Chapter 4
New Antimicrobial Agents of Plant Origin

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Abstract Plants are constantly under attack by microbial pathogens. As part of their defensive arsenal, they use antimicrobial peptides such as thionins, defensins, lipid transfer proteins, hevein-like peptides, knottins, cyclotides, β-barrelins, and others. In addition, they produce a diversity of antimicrobial metabolites. Those where the evidence for a role in plant defense is stronger include benzoxazinoids, camalexin, and glucosinolates among the alkaloids; flavonoids and stilbenes among the phenylpropanoids; and also terpenoids such as saponins. Our understanding of these plant antimicrobial agents has increased significantly in recent years with new information on their distribution, synthesis, regulation, in vivo function, and mechanism of action. Plant antimicrobial agents have a large potential for biotechnological applications. Engineered plants with increased disease resistance have been achieved using almost every family of antimicrobial peptides. There have been also been successes in using metabolic engineering to increase the production of antimicrobial compounds, as in the case of stilbenes and glucosinolates. Commercial applications using both approaches are likely to appear soon. The use of plant antimicrobials in human medicine is probably further in the future, although there are promising antifungal agents like defensin peptides, and saponins. With more than a quarter million species and a particularly diverse specialized metabolism, the richness of plant antimicrobials has barely been explored.

4.1 Plant Antimicrobial Defenses

Wild plants are quite successful at keeping bacterial and fungal pathogens at bay. In addition to physical barriers, they have an immune system capable of detecting and responding to the presence of pathogens. The first layer of defense is based on

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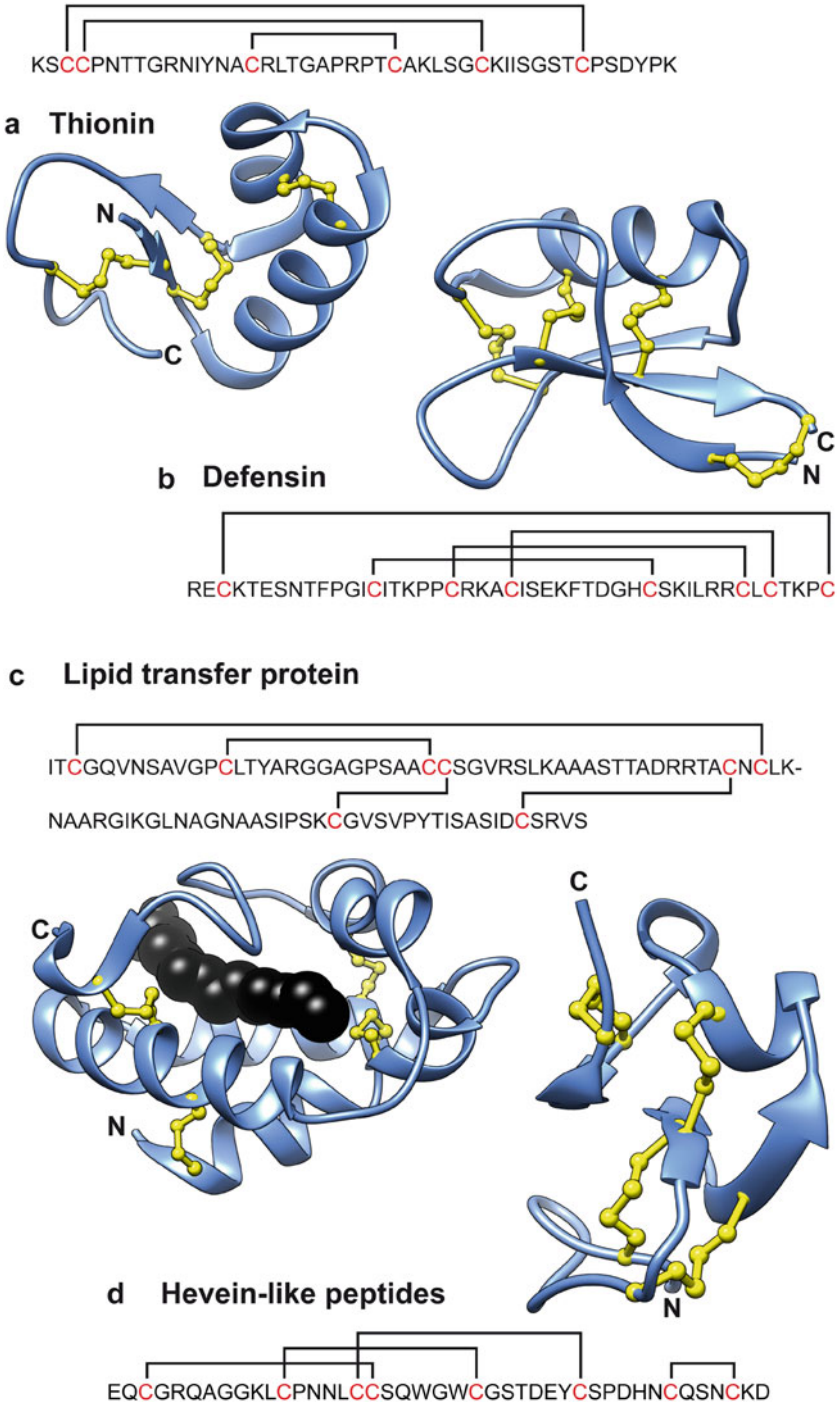
a series of receptors capable of recognizing conserved microbial products, like bacterial flagellin or fungal chitin, as well as signs of compromised cell wall integrity, such as lytic polysaccharide fragments (Schwessinger and Ronald 2012). Activation of these receptors triggers a series of responses called pattern-triggered immunity which includes the production of broad-spectrum antimicrobial compounds. Pathogens can overcome this barrier by producing a range of specific effectors, proteins, and other molecules that suppress pattern-triggered immunity or otherwise enhance their virulence. A second layer of defense is activated when the plant detects directly or indirectly the presence of these effectors through specific receptors called R proteins (Spoel and Dong 2012). The effector-triggered immune response is stronger than the pattern-triggered response and can activate programmed cell death in the affected area. Plants can also produce multiple mobile immune signals that are transported through the vasculature, usually after an effector-triggered response. These signals preventively activate defenses in unaffected organs and can also induce chromatin modifications that establish immune memory and lead to stronger responses in subsequent attacks.

Plant antimicrobial peptides and secondary or specialized metabolites are a critical part of the immune response, even if some of them are also produced constitutively. While some groups are ubiquitous, many are restricted to specific families. Secondary metabolites involved in defense responses are called phytoalexins when are synthesized in response to an attack and phytoanticipins if they are produced as inactive precursors and are activated upon attack (Morant et al. 2008; Ahuja et al. 2012). According to their biosynthetic pathway they can be separated into alkaloids, phenylpropanoids, and terpenoids, which is the classification that will be used in this chapter.

Increasing the production of antimicrobial peptides or specialized metabolites through genetic engineering has lead to enhanced resistance in many cases and will probably result soon in improved commercial crops (Collinge et al. 2010). While introducing a new antimicrobial peptide is relatively easy, engineering the synthesis of specialized metabolites is much more complex, but has now become a real possibility (Großkinsky et al. 2012). Regarding potential uses in human medicine, large-scale screens have failed to find potent and safe plant antibiotics (Lewis and Ausubel 2006). However, there is evidence that some plant antimicrobials act synergistically and may need to be combined to produce effective treatments.

4.2 Ribosomal Peptides

Antimicrobial peptides belong to a number of unrelated families, but in many cases they share some common characteristics. Despite their small size, below 10 kD, most of them have several disulfide bridges that increase their stability. The pattern of cysteines is characteristic of each family and is sometimes the easier way to identify new peptides, due to poor sequence conservation (Fig. 4.1).


KSCCPNTTGRNIYNACRLTGAPRPTCAKLSGCKIISGSTCPSDYPK
a Thionin
N
C
b Defensin
C
N
RECKTESNTFPGICITKPPCRKACISEKFTDGHCSKILRRCLCTKPC
c Lipid transfer protein
ITCGQVNSAVGPCLTYARGGAGPSAACCSGVRSLKAAASTTADRRTACNCLK-
NAARGIKGLNAGNAASIPSKCGVSVPYTISASIDCSRVS
C
N
C
N
d Hevein-like peptides
EQCGRQAGGKLCPNNLCCSQWGWCGSTDEYCSPDHNCQSNCKD

◀ **Fig. 4.1** Sequence and structure of plant antimicrobial peptides from different families with disulfide bridges indicated. **a** Viscotoxin A3 from *Viscum album* (PDB identifier 1ED0), **b** NaD1 from *Nicotiana alata* (1MR4), **c** nsLTP1 from *Oryza sativa* complexed with myristic acid (1UVA), **d** Hevein from *Hevea brasiliensis* (1HEV), **e** PAFP-S from *Phytolacca americana* (1DKC), **f** Kalata B1 from *Oldenlandia affinis* (1NB1), **g** MiAMP1 from *Macadamia integrifolia* (1C01)

Peptides from several different families are basic and amphiphilic, with both hydrophobic and positively charged hydrophilic areas. Many of these seem to interact with lipids as part of their mechanism of action and in some cases, they seem to penetrate and disrupt lipid membranes. An online database of plant antimicrobial peptides called PhytAMP is available (Hammami et al. 2009).

4.2.1 Thionins

Thionins, whose name comes from a high proportion of sulfur-containing cysteines, are also known as family 13 of pathogenesis-related proteins (PR-13). Wheat thionins were the first plant antimicrobial peptide to be isolated, when they were identified as the component of wheat flour toxic to yeast cells (Balls et al. 1942).

Thionins have been characterized in a limited range of angiosperm species (Stec 2006; Hammami et al. 2009). Most of those with proven antimicrobial activity were obtained from monocot species of the grass (*Poaceae*) family (Oard et al. 2004; Fujimura et al. 2005) or dicot plants of the mistletoe (*Viscaceae*) family (Giudici et al. 2004). However, related sequences are present in many other plants suggesting that many more thionins with antimicrobial properties remain to be discovered (Silverstein et al. 2007).

In vitro thionins are toxic at micromolar concentrations to a wide range of organisms from bacteria and fungi to protozoa and human cells (Fujimura et al. 2005; Loeza-Ángeles et al. 2008; Berrocal-Lobo et al. 2009). Thionins have been found in both seeds and vegetative organs, such as leaves and stems (Stec 2006). While some are expressed constitutively, others are induced by fungal infections, supporting an in vivo defensive function for these peptides. It has also been proposed that they could have a role in redox regulation and that seed thionins could function as storage proteins (Castro and Fontes 2005).

Mature thionin peptides have 45–47 amino acids and are processed from longer pre-pro-proteins with a signal peptide and an acidic C-terminal domain of unknown function (Abbas et al. 2013). The degree of sequence similarity in mature thionins is quite high and those with proven antimicrobial activity are basic and amphiphilic (Stec 2006). The three-dimensional structure of more than 10 thionins from different groups has been resolved (Stec 2006). They have the shape of an L with two α-helixes on one arm and a two-stranded antiparallel β-sheet on the other (Fig. 4.1a). This structure is stabilized by three conserved disulfide bridges, which are supplemented by a fourth one in some groups.

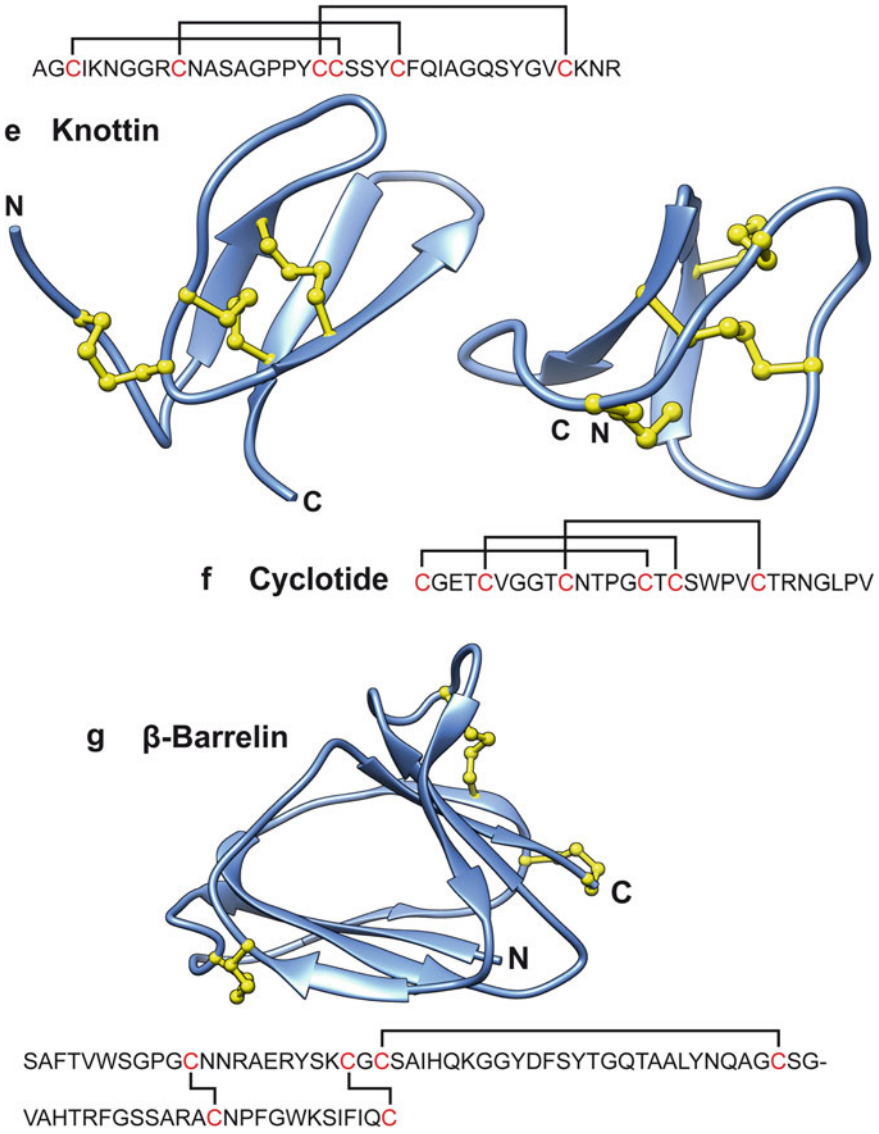


Fig. 4.1 continued

The toxicity of thionins is associated with an increase in membrane permeability that causes depolarization and cell death (Giudici et al. 2004; Berrocal-Lobo et al. 2009). Their positive charges allow them to bind negatively charged phospholipid heads while a hydrophobic region could interact with the lipid tails. Several mechanisms have been proposed to explain the interaction of thionins with membranes (Stec 2006). In the first hypothesis, a complex of thionin molecules

forms transmembrane channels that allow the selective passage of ions (Hughes et al. 2000). Another proposed mechanism is that binding of thionins to the outer membrane surface creates rigid patches that allow the formation of transient pores at their margins (Coulon et al. 2002). It has also been suggested that thionins could bind and solubilize individual lipid molecules contributing in the process to membrane disruption (Stec et al. 2004). A recent proposal suggests that thionins become inserted in the outer leaflet of the membrane where they form half-channels that allow water to reach the interior of the membrane, disrupting its organization and creating transient pores (Oard 2011). Truncation of a thionin sequence showed that the α-helixes are sufficient for antimicrobial activity and membrane permeabilization (Vila-Perelló et al. 2005). In addition to this primary effect, additional mechanisms of toxicity have been suggested, such as DNA or RNA binding, although the evidence is limited (Stec 2006).

There have been some successes in engineering transgenic crops with improved resistance against pathogens by overexpressing thionins. Expression of a barley thionin in sweet potato, for instance, increased resistance against a fungal disease (Muramoto et al. 2012) and an oat thionin protected rice seedlings against bacterial infections (Iwai et al. 2002). However, the broad-spectrum toxicity of these peptides has to be taken into account, as is the case too with the possibility of using thionins to treat infectious human diseases. An Arabidopsis thionin was found to be active against human pathogens, both bacterial and fungal, but it also reduced the viability of mammalian cells (Loeza-Ángeles et al. 2008). The same is likely to be the case for a wheat thionin that has shown strong activity against the protozoan parasite responsible for human leishmaniasis (Berrocal-Lobo et al. 2009).

4.2.2 Defensins

Defensins were originally called γ-thionins (Colilla et al. 1990), but were later separated in their own family because of the structural differences between the two groups of antimicrobial peptides (Terras et al. 1995). The new name was based on their similarity to animal defensins, which are also antimicrobial peptides. Plant defensins are also known as family 12 of pathogenesis-related proteins (PR-12). There is an online database for defensins which includes both plant and animal sequences (defensins.bii.a-star.edu.sg/).

Defensins are ubiquitous in plants, and a single species can have hundreds of defensin-like sequences in its genome (Silverstein et al. 2005). Sequence conservation is generally poor, but these peptides share a common pattern of disulfide bridges. A recent phylogenetic analysis of 139 defensins has divided the family in 18 groups (van der Weerden and Anderson 2013). Many defensins from different groups have been shown to have in vitro antifungal activity at micromolar concentrations (Carvalho and Gomes 2011). Antibacterial activity against both gram-positive and gram-negative species has been proven for a smaller number of

defensins, many of which belong to divergent groups (van der Weerden and Anderson 2013).

There is abundant evidence that plants use defensins in vivo to protect themselves from the attack of fungal pathogens (De Coninck et al. 2013). Some of them are produced constitutively during seed germination and others accumulate in vegetative tissues in response to pathogen inoculation. The best understood activation pathway is that of Arabidopsis PDF1.2a and closely related defensins which are induced by necrotrophic fungi. This pathway involves the production of the hormones ethylene and methyl jasmonate and a number of transcription factors have been identified (Çevik et al. 2012). Some defensins seem to have additional functions not related to antimicrobial defense such as a role in pollen recognition (Carvalho and Gomes 2011; van der Weerden and Anderson 2013).

Mature defensins have approximately 50 aminoacids. Their precursors have a signal peptide and in some cases additional domains of unknown function (De Coninck et al. 2013). Defensins share a common structure which has been determined for many different peptides (van der Weerden and Anderson 2013). Three disulfide bridges connect an α-helix with an antiparallel β-sheet formed by three strands. A fourth bridge further stabilizes the structure by joining the N terminus with the C terminus (Fig. 4.1b). The second and third strands of the β-sheet together with the intermediate loop form the γ-motif or γ-core which is critical for antimicrobial activity and can indeed maintain this activity on its own (Sagaram et al. 2011).

The mechanism of action of antifungal defensins has been studied extensively (Kaur et al. 2011; De Coninck et al. 2013). NaD1, a defensin from *Nicotiana alata*, appears to bind a receptor in the outer cell wall layer (van der Weerden et al. 2010). This leads to the formation of membrane pores that allow it to enter the cytoplasm, where it could reach intracellular targets (van der Weerden et al. 2008). RsAFP2, a radish defensin, also interacts with a cell wall receptor, but it does not appear to be internalized (Thevissen et al. 2012). In this case, the receptor was identified as a sphingolipid that is present in both membrane and cell wall. Other defensins have also been shown to interact specifically with different fungal sphingolipids (De Coninck et al. 2013).

Membrane permeabilization has been demonstrated for a number of defensins from different species, including RsAFP2 and NaD1 (Sagaram et al. 2011; De Coninck et al. 2013). An interesting result is that NaD1 cannot induce permeabilization in liposomes, suggesting that it does not act directly on the membrane (van der Weerden et al. 2010). In addition to membrane permeabilization, the antifungal activity of several defensins appears to involve formation of reactive oxygen species and activation of apoptosis (De Coninck et al. 2013). One of these is RsAFP2 which appears to act extracellularly by compromising cell wall integrity (De Coninck et al. 2013). On the other hand, a pea defensin that, like NaD1, enters the cytoplasm, has been shown to localize to the nucleus where it interferes with the cell cycle (Lobo et al. 2007). These results clearly indicate that defensins can have different modes of action.

There is a large potential for biotechnological applications of defensins (Carvalho and Gomes 2011). Numerous groups have reported the use of constitutively expressed defensins to obtain crops with improved resistance to diverse fungal pathogens and commercial applications are under development. Among the plants where this approach has been successful are potato, tomato, rice, wheat, and banana (Kaur et al. 2011; Ghag et al. 2012; De Coninck et al. 2013). While most experiments have been carried out in growth chambers or greenhouses, two different transgenic potatoes have shown effective resistance in field tests to the agronomically important pathogens *Verticillium dahliae* and *Phytophthora infestans* (Gao et al. 2000; Portieles et al. 2010). Using NaD1, the Australian biotechnology company Hexima developed and field-tested cotton plants with increased resistance to *Fusarium* and *Verticillium* wilt (Kaur et al. 2011).

Regarding medical applications, several plant defensins have been considered promising candidates for the treatment of *Candida* infections in human (Thevissen et al. 2007). A radish defensins has already been shown to be effective in vivo as a treatment against systemic candidiasis in mice (Tavares et al. 2008). Unlike thionins, defensins do not appear to be toxic to human cells. While commercial-scale production of large defensin peptides would require heterologous systems, the small γ-motif can be chemically synthesized. It has been reported recently that a tomato defensin γ-motif has strong activity against bacterial human pathogens, both gram-positive and gram-negative, with no cytotoxic effect on human cells (Rigano et al. 2012).

4.2.3 Lipid Transfer Proteins

Antimicrobial lipid transfer proteins (LTPs) are also called nonspecific lipid transfer proteins (nsLTPs), because they can associate with a broad range of phospholipids and transfer them from one membrane to another. LTPs have also been classified as family 14 of pathogenesis-related proteins (PR-14).

Dozens of different LTPs are present in a single plant genome and many more if we also include LTP-like sequences (Silverstein et al. 2007; Boutrot et al. 2008). Phylogenetic analyses of LTPs recognize up to nine different types, some specific to particular plant groups (Boutrot et al. 2008). Type I genes are widespread in seed plants and seem to be the most numerous in plant genomes. LTPs with proven antimicrobial activity belong to this group. They were discovered in the 1990s and have been purified from a number of species including radish, barley, spinach, onion, or coffee (Yeats and Rose 2008; Ng et al. 2012). Type I LTPs have shown activity against diverse fungi, as well as gram-positive and gram-negative bacteria, although the specificity varies among them (Carvalho and Gomes 2007). Onion Ace-AMPl, for instance, has activity against fungi and gram-positive bacteria at nanomolar concentrations, but shows no activity against gram-negative bacteria or

human cells (Cammue et al. 1995). In the same work, maize and wheat type I LTPs showed no antimicrobial activity.

Lipid transfer proteins were originally thought to be involved in the transport of lipids among different cellular compartments, but they are generally located extracellularly. A role for type I LTPs in plant defense is suggested by their in vitro activity against plant pathogens, but also by the induction of a number of LTPs upon pathogen attack (Carvalho and Gomes 2007; Yeats and Rose 2008). However, other possible functions have been suggested for type I LTPs, such as transportation of waxes to the cuticle or regulatory functions that involve interaction with hydrophobic molecules (Carvalho and Gomes 2007; Yeats and Rose 2008). In this regard, an Arabidopsis LTP-like gene has been shown to be necessary for the correct formation of the stem cuticle (DeBono et al. 2009). Even if lipid transport is the primary function of LTPs, their antimicrobial activity could also be beneficial to the plant.

Mature type I LTPs are basic peptides of 90–95 amino acids, preceded by a signal peptide in the pre-protein (Carvalho and Gomes AdO and Gomes 2007; Yeats and Rose 2008). Sequence conservation is not very high, but all share eight strictly conserved cysteines. The three-dimensional structure reveals four α-helixes that surround a hydrophobic tunnel and are connected by the four disulfide bridges (Fig. 4.1c). The tunnel can bind different types of lipids, as can be seen in several of the structures that have been resolved (Yeats and Rose 2008). Interestingly, in onion Ace-AMPl, the hydrophobic tunnel is blocked by the presence of voluminous side-chains, but the antimicrobial activity is not affected (Tassin et al. 1998).

Not much is known about the antimicrobial mechanism of action of LTPs. Several groups have observed membrane permeabilization, although the amount of leakage was small in some cases (Tassin et al. 1998; Regente et al. 2005; Zottich et al. 2011). Onion Ace-AMPl is one of the LTPs that causes membrane leakage, despite its inability to bind isolated lipids or transport them between membranes (Cammue et al. 1995; Tassin et al. 1998).

A small number of studies have found increased pathogen resistance in plants overexpressing antimicrobial LTPs. A barley LTP reduced the symptoms caused by the bacterial pathogen *Pseudomonas syringae* in tobacco and Arabidopsis (Molina and García-Olmedo 1997). Similarly, overexpression of Ace-AMPl from onion resulted in increased resistance to a fungal pathogen in wheat, as had previously been found also in geranium and rose (Roy-Barman et al. 2006). An LTP from motherwort also improved resistance against fungal pathogens in tobacco and poplar (Jia et al. 2010). It is possible that some of these results are caused by indirect effects such as alterations in defense signaling or cuticle properties, as suggested by an Arabidopsis mutant in an LTP-like gene with a defective cuticle and increased susceptibility to fungal attack (Lee et al. 2009). Biotechnological applications of LTPs also need to consider the fact that these peptides are potent allergens (Egger et al. 2010).

4.2.4 Hevein-Like Peptides

Hevein is an antifungal peptide that was isolated from rubber-tree latex using chitin-affinity chromatography (Parijs et al. 1991). Sequences homologous to hevein and capable of binding chitin have been found as peptides in other species, but also as domains that form part of larger proteins, such as chitinases (Porto et al. 2012).

Database searches show that hevein-like peptides are present in dicots and monocots and even in spikemosses, suggesting that they are widespread in vascular plants (Porto et al. 2012). Hevein-like peptides with antifungical activities at micromolar or lower concentrations have been purified from *Eucommia ulmoides* bark, ginkgo leafs, elderberry fruits and seeds of amaranth, morning glory, or wheat (Koo et al. 1998; Van Damme et al. 1999; Huang et al. 2000; Huang et al. 2002; Lipkin et al. 2005; Odintsova et al. 2009). These peptides appear to be expressed constitutively, suggesting a possible preventive role against fungal attacks. A hevein-like peptide from spindle tree bark, in addition to inhibiting the growth of several fungi, also showed activity against gram-positive bacteria, but not gram-negative species or yeasts (Van den Bergh et al. 2002).

There are two types of hevein-like peptides. The larger ones, like hevein itself, have 40–45 amino acids, while the shorter ones are only 30 amino acids long with a truncated C-terminal region (Egorov and Odintsova 2012). The three-dimensional structure of the longer peptides is anchored by a central three-stranded antiparallel β-sheet (Fig. 4.1d). Four conserved disulfide bridges connect the central β-sheet with the rest of the peptide, resulting in high thermal and pH stability. Some sequences have an additional fifth bridge, which can adopt several configurations (Van den Bergh et al. 2002; Odintsova et al. 2009). A cluster of hydrophobic residues forms the chitin-binding site, while the opposite face of the peptide is enriched in charged residues (Xiang et al. 2004). Short hevein-like peptides are missing the last strand of the β-sheet and have only three disulfide bridges (Martins et al. 1996). Precursor proteins include a signal peptide and a C-terminal pro-peptide of variable length, and they can sometimes contain more than one hevein-like peptide (Van Damme et al. 1999; Egorov and Odintsova 2012).

Although hevein-like peptides bind to chitin with high affinity, it is not clear what role this plays in their mechanism of action. Several peptides have shown activity against fungi with no chitin in their walls or against gram-positive bacteria (Koo et al. 1998; Van den Bergh et al. 2002; Huang et al. 2002; Odintsova et al. 2009). A hevein-like peptide from morning glory was shown to cause actin depolimerization resulting in burst hyphae (Koo et al. 2004). Analysis of resistant mutants suggests that the target of this peptide might be a membrane glycoprotein and that it activates the cell wall integrity pathway.

Constitutive expression of a hevein-like peptide from morning glory enhanced resistance of tobacco and tomato against different fungal species (Choon Koo et al. 2002; Lee et al. 2003). Similarly expression of hevein-like peptides from *Stellaria media* increased resistance against phytopathogenic fungi in Arabidopsis, tomato and tobacco (Khaliluev et al. 2011; Shukurov et al. 2012). A possible obstacle to

the biotechnological application of these peptides is that hevein is a major latex allergen (Chen et al. 1997).

4.2.5 Knottins and Cyclotides

Knottin is a generic term for small proteins with a particular type of cysteine knot motif known as inhibitor cysteine knot (Isaacs 1995). Cysteine knots require two disulfide bridges that form a circular structure with the protein backbone. The knot is formed when a third disulfide bridge crosses this circular structure. In the case of inhibitor cysteine knots, this third bridge is the one formed by cysteines III and VI (Fig. 4.1e,f). The knottin structural family encompasses a number of distinct protein families with apparently unrelated sequences. Knottins can be found in plants, animal, and fungi and they have toxic, inhibitory, and regulatory functions. The KNOTTIN database is available online and has knottin sequences and structures from different families (http://knottin.cbs.cnrs.fr/).

Two families of plant peptides with an inhibitor cysteine knot have been found to possess antimicrobial activity. The first family is simply referred to as antimicrobial knottins and was discovered when two closely related peptides were purified from *Mirabilis jalapa* seeds (Cammue et al. 1992). These peptides showed in vitro activity against a diverse range of phytopathogenic fungi and gram-positive bacteria, but not against gram-negative bacteria or human cells. Another antimicrobial peptide with a related sequence was purified by two different groups from seeds of *Phytolacca americana*, a species from the same order as *M. jalapa* (Shao et al. 1999; Liu et al. 2000). This peptide also showed activity against fungi and gram-positive bacteria, but not against gram-negative bacteria. The KNOTTIN database indicates that similar sequences exist in other species such as poplar, but it is currently unknown if they also have antimicrobial activity.

The characterized antimicrobial plant knottins are 36–38 amino acids long and have high temperature and pH stability as a result of the cysteine knot. The three-dimensional structure of the *P. americana* peptide was determined by NMR (Gao et al. 2001). It has an antiparallel β-sheet formed by three strands (Fig. 4.1e). This peptide is an amphiphile with a hydrophobic surface next to a patch of hydrophilic residues that includes positive charges. The precursor proteins of knottins include a signal peptide and are expressed specifically in seeds (Bolle et al. 1995; Liu et al. 2000).

There is little information on the mechanism of action of these peptides beyond the fact that the *P. americana* knottin binds to the sphingolipids of fungal membranes and its antifungal activity is critically dependent on the presence of the hydrophobic patch (Peng et al. 2005). As for biotechnological applications, one of the *M. jalapa* peptides increased resistance to the fungus *Alternaria solani* when introduced in tomato (Schaefer et al. 2005).

The cyclotides form the second family of antimicrobial peptides with an inhibitor cysteine knot. They are approximately 30 amino acids long and most are

cyclic peptides, with the N-terminal and C-terminal amino acids connected by a peptide bond (Fig. 4.1f). This increases their stability and makes them resistant to exoproteases (Craik 2009; Pinto et al. 2012). Cyclotides seem to be widespread in the violet (*Violaceae*) family and are common in the coffee (*Rubiaceae*) and dogbane (*Apocynaceae*) families, with individual species producing 10–100 different peptides (Gruber et al. 2008). They have also been found in isolated species of the potato (*Solanaceae*) and pea (*Fabaceae*) families, although genomic analyses suggest that they are not common (Poth et al. 2012). Sequences related to cyclotides are present in the grass (*Poaceae*) family but the peptides appear to be exclusively linear (Nguyen et al. 2012). Linear cyclotides, also called acyclotides or uncyclotides, are rare in species from other families, where they are always found together with the more abundant circular ones.

Cyclotides were discovered in a medicinal herb used in DR Congo to accelerate delivery and were later found to be toxic to a wide range of organisms (Craik 2009). Four cyclotides from different species showed moderate antifungal activity, but strong antibacterial activity against a range of gram-positive and gram-negative human pathogens (Tam et al. 1999). In another study, a cyclotide from violet was found to have strong activity against gram-negative human pathogens, but not gram-positive *Staphylococcus* species (Pränting et al. 2010). Finally, a linear grass cyclotide showed activity against *Escherichia coli* (Nguyen et al. 2012). All these cyclotides were also found to be cytotoxic to human cells. The function of cyclotides in plants is unclear. Since they accumulate at high concentration, a defensive function is possible and insects have been suggested as their main target (Craik 2009). There are no reports of antimicrobial activity against plant pathogens, but their antibacterial activity does not appear to be highly specific.

Cyclotides are synthesized in ribosomes as part of larger pre-pro-proteins (Pinto et al. 2012). In addition to a signal peptide, they have N and C-terminal domains that are removed during maturation. Precursor proteins can contain up to three cyclotides separated by intermediate sequences. Cyclization appears to be catalyzed by an asparaginyl endopeptidase that cuts the C terminus of the mature peptide (always Asn or Asp) and transfers it to the N-terminal amino acid in a transpeptidation reaction (Craik 2009). This process occurs on the vacuole where cyclotides are stored (Conlan et al. 2011). The main mechanism of action of cyclotides seems to involve the disruption of lipid membranes (Henriques et al. 2011). These peptides appear to bind specifically to phosphoethanolamine creating pores when they become inserted into membranes (Wang et al. 2012).

4.2.6 β-Barrelins

The β-barrelin or MiAMP1 family is present in gymnosperms, angiosperms, and spikemosses, although they seem to be missing in several species with sequenced genomes (Manners 2009). It was discovered when a peptide purified from macadamia nuts, called MiAMP1, showed antimicrobial activity against several fungi

and a gram-positive bacteria, but not gram-negative bacteria or mammalian cells (Marcus et al. 1997). In vitro antifungal activity against several phytopathogens, as well as yeast, has also been demonstrated for homologous peptides from three different pine species (Sooriyaarachchi et al. 2011; Canales et al. 2011; Zamany et al. 2011). The β-barrelin precursor proteins have a signal peptide and in gymnosperms expression is induced by fungal attack, supporting an in vivo defensive role (Manners 2009; Sooriyaarachchi et al. 2011).

The family has been named β-barrelins due to the three-dimensional structure of MiAMP1 (Fig. 4.1g). This protein, which is 76 amino acids long, forms a barrel of eight β-strands that includes three disulfide bonds (McManus et al. 1999). Sequence conservation is high in the family and basic amino acids are abundant.

The mechanism of action of these peptides against fungi appears to involve binding specifically to β-(1,3)-glucans in fungal cell walls through conserved polar and aromatic residues (Sooriyaarachchi et al. 2011). The three-dimensional structure of β-barrelins is very similar to that of the fungal killer toxin HM-1, which interacts with β-(1,3)-glucans and blocks their synthesis (Kasahara et al. 1994; McManus et al. 1999). Regarding biotechnological applications, expression in canola of β-barrelins from macadamia and pine resulted in increased resistance to fungal pathogens (Kazan et al. 2002; Verma et al. 2012).

4.2.7 Other Peptides

An antibacterial peptide from maize kernels, called MBP-1, showed activity against several fungi and both gram-positive and gram-negative bacteria (Duvick et al. 1992). Closely related peptides were purified from seeds of two other grass species and antifungical activity was demonstrated for one of them (Egorov et al. 2005; Nolde et al. 2011). These peptides have approximately 35 amino acids and their three-dimensional structure consists of two α-helixes that form a hairpin stabilized by two disulfide bridges (Nolde et al. 2011). A similar cysteine pattern, but with low sequence similarity, was found in several macadamia nut peptides with activity against diverse fungi and a gram-positive bacterial phytopathogen (Marcus et al. 1999). The macadamia peptides are all produced from a large pre-pro-protein precursor that belongs to the vicilin family of seed storage proteins. Vicilins from other species include similar sequences which could be released as antimicrobial peptides. The names α-helical hairpin and 4-cys peptides have been proposed for this family, which also includes peptides that inhibit trypsin or ribosomes (Egorov and Odintsova 2012). The peptide from barnyard grass is internalized in fungal spores and hyphae suggesting an intracellular target, but it does not seem to disrupt the cell membrane (Nolde et al. 2011).

Other antimicrobial peptides have been identified in a single species. The seeds of *Impatiens balsamina*, for example, accumulate four antimicrobial peptides that are produced from the same pre-pro-protein (Tailor et al. 1997). They were shown to be highly effective against a range of phytopathogenic fungi and gram-positive

bacteria. The mature peptides have only 20 amino acids, are highly basic and form a loop stabilized by two disulfide bridges that contains a hydrophobic patch (Patel et al. 1998). The cysteine pattern is clearly different from that of the 4-cys peptides discussed above and no homologs have been identified in other species. A recent study that evaluated the potential use of one of these peptides to control foodborne pathogens found it promising, although toxicity against some types of human cells was significant (Wu et al. 2013).

Two peptides of about 7 kD were purified from potato tubers on the basis of their ability to inhibit bacterial growth (Segura et al. 1999; Berrocal-Lobo et al. 2002). They belong to the snakin/GASA family of small proteins, which is widely distributed in angiosperms and appears to be involved in the regulation of plant development and stress responses (Nahirñak et al. 2012). The potato sequences are highly divergent, suggesting a possible change of function. The mature peptides are derived from the C-terminal region of the precursor protein, have a positive charge and include 12 conserved cysteines. They showed antifungal and antibacterial activity in vitro and overexpression of the corresponding genes resulted in transgenic plants with enhanced resistance to fungal and bacterial pathogens (Almasia et al. 2008; Balaji and Smart 2012).

4.3 Alkaloids

Alkaloid can be used either as a generic term for all nitrogen containing specialized metabolites or just for those derived from amino acids. In both definitions, alkaloids are a highly diverse group of compounds that that do not share a common biosynthetic pathway. The best understood classes of plant antimicrobial alkaloids are benzoxazinoids, mostly found in grasses, and two groups of sulfur-containing alkaloids that are found in many species of the mustard (*Brassicaceae*) family, also known as crucifers (Niemeyer 2009; Bednarek 2012). The two groups of sulfur-containing alkaloids are glucosinolates and indole-type compounds, of which camalexin is the best known.

Both benzoxazinoids and camalexin-type compounds are derived from tryptophan or its precursors. Glucosinolates can be produced from a number of different amino acids. Benzoxazinoids and glucosinolates are produced in inactive form, conjugated with glucose, and only become toxic when this sugar is removed. Camalexin and glucosinolates have been extensively studied because they are both produced by *Arabidopsis thaliana*, the most popular model species for plant molecular biology. Both types of compounds most likely acquire their sulfur from the tripeptide glutathione and also share some other biosynthetic steps (Bednarek 2012). They act synergistically against fungal attacks with constitutively produced glucosinolates offering a first line of defense, while induction of camalexin biosynthesis at later stages creates a second barrier against infection (Schlaeppi et al. 2010).

4.3.1 Benzoxazinoids

Benzoxazinoids are a small group of glucosylated derivatives of indole-3-glycerol phosphate, a precursor of tryptophan (Fig. 4.2). They are found in the grass (*Poaceae*) family and in isolated dicotyledonous species. This distribution seems to be the result of convergent evolution (Frey et al. 2009; Dick et al. 2012).

The entire synthesis pathway of benzoxazinoids has been established in maize, where nine genes code for the enzymes that participate in the process, including two partially redundant glucosyltransferases (Frey et al. 2009). The synthesis pathway starts in the plastid, continues in the microsomes, and ends in the cytosol, where active benzoxazinoids are glucosylated to avoid autotoxicity before they are stored in the vacuole.

In vitro antifungal and antibacterial activity against plant and human pathogens has been reported for benzoxazinoids and their decomposition products, such as benzoxazolinones (Bravo et al. 1997; Glenn et al. 2001; Maresh et al. 2006; Rostás 2007). Other plant fungal pathogens can tolerate these compounds only because they have developed the ability to metabolize them (Glenn and Bacon 2009). The best evidence for an in vivo function in plant antimicrobial defense is a maize mutant unable to produce benzoxazinoids that showed increased susceptibility to a fungal pathogen (Ahmad et al. 2011). However, this mutant also showed altered defense responses suggesting that benzoxazinoids could have an additional regulatory function. Further support for an antifungal function comes from several studies that have found significant correlations between benzoxazinoid content and resistance to fungal pathogens (Niemeyer 2009). In addition benzoxazinoids seem to have an important role in defense against insects and neighboring plants (Rostás 2007; Niemeyer 2009; Ahmad et al. 2011).

Benzoxazinoids stored in the vacuole can be activated by plastid glucosidases upon tissue disruption, but in root exudates or in case of fungal attack benzoxazinoids appear to be exported directly to the apoplast, where they are activated by unknown glucosidases (Niemeyer 2009; Ahmad et al. 2011). Removal of the glucose generates an unstable compound with a highly electrophilic carbon that can easily react with nucleophiles, such as thiol groups of proteins, forming stable conjugates (Niemeyer 2009; Dixon et al. 2012). Benzoxazinoids can also decompose to toxic benzoxazolinones, whose mechanism of action is less clear. In the case of the plant pathogen *Agrobacterium tumefaciens* benzoxazinoids do not affect viability but appear to target instead the host recognition system (Maresh et al. 2006).

4.3.2 Camalexin and Related Indole-Type Metabolites

Camalexin is a sulfur-containing derivative of tryptophan produced by *Arabidopsis thaliana* (Fig. 4.2). It forms part of a group of related phytochemicals produced by species in the *Brassicaceae* family. More than 44 different compounds are known,

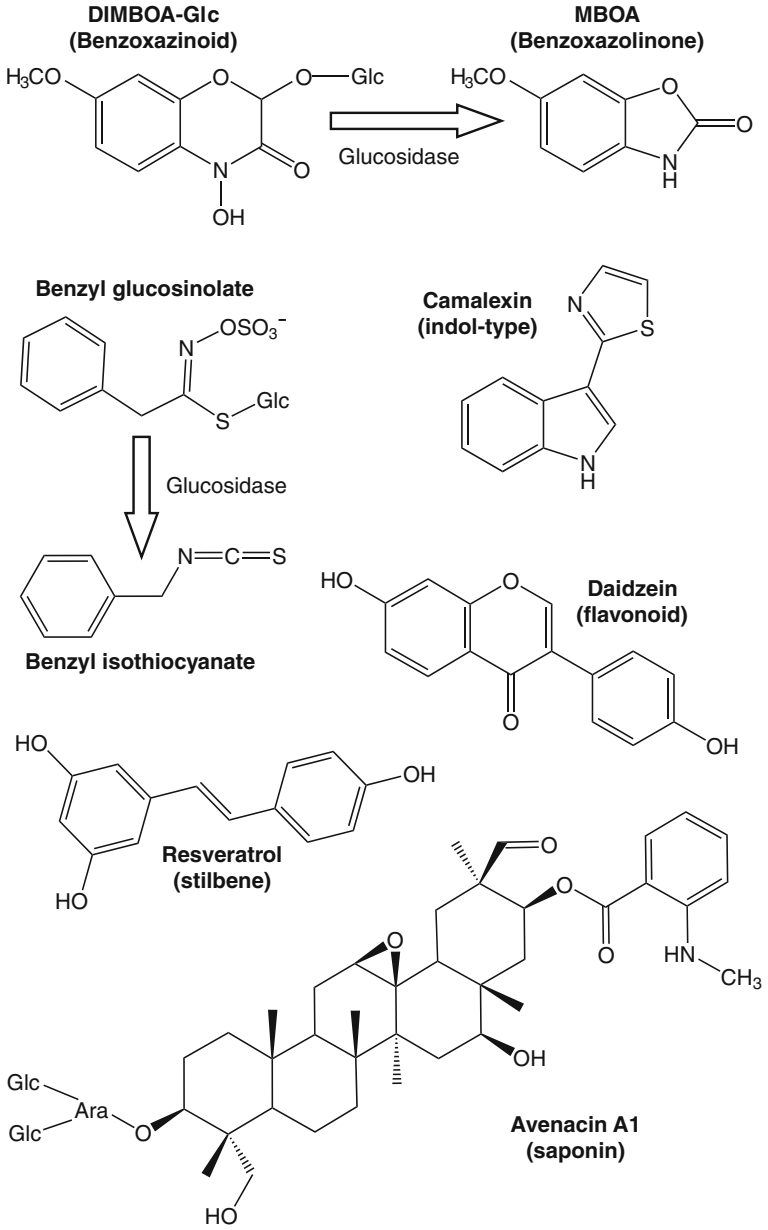


Fig. 4.2 Structure of representative plant antimicrobial metabolites from the main groups discussed in the text

although many others are likely to exist, since only a minority of species in the family have been analyzed (Pedras et al. 2011).

The synthesis of camalexin and related compounds is quite well understood (Pedras et al. 2011; Bednarek 2012; Ahuja et al. 2012). Thanks to the availability of Arabidopsis mutants, most of the genes involved in the seven-step camalexin pathway have been identified, as well as some of the transcription factors that regulate the process. A glutathione S-transferase is most likely responsible for the incorporation of sulfur and most of the remaining steps are catalyzed by cytochrome P450 enzymes.

There is evidence of in vitro antimicrobial activity for 44 of these indole-type metabolites, mainly against fungi but in some cases against gram-positive and gram-negative bacteria too (Pedras et al. 2011). Production of these compounds, which are also toxic to plant cells, is only activated in case of stress, including bacterial and fungal infections (Pedras et al. 2011; Ahuja et al. 2012). In vivo antimicrobial activity has only been demonstrated for camalexin in Arabidopsis, with enhanced susceptibility to fungal pathogens in mutants unable to produce this compound (Schlaeppi et al. 2010; Stotz et al. 2011). Camalexin is a lipophilic molecule and its mechanism of action, while not well understood, appears to involve membrane disruption in both fungi and bacteria (Rogers et al. 1996; Joubert et al. 2011; Ahuja et al. 2012). In fungi camalexin treatment activates cell wall integrity and high osmolarity pathways that contribute to pathogen virulence.

4.3.3 Glucosinolates

Glucosinolates can derived from six to nine different amino acids (Ala, Val/Leu, Ile, Met, Phe/Tyr, Trp, and possibly Glu) and include a sulfur atom linked to the anomeric carbon of a β-glucose residue (Fig. 4.2). More than 100 different glucosinolates have been identified in the *Brassicaceae* and closely related families (Agerbirk and Olsen 2012).

The synthesis of glucosinolates shares some steps with the synthesis of camalexin and related compounds (Sønderby et al. 2010; Bednarek 2012). Glutathione is again the most likely donor of the first sulfur. The rest of the tripeptide is removed and the remaining thiol group is then glucosylated. A sulfate group is later added from 3′-phosphoadenosine 5′-phosphosulfate.

The glucose residue can be removed by thioglucosidases called myrosinases (Halkier and Gershenzon 2006; Bednarek 2012). Some myrosinases are stored in specialized cells and become mixed with glucosinolates when the tissue is damaged by herbivores, but others are produced in cells under pathogen attack. The resulting aglycone is unstable and it rapidly decomposes. The main direct products are isothiocyanates (Fig. 4.2), which are responsible for the bitter and pungent flavors of crucifers like cabbage, mustard or wasabi. Isothiocyanates are toxic in vitro to a wide variety of organisms, including fungi and bacteria (Aires et al. 2009; Schlaeppi et al. 2010; Shin et al. 2010; Wu et al. 2011; Stotz et al. 2011).

An in vivo role for glucosinolates as antimicrobial defense compounds is now well established in Arabidopsis. Mutants deficient in glucosinolate synthesis show

enhanced susceptibility to various fungal pathogens (Schlaeppi et al. 2010; Stotz et al. 2011). In addition to producing toxic compounds, glucosinolate hydrolysis results in molecules that appear to have a regulatory role in plant defense responses (Bednarek 2012). The mechanism of action of isothiocyanates involves the central carbon of the functional group, which is a reactive electrophile that can readily form stable covalent bonds with nucleophilic atoms in thiol and amine groups of proteins and peptides (Brown and Hampton 2011).

There has been interest in the possibility of using isothiocyanates as natural preservatives, to prevent bacterial and fungal growth on diverse food products, from chicken meat to apples (Shin et al. 2010; Wu et al. 2011; Wilson et al. 2013). While they are generally considered safe for human consumption, the strong odor and flavor of these chemical can be unpleasant. The most promising approach is to apply them as a vapor, as they are volatile and can be effective at low concentrations.

The biosynthetic pathway of glucosinolates is quite well understood and dozens of genes have been identified in Arabidopsis, opening the way for plant genetic engineering (Sønderby et al. 2010). The introduction in Arabidopsis of different cytochrome P450 genes, responsible for the first committed step, resulted in the production of two different types of glucosinolates and led to increased resistance to specific bacterial pathogens (Brader et al. 2006). While this approach would work in crucifer crops, engineering glucosinolate production in species from other families is a more complex task. The simultaneous expression of six heterologous genes was sufficient to obtain stable tobacco transformants capable of synthesizing benzylglucosinolate from phenylalanine (Møldrup et al. 2012). Production of a the glucosinolate glucoraphanin, on the other hand, was achieved by transient expression of 13 different genes in tobacco leafs (Mikkelsen et al. 2010). Recently the synthesis of glucosinolates in yeast was achieved by introducing a seven-step pathway (Mikkelsen et al. 2012).

4.4 Phenylpropanoids

Phenylpropanoids, also referred to as phenolics or polyphenols, are compounds with aromatic rings derived from phenylalanine, and to a lesser extent tyrosine, by the loss of their amine group (Vogt 2010). This pathway is responsible for the formation of lignin monomers as well as a wide variety of other specialized metabolites, some of which have antimicrobial activities (Daglia 2012). Flavonoids and stilbenes are the two groups of phenylpropanoids where evidence for an in vivo role in plant defense is clearest. It is also likely that a tobacco coumarin with in vitro antimicrobial activity plays an important role in resistance to bacterial and fungal pathogens (El Oirdi et al. 2010; Großkinsky et al. 2011). Flavonoid, stilbenes and coumarins are all derived by different routes from p-coumaroyl-CoA, a key branching point in the phenylpropanoid pathway.

4.4.1 Flavonoids

The flavonoids are generally tricyclic compounds with two aromatic rings (Fig. 4.2). They are a very large group of specialized metabolites with more than 6,000 known molecules (Falcone Ferreyra et al. 2012). They are present in all land plants and have a diversity of functions, including providing color to flowers, fruits and seeds.

The synthesis of flavonoids starts with one molecule of p-coumaroyl-CoA and three of malonyl-CoA. The enzyme chalcone synthase catalyzes a series of decarboxylation, condensation, and cyclization reactions that produce naringenin chalcone, a C6-C3-C6 compound with two aromatic rings connected by a chain of three carbons (Winkel-Shirley 2001). With the collaboration of chalcone reductase, chalcone synthase can produce isoliquiritigenin, also known as trihydroxychalcone, which has one less OH group. Chalcone isomerase can then act on both products to form the tricyclic molecules naringenin and liquiritigenin, precursors to most flavonoids. Glycosylation is common in flavonoids, usually with one or two sugar residues.

A large number of flavonoid compounds have shown in vitro antifungal and antibacterial activity, sometimes at very low concentrations, and against a wide range of species that includes plant and human pathogens (Rivera-Vargas et al. 1993; Cushnie and Lamb 2011; Pistelli and Giorgi 2012). The clearest evidence for an in vivo role in antimicrobial plant defense comes from studies of isoflavonoids or its pterocarpan derivatives in the pea (*Fabaceae*) family. The synthesis of these compounds starts with isoflavone synthase acting on naringenin or liquiritigenin to change the position of the aryl group (Du et al. 2010). Isoflavonoid synthesis is induced upon fungal infection in many *Fabaceae* species (Ahuja et al. 2012). Overexpression of an isoflavone biosynthetic enzyme in alfalfa resulted in increased accumulation of these compounds and enhanced resistance to a fungal pathogen (He and Dixon 2000). On the other hand silencing of another biosynthetic enzyme in pea led to increased susceptibility to a different fungal pathogen (Wu and VanEtten 2004). The same result was obtained when isoflavone synthase or chalcone reductase were silenced in soybean (Subramanian et al. 2005; Graham et al. 2007). Silencing of another enzyme required for isoflavone synthesis resulted in increased susceptibility of soybean to the bacterial pathogen *Pseudomonas syringae*, (Zhou et al. 2011). *P. syringae*, unlike fungal pathogens, was not inhibited by isoflavones in vitro, suggesting that isoflavones could also have a role in defense signaling (Rivera-Vargas et al. 1993; Zhou et al. 2011). In particular soy isoflavonoids appear to activate programmed cell death around the infected area (Graham et al. 2007).

The antimicrobial mechanism of action of flavonoids is currently unclear. Various hypotheses have been proposed for their antibacterial activity, including membrane alterations, inhibition of DNA modifying enzymes and interference with energy metabolism. However, a recent review concluded that many published studies, in addition to presenting methodological problems, cannot discriminate

between causes and consequences (Cushnie and Lamb 2011). A proposed mechanism that could that could explain toxicity of flavonoids to both eukaryotes and prokaryotes is direct inhibition of ATP synthase, as flavonoids can bind the γ-subunit and block its rotation (Gledhill et al. 2007; Dadi et al. 2009). Several studies also indicate that flavonoids could reduce the pathogenicity of bacteria by interfering with quorum-sensing mechanisms (Vandeputte et al. 2011; Cushnie and Lamb 2011).

There has been several successful attempts to manipulate flavonoid biosynthesis in plants, introducing for example isoflavone synthase in species that do not normally produce isoflavonoids (Du et al. 2010). However these studies have been centered on the possible health benefits of dietary isoflavonoids as phytoestrogens, instead of their antimicrobial properties. There have also being significant advances in the production of flavonoids, including isoflavonoids, in bacterial and yeast heterologous systems (Du et al. 2010; Falcone Ferreyra et al. 2012). Finally a number of flavonoids have been considered for clinical use against human pathogens such as antibiotic-resistant bacteria (Liu et al. 2009; Cushnie and Lamb 2011; Pistelli and Giorgi 2012).

4.4.2 Stilbenes

Stilbenes have two aromatic rings connected by a two-carbon bridge (Fig. 4.2). More than 400 stilbenes have been described and they can appear as monomeric or oligomeric structures (Shen et al. 2009). They have been found in several unrelated families of land plants, including mosses, ferns, gymnosperms and angiosperms, in what appears to be a case of convergent evolution (Tropf et al. 1994; Riviere et al. 2012). Among economically important plants stilbenes have been found in grapevine, pines, peanut and sorghum (Chong et al. 2009).

Stilbene synthases are the enzymes responsible for the first committed synthesis step. These enzymes have independently evolved several times from chalcone synthases. They produce a C6-C2-C6 skeleton instead of the C6-C3-C6 skeleton of flavonoids (Chong et al. 2009). Like chalcone synthases, stilbene synthases can use three molecules of malonyl-CoA and one of p-coumaroyl-CoA as substrates, resulting in the production of resveratrol. Some stilbene synthases can also utilize cinnamoyl-CoA instead of p-coumaroyl-CoA to produce pinosylvin. Similarly to flavonoids, stilbenes are frequently glycosylated.

There are numerous reports of significant in vitro antifungal activity for different stilbenes (Jeandet et al. 2002; Chong et al. 2009). Antibacterial activity against gram-positive and gram-negative species has also been reported (Jeandet et al. 2012; Plumed-Ferrer et al. 2013). Some stilbenes are cytotoxic to human cells and have antitumoral activities (Jane Lunt et al. 2011; Jeandet et al. 2012). Regarding their physiological role in vivo, resveratrol and its derivatives are produced at higher levels during fungal infections in grapevine, while in conifers pinosylvin derivatives accumulate in the heartwood constitutively, probably

protecting it from decay (Jeandet et al. 2002; Chong et al. 2009). The best evidence for the effectiveness of stilbenes in vivo is the enhanced resistance of different species transformed with stilbene synthase and the linkage between fungal pathogenicity and the capacity to metabolize stilbenes (Chong et al. 2009; Jeandet et al. 2012). As for the mechanism of action of stilbenes, one possibility, as in the case of flavonoids, is inhibition of ATP synthase by direct binding (Gledhill et al. 2007; Dadi et al. 2009).

The first demonstration of enhanced disease resistance in a transgenic plant producing a new antimicrobial compound was achieved by introducing stilbene synthase in tobacco (Hain et al. 1993). The transgenic tobacco produced the stilbene resveratrol and showed increase resistance to a fungal pathogen. The same experiment has been repeated in numerous other species including rice, barley, wheat, apple, papaya, banana, tomato, lettuce and pea (Jeandet et al. 2012). In many cases increased resistance against fungal pathogens was observed. Tobacco plants producing pterostilbene, a derivative of resveratrol with higher antifungal activity, were obtained by introducing an *O*-methyltransferase in addition to stilbene synthase (Rimando et al. 2012). Synthesis of resveratrol from amino acids phenylalanine and tyrosine has been achieved in yeast (Jeandet et al. 2012). It is also possible to produce resveratrol in *E. coli*, but the culture needs to be fed p-coumaric acid (Lim et al. 2011).

4.5 Terpenoids

Terpenoids, also called isoprenoids, are the most diverse class of specialized metabolites, but they also include core metabolites, such as sterol lipids. They are derived from the five carbon isomers isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAP). These compounds are produced in the cytosol and chloroplast by different pathways. Addition of one to three IPP subunits to DMAP produces skeletons of 10 (geranyl diphosphate), 15 (farnesyl diphosphate) or 20 carbons (geranylgeranyl diphosphate) that are further modified to produce the huge diversity of plant terpenoids (Vranová et al. 2012).

Terpenoids are usually classified according to the number of IPP or DMAP units used in their synthesis. Sesquiterpenes (C15 or three subunits) appear to have a role as antimicrobial metabolites in species from the potato (*Solanaceae*) family. In addition to antimicrobial activity in vitro, transgenic tobacco and cotton that produced higher amounts of sesquiterpenes, due to altered pathogen response pathways, show enhanced resistance against fungal pathogens (Parkhi et al. 2010; Großkinsky et al. 2011). A novel family of sesquiterpenes induced by fungal infection and with antifungal activity in vitro has also being discovered in maize (Huffaker et al. 2011). There is also evidence for a role of diterpenes (C20) as antifungal compounds in plants from the grass (*Poaceae*) family. Mutants or transgenic rice plants with altered defense pathways that have increased or

decreased levels of diterpenes shows correlated changes in fungal disease resistance (Mori et al. 2007; Kim et al. 2009). Direct proof of an in vivo role, however, is only available for a group of triterpenes (C30) called saponins.

4.5.1 Saponins

Saponins are glycosylated triterpenoids whose name derives from their ability to form soap-like foams in solution due to the combination of hydrophilic and hydrophobic moieties (Fig. 4.2). Saponins appear to be widespread in angiosperms and are found in both monocots and dicots (Vincken et al. 2007).

The synthesis of saponins starts with two farnesyl diphosphate molecules that are linked and oxidized to produce oxidosqualene. The cyclization of this linear compound can be carried out by a number of oxidosqualene cyclases that generate different saponin precursors, which are further modified and glycosylated, usually with 2–5 monosaccharide units (Vincken et al. 2007; Sawai and Saito 2011; Augustin et al. 2011; Osbourn et al. 2011). The two main types of saponin are C27 steroidal saponins and C30 triterpenoid saponins, but the latter can be subdivided in 10 different groups according to their skeleton (Vincken et al. 2007). Although saponins accumulate constitutively in many species, saponin levels can also increase in response to fungal attacks (Augustin et al. 2011).

There are numerous reports of saponins with in vitro toxicity against diverse fungi including plant and human pathogens (Coleman et al. 2010; Saha et al. 2010). Antibacterial activity against gram-positive and gram-negative species has also being reported (Sung and Lee 2008). Many saponins are also hemolytic, but this property does not seem to correlate with antifungal activity (Augustin et al. 2011).

The best evidence for an in vivo defensive function for saponins comes from oat. This plant constitutively accumulates in roots fluorescent saponins called avenacins. This has made it possible to identify a number of saponin-deficient mutants that showed reduced resistance against fungal pathogens (Papadopoulou et al. 1999). Five of the genes directly involved in the synthesis of avenacins have already been identified, with mutants in four of them resulting in enhanced disease susceptibility (Mugford et al. 2013). In addition to active avenacins in the root, oat accumulates inactive forms in the leaves called avenacosides (Morant et al. 2008). The inactive saponins have an additional β-glucose residue at C26 that can be removed by a specific β-glucosidase.

The main mechanism of action of saponins seems to involve membrane disruption leading to an increase in permeability (Coleman et al. 2010; Augustin et al. 2011). It appears that the triterpenoid moiety of saponins binds sterol lipids in the membrane. The attached sugars can force the membrane to curve when the local saponin concentration is high. This could lead to disruption of lipid rafts or even pore formation. It is unclear how plants avoid these effects when they accumulate

active saponins, possibly in vacuoles, but differences is sterol concentration or composition might be responsible (Augustin et al. 2011).

Triterpenoid saponins have not been found in cereals other than oat. Engineering other species to synthesize these compounds could enhance their resistance against fungal pathogens. A first step has been taken with the introduction in rice of a β-amyrin synthase from oat, an oxidosqualene cyclase that catalyzes the first committed step in avenacin biosynthesis (Inagaki et al. 2011). There have also being some successes at engineering yeast for the production of saponin precursors (Sawai and Saito 2011). The achievement of this objective would first require the identification of all the steps in the pathway. Regarding clinical applications, saponins have been found to be effective against *Candida albicans* using a nematode model, showing no hemolytic activity at inhibitory concentrations (Coleman et al. 2010).

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