major mechanism of resistance to saponins is likely to reside in membrane composition (Steel and Drysdale, 1988; Keukens et al., 1995). Plants may protect themselves from their own saponins by compartmentalizing them in the vacuole or in other organelles, the membranes of which may avoid lysis due to low or altered sterol composition. Similarly, fungi such as the oomycetes *Pythium* and *Phytophthora* contain little or no sterols in their membranes and are resistant to saponins (Arneson and Durbin, 1968).

The importance of membrane composition is further emphasized by the demonstration that sterol-deficient mutants of a tomato-attacking isolate of *F. solani* showed increased resistance to the tomato steroidal glycoalkaloid α -tomatine and gained the ability to infect green fruits of tomato, which are particularly rich in α -tomatine (Défago and Kern, 1983). The wild type was pathogenic only to ripe tomato fruits, which contain relatively low levels of α -tomatine. Analysis of the progeny from crosses between mutant and wild-type fungi showed that pathogenicity to green tomato fruits, low sterol content, and

insensitivity to α -tomatine were always inherited together, suggesting that α -tomatine may be an important disease resistance determinant (Défago et al., 1983).

Saponin-Detoxifying Enzymes

Whereas modified membrane composition may allow some phytopathogenic fungi to tolerate saponins, a second major mechanism of resistance involves enzymatic detoxification (Osbourn, 1996). A number of fungal pathogens of oats or tomato produce specific glycosyl hydrolases that remove sugars from the sugar chain attached to C-3 of the saponin backbone. Removal of these sugars destroys the ability of the saponin to complex with membrane sterols; therefore, the products of hydrolysis have little or no antifungal activity. Thus, for at least some phytopathogenic fungi, the production of saponin-detoxifying enzymes may be a determinant of pathogenicity to saponin-containing hosts.

Avenacinase as a Host Range Determinant. The ability to detoxify avenacin A-1 has been shown to be essential for the interaction of *G. g. avenae* with oats (Bowyer et al., 1995), providing further evidence that avenacin A-1 may play a role in protecting oats against fungal attack. Isolates of *G. g. avenae* are avenacin resistant and produce the saponin-detoxifying enzyme avenacinase, a β -glucosyl hydrolase that removes β ,1-2- and β ,1-4-linked terminal D-glucose molecules from avenacin A-1 (Figure 1A; Turner, 1961; Crombie et al., 1986; Osbourn et al., 1991). Glycosyl hydrolases have been grouped into ~40 families on the basis of sequence similarity (Henrissat, 1991), and within this classification, the majority of β -glucosyl hydrolases fall into two of these families (family 1 and family 3). *G. graminis* avenacinase belongs to the latter family (Osbourn et al., 1995), which also includes a number of cellobiose-degrading β -glucosyl hydrolases from yeasts and *Trichoderma*.

The isolation of the gene encoding avenacinase allowed the generation of specific fungal mutants defective in saponin detoxification by targeted gene disruption (Bowyer et al., 1995). Fungal mutants lacking avenacinase showed increased sensitivity to avenacin A-1 and importantly were no longer able to infect oats, indicating that avenacinase is a determinant of pathogenicity to this host. However, the enzyme is not a general pathogenicity factor because the mutants retained full pathogenicity to the alternative host, wheat, which does not contain saponins. These experiments indicate that at least for the interaction of *G. g. avenae* with oats, the ability of fungi to detoxify saponins can determine host range.

Detoxification of α -Tomatine. A number of tomato-infecting fungi also produce saponin-detoxifying glycosyl hydrolases, which in this case act on α -tomatine. These enzymes are collectively referred to as tomatinases, but their mechanisms of action differ. Some remove a single terminal D-glucose molecule, whereas others release the intact β -lycotetraose group or its four individual monosaccharide components (Figure 1C;

reviewed in Osbourn, 1996). Two of these tomatinase enzymes (from *Septoria lycopersici* and *F. o. lycopersici*) have been studied in detail and are described below.

The *S. lycopersici* tomatinase enzyme removes a single terminal D-glucose molecule from β -lycotetraose by hydrolysis of a β ,1-2 linkage (Arneson and Durbin, 1967); therefore, its mode of action is similar to that of *G. graminis* avenacinase. However, the two enzymes are highly specific for their respective host plant saponins and until recently were not believed to be related. Purification of *S. lycopersici* tomatinase has now revealed that in fact this enzyme shares many properties (including immunological cross-reactivity) with avenacinase from *G. g. avenae* (Osbourn et al., 1995; Sandrock et al., 1995). A cDNA clone encoding tomatinase from *S. lycopersici* was isolated by using an avenacinase cDNA clone as a heterologous probe (Osbourn et al., 1995). The predicted amino acid sequence of tomatinase revealed that this enzyme is also a family 3 glycosyl hydrolase and that it shares 68% amino acid similarity with avenacinase, allowing for conservative substitutions. The potential now exists to test the role of this tomatinase enzyme in pathogenicity of *S. lycopersici* to tomato by targeted gene disruption, following the approach that was taken for *G. g. avenae* avenacinase.

The tomatinase enzyme from *F. o. lycopersici* has also been purified recently (Lairini et al., 1996). This enzyme has a different mechanism of action from the *S. lycopersici* tomatinase, releasing the intact β -lycotetraose group to give the aglycone tomatidine. In contrast to *S. lycopersici* tomatinase and *G. g. avenae* avenacinase, which both have molecular masses >100 kD, this tomatinase has a molecular mass of 50 kD. Determination of the sequence of the first seven N-terminal amino acids was insufficient to indicate any relatedness with the other saponin-detoxifying enzymes described above or with other known glycosyl hydrolases (Lairini et al., 1996).

Detoxification of Oat Leaf Saponins by *S. avenae*. The foliar pathogen of oats, *S. avenae*, is also capable of detoxifying saponins and produces an enzyme that is capable of sequential hydrolysis of all of the sugars from the sugar chains attached to C-3 of 26-desglucoavenacosides A and B, ultimately to give the corresponding aglycone, nuatigenin (Figure 1B; Wubben et al., 1996). This enzyme shares the trivial name of avenacosidase with the oat enzyme involved in avenacoside activation (Gus-Mayer et al., 1994a, 1994b), because avenacosides are substrates for both enzymes. However, the mechanisms of action of the two enzymes are clearly different because the oat avenacosidase is specific for the D-glucose molecule attached to C-26 and does not remove sugars from the sugar chain at C-3 (Lüning and Schlösser, 1975; Nisius, 1988), whereas the *S. avenae* avenacosidase removes L-rhamnose, followed by the remaining two (26-desglucoavenacoside A) or three (26-desglucoavenacoside B) D-glucose molecules (Wubben et al., 1996). *S. avenae* avenacosidase is highly specific for the steroidal foliar oat saponins and has no detectable activity toward the triterpenoid saponin avenacin A-1 (which is present in oat roots). Amino acid sequence anal-

ysis of purified *S. avenae* avenacosidase indicates that the enzyme clearly belongs to the same family of β -glycosyl hydrolases as *G. g. avenae* avenacinase and the *S. lycopersici* tomatinase (Osbourn et al., 1995; J.P. Wubben, M.J. Daniels, and A.E. Osbourn, unpublished data).

CYANOGENIC GLYCOSIDES

Over 2000 plant species are known to be cyanogenic and are able to form hydrocyanic acid (HCN) in response to tissue damage (Davis, 1991). Because of the toxicity of HCN to most organisms, primarily as a respiratory poison, HCN has generally been associated with defense against herbivores and pathogens (Fry and Evans, 1977; Hughes, 1991). HCN release is a consequence of the degradation of precursors (usually cyanogenic glycosides) by plant hydrolytic enzymes (Poulton, 1988). The hydrolysis of cyanogenic glycosides is initiated by cleavage of the carbohydrate moiety by one or more β -glycosyl hydrolases to give the corresponding α -hydroxynitrile. The resulting intermediate is then converted, either spontaneously or enzymatically in the presence of an α -hydroxynitrile lyase, to give HCN and an aldehyde or ketone. In all cyanogenic glycosides, the monosaccharide unit attached directly to the aglycone is β -D-glucose, although this glucose molecule may sometimes be modified by linkage to a second monosaccharide or by formation of a sugar ester (Davis, 1991). The release of hydrogen cyanide from a cyanogenic glucoside precursor is illustrated in Figure 2.

The tissues of healthy cyanogenic plants do not contain detectable HCN, suggesting that the cyanogenic glycosides are normally separated from the enzymes that catalyse HCN release. Compartmentalization may be at the tissue level or at the cellular or subcellular level (Poulton, 1988; Poulton and Li, 1994). The highest glycoside levels are often found in outer cell layers, suggesting a role in defense (Poulton, 1988). Plants are able to combat the toxic effects of the HCN that they produce by detoxification involving enzymes such as β -cyanoalanine synthase and rhodanese (Miller and Conn, 1980).

Over 300 cyanoglycosides have been identified, representing >50 distinct structures (Davis, 1991). Their biosynthesis involves the formation of hydroxynitriles from amino acid precursors (Hughes, 1991). The hydroxynitriles are then glycosylated to give a cyanoglycoside. The best known of these is amygdalin, which is responsible for the toxicity of bitter almonds and other rosaceous seeds. Two cyanoglycosides that are structurally related to each other, linamarin and lotaustralin, are found in a number of important plant species, including cassava (*Manihot esculenta*), flax (*Linum usitatissimum*), rubber (*Hevea brasiliensis*), and lima bean (*Phaseolus lunatus*), and in forage crops such as white clover (*Trifolium repens*) and bird's-foot trefoil (*Lotus corniculatus*; Davis, 1991). A number of grasses, including cereals, also are known to contain cyanoglycosides, the production of dhurrin by *Sorghum* species being a particularly well-known example.

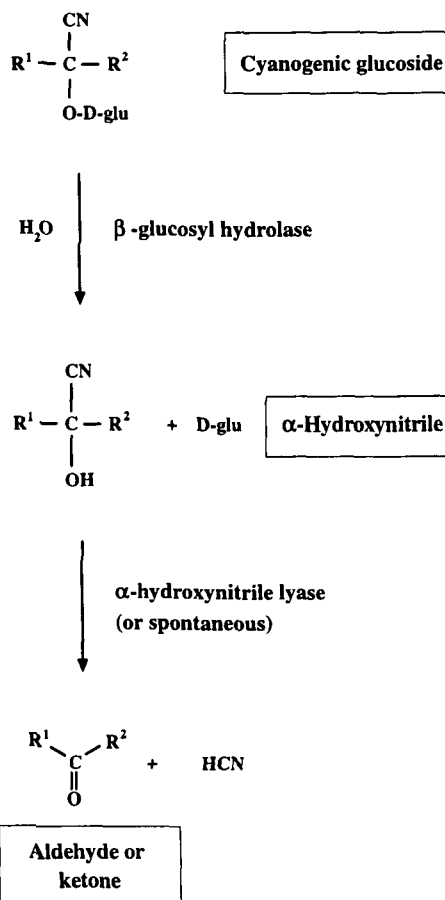


Figure 2. General Scheme for the Generation of Hydrogen Cyanide from a Cyanogenic Glucoside Precursor.

In intact plants, cyanogenic precursors (top) are separated by compartmentalization from the hydrolytic enzymes that activate them. In response to tissue damage, hydrolysis is initiated by cleavage of the carbohydrate moiety by one or more β -glycosyl hydrolases to give the corresponding α -hydroxynitrile (middle). The α -hydroxynitrile intermediate is then converted, either spontaneously or by the action of a plant α -hydroxynitrile lyase, to give hydrogen cyanide and an aldehyde or ketone (bottom). For simplicity, the cyanogenic precursor in this figure is illustrated as a cyanogenic glucoside, although additional sugar linkages have been described in other cyanogenic compounds (see the text).

Resistance of Fungi to Hydrogen Cyanide

Although there is considerable evidence that HCN may act as a feeding deterrent for grazing animals (Jones, 1981; Hughes, 1991), attempts to correlate cyanogenic glycoside levels with resistance to fungal pathogens have proved less conclusive (Fry and Myers, 1981). Indeed, there is evidence from some plant-fungus interactions that highly cyanogenic plant varieties are more susceptible to fungal attack than are low cyanogenic varieties. It has been demonstrated that, for the interaction between the fungus *Microcyclus ulei* and the

rubber tree *H. brasiliensis*, cyanogenesis inhibits the ability of the plant to produce the phytoalexin scopoletin (Lieberei et al., 1989). Thus, release of HCN in response to pathogen attack may cause inhibition of active defense responses in highly cyanogenic plants.

Although there is no consistent correlation between cyanogenic glycoside content and fungal disease resistance, there is a clear association between the ability of fungi to infect cyanogenic plants and their ability to tolerate HCN. For some fungi, such as the rubber tree pathogen *M. ulmi*, this tolerance has been attributed to cyanide-resistant respiration (Lieberei, 1988). However, fungi are also reported to detoxify HCN by various means (Fry and Myers, 1981). The majority of successful pathogens of cyanogenic plants produce the cyanide-inducible enzyme cyanide hydratase (CHT; formerly known as formamide hydrolyase), which detoxifies HCN by converting it to formamide (Fry and Evans, 1977; Wang et al., 1992). This enzyme activity appears to be specific to fungi and has not been found in bacteria or plants. A number of saprophytic fungi as well as some fungi that infect noncyanogenic plants also produce CHT, indicating (not surprisingly) that the ability to metabolize HCN is not the sole determinant of pathogenicity to cyanogenic plants (Fry and Evans, 1977; Wang et al., 1992).

CHT was first identified in the fungus *Stemphylium loti* (a pathogen of the cyanogenic plant bird's-foot trefoil; Fry and Millar, 1972) but has been studied most extensively in the sorghum pathogen *Gloeocercospora sorghi* (Fry and Myers, 1981; Wang et al., 1992) and also in *F. lateritium* (a pathogen of sweet potato and other plants; Cluness et al., 1993). The enzymes from *G. sorghi* and *F. lateritium* are very similar. They both exist as multimeric aggregates of a single protein with a molecular mass of ~45 kD and have relatively high K_m values for cyanide (12 and 43 mM for *G. sorghi* and *F. lateritium*, respectively). The corresponding genes have been cloned (Wang and VanEtten, 1992; Cluness et al., 1993), and the proteins have been found to share 75% identity at the amino acid level. The most closely related protein to these CHT enzymes is a nitrilase from *Klebsiella pneumoniae* that degrades the herbicide bromoxynil (3,5-dibromo-4-hydroxybenzoxynitrile; Stalker et al., 1988). The nitrilase shares ~35% amino acid sequence identity to both the *G. sorghi* and the *F. lateritium* CHT enzymes.

Mutants of *G. sorghi* generated by transformation-mediated disruption of the CHT gene were found to lack CHT activity in vitro and to show the anticipated increase in sensitivity to HCN (P. Wang, R.W. Sandrock, and H.D. VanEtten, personal communication). However, these mutants were still fully pathogenic to the cyanogenic host, sorghum (VanEtten et al., 1995). There was no evidence for CHT activity of the mutants in infected plants, eliminating the possibility that *G. sorghi* may have an alternative CHT enzyme that is expressed in the plant but not in vitro. It appears then that either the ability to tolerate HCN is not important for infection of sorghum by *G. sorghi* or this fungus has some alternative means of cyanide tolerance (for instance, cyanide-resistant respiration) that is expressed only in the plant. The significance of cyanide resistance in other pathogens of cyanogenic plants (including *F. lateritium*) remains to be tested.

GLUCOSINOLATES

Glucosinolates are sulphur-containing glucosides found in members of the family Cruciferae, including the agronomically important genus *Brassica* and the cruciferous weed *Arabidopsis*. The main interest in these compounds relates to their roles as attractants or as feeding deterrents in the interactions of *Brassica* with vertebrate and invertebrate pests (Chew, 1988; Mithen, 1992; Giamoustaris and Mithen, 1995). In contrast to saponins and cyanogenic glycosides, relatively few studies have been directed toward exploring the significance of glucosinolates in the resistance of plants to fungal attack. However, the increase in interest in this area merits the inclusion of these compounds in this review.

Glucosinolates can be subdivided into three major classes, depending on the nature of their side chains, which may be derived from aliphatic, indolyl, or aralkyl α -amino acids (Fenwick et al., 1983; Chew, 1988; Duncan, 1991; Mithen, 1992). Within individual plants, the distribution of glucosinolates is tissue specific. For instance, in oilseed rape, aliphatic glucosinolates predominate in the leaves, but indolyl and phenylethyl glucosinolates are the major glucosinolates in roots and stems (Mithen, 1992). Lines of oilseed rape that have been bred for reduced glucosinolate levels in the seed may retain high levels in the leaves, indicating that leaf and seed glucosinolate levels are under separate genetic control.

Like the bisdesmosidic saponins and cyanogenic glycosides, glucosinolates are also activated in response to tissue damage (Figure 3). Activation is by the plant enzyme myrosinase

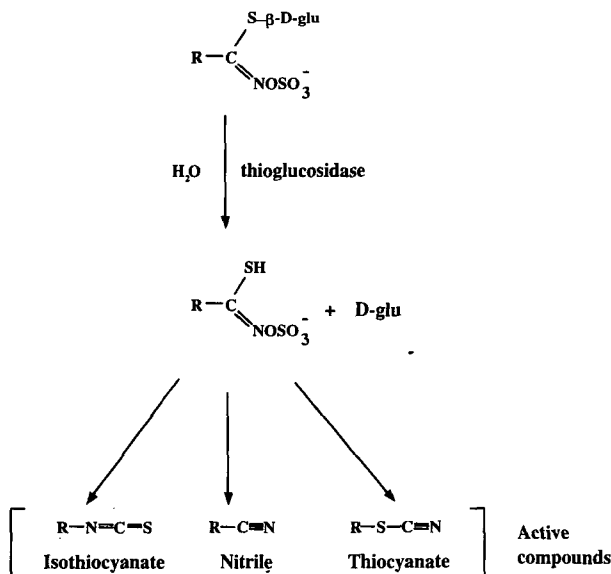


Figure 3. General Scheme for the Hydrolysis of Aliphatic Glucosinolates.

Glucosinolates (top) are activated in response to tissue damage by the plant enzyme myrosinase (a thioglucosidase). The unstable aglycone generated by the action of myrosinase (middle) may then break down into a variety of toxic products, including isothiocyanates, nitriles, and thiocyanates (bottom).

(a thioglucosidase), which in healthy plants is believed to be separated from its glucosinolate substrates by subcellular compartmentalization. The unstable aglycone generated by the action of myrosinase may then form various products, including volatile isothiocyanates (mustard oils), nitriles, and thiocyanates, all of which are highly reactive compounds.

Glucosinolate hydrolysis products have been demonstrated to have toxic activity toward a range of fungi in vitro, including both pathogens and nonpathogens of *Brassica*, although the mechanism of toxicity is not known. Because the nature of the breakdown products depends on the structure of the glucosinolate, the form of myrosinase(s) present, the plant species, and a range of other factors, including pH, temperature, metal ion concentrations, and protein cofactors, it may be difficult, simply by assessing the relative amounts of specific glucosinolates present in the host plant, to predict which toxic products a pathogen is likely to encounter (Fenwick et al., 1983; Chew, 1988).

The major breakdown products generated in leaves of *Brassica* are isothiocyanates, the most fungitoxic of which are allyl-(2-propenyl) and 3-butenyl isothiocyanate (Mithen et al., 1986). Breakdown products of indolyl glucosinolates may also be toxic toward fungi (Mithen et al., 1986). In addition, they may be used as precursors for auxin biosynthesis in infection of *Brassic*as by fungi that cause abnormal tissue growth (such as the root gall fungus *Plasmodiophora brassicae*; Fenwick et al., 1983; Chew, 1988). It has also been suggested that indolyl glucosinolate breakdown products may function as precursors to a class of indole phytoalexins that are induced in *Brassica* (Rouxel et al., 1989). A number of pathogens of *Brassica*, such as *Leptosphaeria maculans* (Mithen et al., 1986), *Peronospora parasitica* (Greenhalgh and Mitchell, 1976), *Mycosphaerella brassicae* (Harthill and Sutton, 1980), and *Alternaria* sp (Milford et al., 1989), have been shown to be sensitive to at least some glucosinolate breakdown products. However, it is unclear whether there is any relationship between resistance of fungi to glucosinolates and ability to cause disease. Glucosinolate breakdown products are also effective against a range of nonpathogens of *Brassica*, leading to in-

terest in the use of these compounds as naturally occurring fungicides for the control of cereal diseases (Angus et al., 1994) and postharvest pathogens of fruit and vegetables (Mari et al., 1993).

High glucosinolate levels have been associated with resistance of oilseed rape and Indian mustard to *L. maculans* (Mithen and Magrath, 1992; R. Mithen, personal communications; B. Howlett, personal communication) and with resistance of cabbages to *P. parasitica* (Greenhalgh and Mitchell, 1976). However, in studies of crosses of *B. napus* lines with high and low glucosinolate levels in their leaves, resistance to *L. maculans* and glucosinolate profiles did not cosegregate (Mithen and Magrath, 1992). Further studies with *B. napus* lines that had contrasting glucosinolate profiles indicated that high levels of glucosinolates are unlikely to confer greater resistance to *L. maculans* and *Alternaria* spp in oilseed rape and may even give enhanced susceptibility (A. Giamoustaris and R. Mithen, personal communication).

CONCLUSION AND FUTURE PROSPECTS

Because preformed inhibitors may be harmful to the plants that produce them, they are usually sequestered within plant cells or tissues, often as inactive glycosides. With the exception of the monodesmosidic saponins, the preformed inhibitors considered in this review all require the action of plant hydrolytic enzymes for release of the corresponding antifungal agent (Table 1). Interestingly, the oat avenacosidase enzyme (Gus-Mayer et al., 1994a, 1994b), linamarase (Oxtoby et al., 1991), and myrosinase (Lenman et al., 1993) (which activate the bisdesmosidic avenacoside saponins, cyanogenic glucosides, and glucosinolates, respectively; Table 1) are all related, suggesting that these enzymes may share a common ancestor (Lenman et al., 1993; Gus-Mayer et al., 1994a, 1994b). These three enzymes are all β -glucosyl hydrolases belonging to the family 1 group of glycosyl hydrolases, in contrast to the saponin-detoxifying enzymes, which have been characterized from

Table 1. Preformed Antifungal Compounds in Plants and Resistance Mechanisms in Phytopathogenic Fungi

Preformed Compound	Antifungal Agent	Plant Activating Enzyme(s)	Major Mechanisms of Resistance in Fungi
Monodesmosidic saponins	Monodesmosidic saponins	None	C-3 specific saponin glycosyl hydrolases; membrane composition
Bisdesmosidic saponins	Monodesmosidic saponins	C-26/C-28 specific β -glucosyl hydrolases (e.g., oat avenacosidase)	C-3 specific saponin glycosyl hydrolases; membrane composition
Cyanogenic glycosides	HCN	β -glucosyl hydrolase (e.g., linamarase); α -hydroxynitrile lyase	Cyanide hydratase; cyanide-resistant respiration
Glucosinolates	Isothiocyanates Nitriles Thiocyanates	Thioglucosidase (e.g., myrosinase)	Unknown

G. g. avenae, *S. lycopersici*, and *S. avenae*, which all belong to family 3. Another plant β -glucosyl hydrolase, which activates the bisdesmosidic saponin protogracillin, has recently been purified from the herbaceous Asian plant *Costus speciosus* and also has been shown to be a member of family 1 (Inoue et al., 1996).

Although studies of inhibition of growth of phytopathogenic fungi by isolated plant compounds suggest a role in plant defense, such in vitro tests may not always give an accurate indication of the significance of these compounds in restricting fungal growth in the plant (Schönbeck and Schlösser, 1976; Mansfield, 1983). The characterization of mechanisms of resistance of phytopathogenic fungi to preformed antifungal compounds offers a means of testing whether antifungal compounds play a role in protecting plants against disease. Such resistance mechanisms have been studied in the most detail for fungi that encounter saponins or cyanogenic glycosides, whereas little is known about fungal resistance to glucosinolate breakdown products (Table 1).

A number of fungi actively detoxify preformed inhibitors by producing specific enzymes. Targeted gene disruption has allowed stringent genetic tests of the role in pathogenicity of two of these—the saponin-detoxifying enzyme avenacinase, produced by *G. g. avenae* (which detoxifies the oat root saponin avenacin A-1), and the cyanide-detoxifying enzyme CHT, produced by the sorghum pathogen *G. sorghi*. These experiments have demonstrated that avenacinase is an essential determinant of host range for *G. g. avenae*, because mutants lacking the enzyme are unable to infect the saponin-containing host, oat (Bowyer et al., 1995). In contrast, CHT does not appear to be required for pathogenicity of *G. sorghi* to cyanogenic plants (VanEtten et al., 1995). However, the possibility that *G. sorghi* may have an alternative mechanism of hydrogen cyanide resistance in the plant (such as cyanide-resistant respiration) cannot be ruled out.

In the future, we are likely to see the continued characterization of more fungal enzymes involved in the detoxification of preformed inhibitors, especially of saponins. The recent progress in purification and characterization of saponin-detoxifying enzymes from *S. lycopersici* (Osbourn et al., 1995; Sandrock et al., 1995), *F. o. lycopersici* (Lairini et al., 1996), and *S. avenae* (Wubben et al., 1996) now presents an opportunity to test the importance of saponin detoxification in pathogenicity, for fungi other than *G. g. avenae*, by isolating the genes encoding these enzymes and generating specific mutants.

The relatedness of at least three out of the four saponin-detoxifying enzymes that have been described in this review suggests that many fungi may employ related enzymes to detoxify host plant saponins. DNA gel blot analyses indicate that DNA sequences related to those encoding avenacinase and *S. lycopersici* tomatinase exist in a wide range of fungi, including pathogens of plants other than oat and tomato (Osbourn et al., 1995). However, it remains to be seen whether these sequences encode other saponin-detoxifying enzymes or simply β -glucosyl hydrolases required for saprophytic growth. If saponin-detoxifying enzymes do have widespread

significance in fungal phytopathogenicity, they may present attractive targets for inhibitor-based disease control strategies (Osbourn, 1996). Their extracellular location should make them particularly amenable to such an approach.

The demonstration that avenacinase is required for pathogenicity of *G. g. avenae* to oats provides an indirect test of the importance of the oat root saponin avenacin A-1 in plant defense. Further direct evidence to indicate that the saponin is likely to play a protective role is now emerging from studies of natural variants of oat that lack the saponin and from preliminary studies of oat mutants. In the future, it may be possible to address the significance of other preformed antifungal compounds in plant defense by generating plant material with altered levels of preformed inhibitors, either by mutagenesis or by manipulation of key steps in the biosynthetic pathways. If such compounds do prove to be important in protecting plants against pathogen attack, as appears to be the case for avenacin A-1, this approach may pave the way for the development of novel strategies for disease control. Such strategies may be based on interference with mechanisms of resistance of phytopathogenic fungi to preformed inhibitors or on the production of plants that make novel or altered antifungal compounds. However, in taking the latter approach, the potential effects of the modification of plant secondary metabolites on the resistance of plants to pests and grazing animals and on the value of the crop as a food source must also be considered.

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