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# Phytoalexins in defense against pathogens

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**Plants use an intricate defense system against pests and pathogens, including the production of low molecular mass secondary metabolites with antimicrobial activity, which are synthesized *de novo* after stress and are collectively known as phytoalexins. In this review, we focus on the biosynthesis and regulation of camalexin, and its role in plant defense. In addition, we detail some of the phytoalexins produced by a range of crop plants from Brassicaceae, Fabaceae, Solanaceae, Vitaceae and Poaceae. This includes the very recently identified kauralexins and zealexins produced by maize, and the biosynthesis and regulation of phytoalexins produced by rice. Molecular approaches are helping to unravel some of the mechanisms and reveal the complexity of these bioactive compounds, including phytoalexin action and metabolism.**

## Phytoalexins: part of the plant response repertoire

Crop loss due to pest and pathogen attack is a serious problem worldwide. Plants are constantly attacked by many potential pathogens and respond by the activation of defense genes, the formation of reactive oxygen species (ROS), the synthesis of pathogenesis-related (PR) proteins, localized cell-wall reinforcement and the production of antimicrobial compounds. Low molecular mass secondary metabolites with antimicrobial activity that are induced by stress are collectively named phytoalexins, and are an important part of the plant defense repertoire [1,2]. Phytoalexins are a heterogeneous group of compounds (Figure 1) [3–5] that show biological activity towards a variety of pathogens and are considered as molecular markers of disease resistance.

The concept of phytoalexins was introduced over 70 years ago [6] based on the finding that potato (*Solanum tuberosum*) tuber tissue that had previously been infected with an incompatible race of *Phytophthora infestans* induced resistance to a compatible race of *P. infestans*. It was hypothesized that the tuber tissue, in response to the incompatible interaction, produced substances (phytoalexins) that inhibited the pathogen and protected the tissue against later infection by other compatible races of the pathogen [2,6,7]. Since then, the field has evolved extensively, not only with respect to studying the roles of phytoalexins in defense against pathogens and pests, but also with respect to their health-promoting effects [2,8–13]. For example, indole phytoalexins contribute to the antioxidant, anticarcinogenic and cardiovascular protective activities of *Brassica* vegetables [2,12]. Peanut (*Arachis hypogea*) phytoalexins have antidiabetic, anticancer and vasodilator effects [11]. The biological activities of glyceollin, a soybean (*Glycine max*) phytoalexin, include antiproliferative and antitumor actions [9]. The sorghum (*Sorghum bicolor*) phytoalexins, 3-deoxyanthocyanins, might be useful in helping to reduce incidence of gastrointestinal cancer [13]. The phytoalexin resveratrol from grapevine (*Vitis vinifera*) has anti-aging, anticarcinogenic, anti-inflammatory and antioxidant properties that might be relevant to chronic diseases and/or longevity in humans [10].

However, the biosynthesis of most phytoalexins, the regulatory networks involved in their induction by biotic and abiotic stress, and the molecular mechanisms behind their cytotoxicity are largely unknown. In this review, we detail some of the recent advances in this field, focusing on the model plant *Arabidopsis* (*Arabidopsis thaliana*) and crop plants from Brassicaceae, Fabaceae, Solanaceae, Vitaceae and Poaceae. The substantial progress that has recently been made in identifying the biosynthetic steps of camalexin, a phytoalexin produced by *Arabidopsis*, and the attempts to decipher its regulation and to understand its role in resistance to pathogens will be covered first. *Arabidopsis* mutants affected in their capacity to produce camalexin upon challenge with pathogens (see Table S1 in the supplementary material online), their biochemical characterization and their use in pathogenicity tests have been of great importance in this respect. To develop disease protection strategies, plant pathogen research in the field of phytoalexins has also focused on interpreting their biosynthesis pathways and regulation in different crop plants by using different cultivars, transgenic plants and mutants, and by applying -omics, molecular biology and biochemical approaches. Most of the reviews in this direction so far have been written on phytoalexins belonging to a particular plant or family or focused on a particular group of phytoalexins. However, in this review, we provide a broader perspective on the research on phytoalexins by covering their diversity, biosynthesis and regulation, and their accumulation or enhancement after pathogen infection or elicitor treatment in some major crop plants.

## Camalexin: the major phytoalexin in *Arabidopsis*

Camalexin (3-thiazol-2′-yl-indole), a phytoalexin that was first isolated from a plant in the Brassicaceae family,

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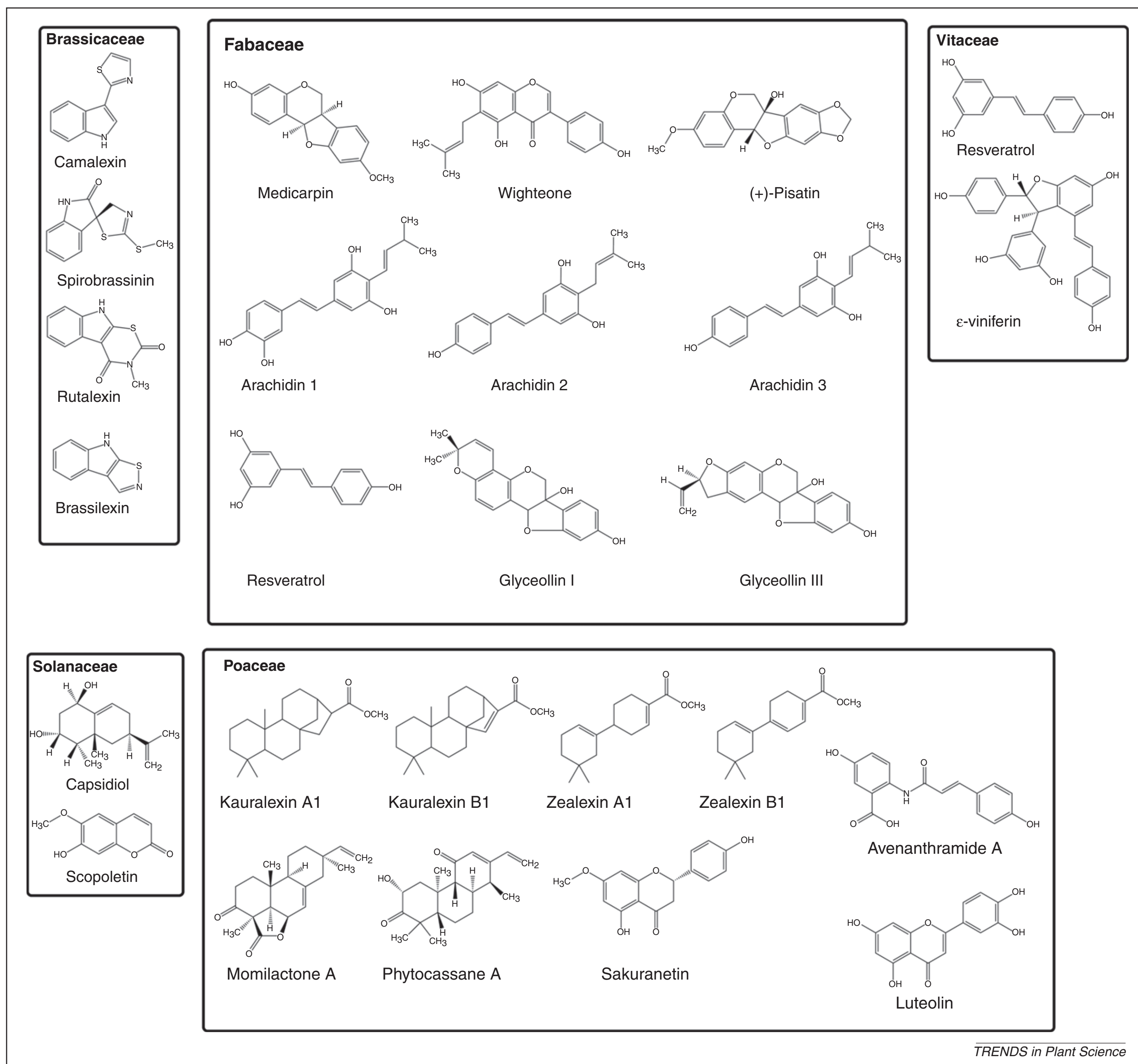


**Figure 1.** Structures of selected phytoalexins produced by members of the Brassicaceae, Fabaceae, Solanaceae, Vitaceae and Poaceae.

camelina (*Camelina sativa*), after which it was named [14], has also been detected in *Arabidopsis* and a few related Brassicaceae species [15]. Although camalexin biosynthesis in *Arabidopsis* has not yet been fully elucidated, several of the steps in the pathway have been characterized over recent years (Box 1). Camalexin was long thought to be the only phytoalexin produced by *Arabidopsis*, but another, rapalexin A, has also been detected in this species [16].

### *Camalexin-inducing conditions and its natural variation in* Arabidopsis

The production of camalexin can be induced in *Arabidopsis* leaves by a range of biotrophic and necrotrophic plant pathogens (bacteria, oomycetes, fungi and viruses). Some examples are listed in Figure 2 and Table S1 in the supplementary material online. Camalexin can be induced in *Arabidopsis* by the recognition of a range of different pathogen-derived substances known as microbe-associated molecular patterns (MAMPs), such as the oomycete necrosis and ethylene-inducing peptide1 (Nep1)-like proteins and bacteria-derived peptidoglycan [17,18]. Although other pathogen-mimicking stimuli, such as plant cell wall-derived oligogalacturonides, chitosan and the bacterial flagellin peptide Flg22, induced the expression of camalexin biosynthetic genes [19–21], triggering of camalexin biosynthesis has not been observed in all instances [18,22–24]. Treatment of *Arabidopsis* with autoclaved baker's yeast (*Saccharomyces cerevisiae*) suspension and fungal toxins (victorin produced by *Cochliobolus victoriae* or fusaric acid produced by *Fusarium* spp.) also induced the production of camalexin [25–27].

Abiotic stresses, such as UV-B, UV-C, chemicals (e.g. acifluorfen, paraquat, chlorsulfuron and α-amino butyric acid) and heavy metal ions (e.g. silver nitrate), can also induce camalexin in *Arabidopsis* leaves [28,29]. Treatment with C6-aldehydes, which are plant volatiles typically released upon wounding, has been reported to elevate levels of camalexin [30]. However, another study showed that wounding alone did not enhance the production of camalexin, although wounding primed the plant for quicker camalexin production upon subsequent *Botrytis cinerea* inoculation, and hence contributed to enhanced resistance [31].

Although most camalexin measurements reported in the literature are performed on whole leaves or seedlings, it has been shown that the increase in camalexin levels is largely limited to the area surrounding the lesion [32,33]. There is little information about the induction of camalexin production in organs other than rosette leaves in *Arabidopsis*. The root-pathogenic oomycete *Pythium sylvaticum* induced the synthesis of camalexin in, and its excretion from, roots [34]. Increased levels of camalexin have also been detected in the root exudates of roots treated with Flg22 [23].

All *Arabidopsis* ecotypes analyzed so far seem to be able to produce camalexin after induction. Some natural variation in camalexin production between *Arabidopsis* ecotypes has been reported, but this has only been comprehensively studied in response to a limited number of pathogens, such as *Botrytis cinerea* [35], *Alternaria brassicicola* and *Cochliobolus carbonum* [36]. The Col-0 ecotype produced higher camalexin levels than did L*er*-0 upon inoculation with *Leptosphaeria maculans*, although both ecotypes are resistant to this fungal pathogen [37]. Upon inoculation with the biotroph *Puccinia triticina*, higher levels of camalexin were induced in the more susceptible ecotype Wa-1 compared with those induced in the Col-0 ecotype. This was probably because of a more powerful plant defense response triggered by enhanced growth of the fungus in Wa-1 [38]. In another study, Col-0 plants were shown to produce camalexin 4 days post inoculation with *Colletotrichum higginsianum* but camalexin could hardly be detected in the more resistant ecotype Eil-0, suggesting that camalexin is not important for the resistance of Eil-0 to this fungus [39].

### *Regulation of camalexin biosynthesis*

Studies on the major signaling pathways controlling the induction of camalexin in *Arabidopsis* indicated that their contribution might depend on the infecting pathogen. Whereas studies looking at the response of *Arabidopsis* jasmonic acid (JA) signaling mutants to *Alternaria brassicicola* infection led to the conclusion that camalexin synthesis is under the control of a JA-independent pathway [40,41], recent studies with *Botrytis cinerea* have concluded that JA signaling controls camalexin synthesis to a large extent [42]. Different studies have proposed that camalexin production is controlled by salicylic acid (SA)-independent [43,44] and SA-dependent [45] signaling pathways. Similarly, ethylene signaling might be involved because *ein2* and *etr1* mutants, which are impaired in ethylene signaling, accumulated less camalexin after challenge with *Pseudomonas syringae* or *Alternaria brassicicola* than did the wild type [41,46]. By contrast, camalexin induction after mitogen-activated protein kinase (MAPK) MPK3/MPK6 activation (see below) is considered to be independent of ethylene [47]. It has recently been suggested that miR393, a plant miRNA induced by Flg22, is able to regulate camalexin production by affecting auxin signaling. miR393 targets the auxin receptors and thereby prevents activation of the auxin response factor 9 (ARF9) transcription factor, a positive regulator of camalexin biosynthesis. This allows *Arabidopsis* to redirect its metabolic flow from camalexin to glucosinolates, which are more effective in biotroph resistance. In addition, repression of auxin signaling prevents auxin from antagonizing SA signaling, enabling the plant to mount an SA response [48]. ROS are also generally associated with camalexin production, as shown by the induction of camalexin by oxidative stress-inducing chemicals such as paraquat and acifluorfen [28,29]. However, it has recently been proposed that both hydrogen peroxide ($H_2O_2$) and SA are required for the accumulation of camalexin [49].

Several recent reports have shown that camalexin biosynthesis is regulated through MAPK cascades (Box 1). MPK3/MPK6, through the activation of upstream MAP kinase kinase (MAPKK; MKK4 and MKK5) and MAP kinase kinase kinase (MAPKKK; MEKK1 and MAPKKKα), acts upstream of the cytochrome P450 CYP71B15 (also called PAD3 for PHYTOALEXIN DEFICIENT 3) and the γ-glutamylcysteine synthase PAD2 [47]. MKK9 also activates MPK3/MPK6, inducing camalexin biosynthesis [50]. Expression of genes involved in tryptophan biosynthesis and those encoding the cytochrome P450 s CYP79B2, CYP71A13 and CYP71B15 (PAD3), which are involved in camalexin biosynthesis, are induced through these cascades [47,50]. Activation of MKK9 also led to the accumulation of three phi class glutathione *S*-transferases (GSTs) and higher GST activity in connection with higher camalexin levels [51]. Concomitant with the fact that the MAPK phosphatases MKP1 and PTP1 dephosphorylate MPK3 and MPK6, the *mkp1 ptp1* double null mutant showed constitutively higher camalexin levels [52]. The MKK4/MKK5–MPK3/MPK6 cascade affected camalexin levels through transcriptional activation and phosphorylation of the WRKY transcription factor WRKY33 [53]. WRKY33 was previously shown to control camalexin levels by regulating the expression of genes, such as *PAD3* and *CYP71A13*, through its interaction with another MAP kinase, MPK4 [19]. WRKY33 forms ternary complexes with MPK4 and its substrate MAP kinase substrate 1 (MKS1). MPK4 in turn is activated by MAMPs, which is followed by phosphorylation of MKS1 and release of WRKY33 from MPK4 [19]. Further research is needed to clarify inconsistencies between the described WRKY33-dependent mechanisms and to determine whether different signaling cascades are involved in the response to different pathogens. Recently, WRKY40 and WRKY18 have also been implicated in the regulation of camalexin biosynthesis because the double *wrky18 wrky40* loss-of-function mutant exhibited transcriptional activation of camalexin biosynthetic genes, including *CYP71A13*, and constitutively higher camalexin levels than in wild-type plants [54].

### Camalexin toxicity and pathogen detoxification mechanisms

Studies of *Arabidopsis* mutants affected in their capacity to produce camalexin upon pathogen challenge (Figure 2 and Table S1 in the supplementary material online) revealed that camalexin plays a role in resistance to the necrotrophic fungi *Alternaria brassicicola* [55], *Botrytis cinerea* [32,56] and *Plectosphaerella cucumerina* [57] but not to the widely used hemibiotrophic bacteria model *Pseudomonas syringae* [58,59]. This suggested that camalexin was involved in defense against necrotrophic but not biotrophic pathogens. However, it has since become clear that camalexin is also implicated in resistance against the hemibiotrophic oomycete *Phytophthora brassicae* [60], the hemibiotrophic fungus *Leptosphaeria maculans* [37,61] and the adapted biotrophic powdery mildew *Golovinomyces orontii* [54,62]. Camalexin also has an important function in post-invasive defense against the non-adapted powdery mildews *Blumeria graminis* and *Erysiphe pisi* [63]. Insect assays on camalexin-deficient mutants indicated that camalexin is not important for resistance against the generalist insects *Myzus persicae* [64] and *Spodoptera littoralis* [65] but has a negative effect on the fitness of the phloem-sucking specialist insect *Brevicoryne brassicae* [66].

The antimicrobial activity of camalexin has been tested *in vitro* on several species of bacteria, oomycetes and fungi [32,57,60,67–70]. However, the mechanisms by which camalexin exerts its toxicity and the mechanisms conferring resistance in some pathogens towards camalexin are still unknown. Camalexin disrupts bacterial membranes, suggesting that this is why camalexin has a toxic effect on *Pseudomonas syringae* pv *maculicola* strain ES4326 [67]. *Alternaria brassicicola* mutants lacking MAPKs involved in signaling cell-wall compensatory mechanisms were more sensitive to camalexin, suggesting that the toxic effect of camalexin on fungi also involves cell membrane damage [71]. Transcriptional profiling of *Alternaria brassicicola* exposed to camalexin has also pointed towards induced membrane maintenance and reduced cell wall permeability, as well as potential involvement of efflux processes [72]. In addition, the unfolded protein response (UPR), a signaling pathway triggered in response to endoplasmic reticulum (ER) stress to maintain the ER protein folding capacity, is activated in *Alternaria brassicicola* when mycelium is treated with camalexin. An *Alternaria brassicicola* mutant strain impaired in the UPR shows cell wall defects and is more susceptible to camalexin [73]. For *Botrytis cinerea*, it has been shown recently that camalexin treatment induces fungal apoptotic-like programmed cell death (PCD) and that a transgenic strain with enhanced anti-apoptotic capacity is less susceptible to camalexin [74]. *In planta*, camalexin might thus induce fungal PCD, limiting the spread of lesions during the early *Botrytis cinerea* infection stage, while the fungal anti-apoptotic machinery would allow the fungus to recover and subsequently establish infection [74]. When *Botrytis cinerea* is exposed to camalexin, it also induces the expression of *BcatrB*, an ABC transporter that has an efflux function, acting as a protective mechanism against the fungitoxic effect of camalexin [75].

A range of camalexin detoxification mechanisms through metabolization has also been reported; examples include, the production of 5-hydroxycamalexin by *Rhizoctonia solani*, glucosylation of camalexin by *Sclerotinia sclerotiorum* and production of 3-indolecarboxylic acid (and other intermediates) by *Botrytis cinerea* [76]. However, *Leptosphaeria maculans* and *Alternaria brassicae* do not seem to metabolize camalexin [77].

## Phytoalexin production in crop plants

Phytoalexins induced by pathogens in crop plants are much more diverse than those induced in *Arabidopsis*. Here, we review some examples of recent efforts to elucidate the biosynthesis and production of phytoalexins that accumulate in Brassicaceae, Fabaceae, Solanaceae, Vitaceae and Poaceae crops in response to pathogen infection or elicitor treatments and their role in pathogen defense (Figure 1, Tables 1, 2, and Table S2 in the supplementary material online).

Elicitors induce production of phytoalexins by mimicking a pathogen attack or other stress [78], and can be substances of pathogenic origin (exogenous) or compounds released by plants by the action of the pathogen (endogenous). Elicitors have potential uses in sustainable crop production and some of the recent advances made towards attaining this goal are detailed below.

### Phytoalexins in Brassicaceae

To date, 44 phytoalexins have been isolated from cultivated and wild Brassicaceae (which are also known as crucifers): most of the phytoalexins are alkaloids that are biosynthetically derived from the amino acid (*S*)-tryptophan and contain sulfur. The structure, biology and detoxification mechanisms of these phytoalexins have been recently reviewed [2,76] and, therefore, are not discussed here. Some of the phytoalexins that accumulate in Brassicaceae after pathogen infection are presented in Figure 1,

**Box 1. Aspects of camalexin biosynthesis and regulation**

The indole ring of camalexin is derived from tryptophan (Figure I) and early biosynthetic steps are shared with other indolic compounds, such as indole glucosinolates. The cytochrome P450 homologs CYP79B2 and CYP79B3 convert tryptophan to indole-3-acetaldoxime (IAOx); the *cyp79b2 cyp79b3* double mutant is devoid of indole glucosinolates and unable to produce more than trace amounts of camalexin [162]. IAOx is the branching point between the biosynthesis of camalexin, indole glucosinolates and the phytohormone indole-3-acetic acid. Subsequently, IAOx is converted into indole-3-acetonitrile (IAN) by another cytochrome P450, CYP71A13. Whether the residual amount of camalexin in rosette leaves of the *cyp71a13* mutant is derived from IAN provided by indole glucosinolate turnover or the homologous CYP71A12 [55] is not yet known. Recent genetic evidence has demonstrated that CYP71A12 has an important role in camalexin synthesis in roots [23]. The glutathione *S*-transferase GSTF6 has been implicated in the next step: the conjugation of IAN with glutathione (GSH); however, this reaction might also involve the activity of a cytochrome P450. Moreover, substantial levels of camalexin in the *gstf6* mutant point towards redundancy within the GST family for this step [51]. The GSH(IAN) conjugate is metabolized to Cys(IAN) and two

possible routes involving γ-glutamyl transpeptidase (GGT) and phytochelatin synthase (PCS) activities, respectively, have recently been proposed [51,163]. However, Geu-Flores *et al.* [164] have argued against a role of GGTs and have presented evidence of the involvement of γ-glutamyl peptidases 1 and 3 (GGP1 and GGP3) in metabolizing GSH(IAN). The final two steps in camalexin biosynthesis [the thiazoline ring formation and cyanide release from Cys(IAN) leading to dihydrocamalexic acid (DHCA) and the subsequent oxidative decarboxylation of DHCA] are catalyzed by the multifunctional cytochrome P450 CYP71B15 (PAD3) [163,165]. Although the *pad3* mutant accumulates at best trace amounts of camalexin upon biotic stress (Table S1 in the supplementary material online), it does produce Cys(IAN), DHCA and derivatives of these compounds [163]. DHCA and trace amounts of camalexin in *pad3* are likely to be the result of non-enzymatic conversions of their precursors [163]. Overexpression of *CYP79B2* [162] or *PAD3* [165] does not lead to camalexin formation without elicitation, suggesting that the steps catalyzed by these enzymes are not rate-limiting. Alternatively, it could indicate the necessity of co-activation of all genes in the pathway to drive camalexin biosynthesis, as indicated by gene expression profiling after elicitation.

Camalexin can be further modified *in planta*. Methoxylated and methylated derivatives, such as 6-methoxy- and *N*-methyl-camalexin, are produced in some species, but these seem to be lacking from *Arabidopsis* [15,163]. Instead, derivative compounds resulting from sequential hydroxylation, *O*-glycosylation and malonylation of camalexin are detected in *Arabidopsis* [163].

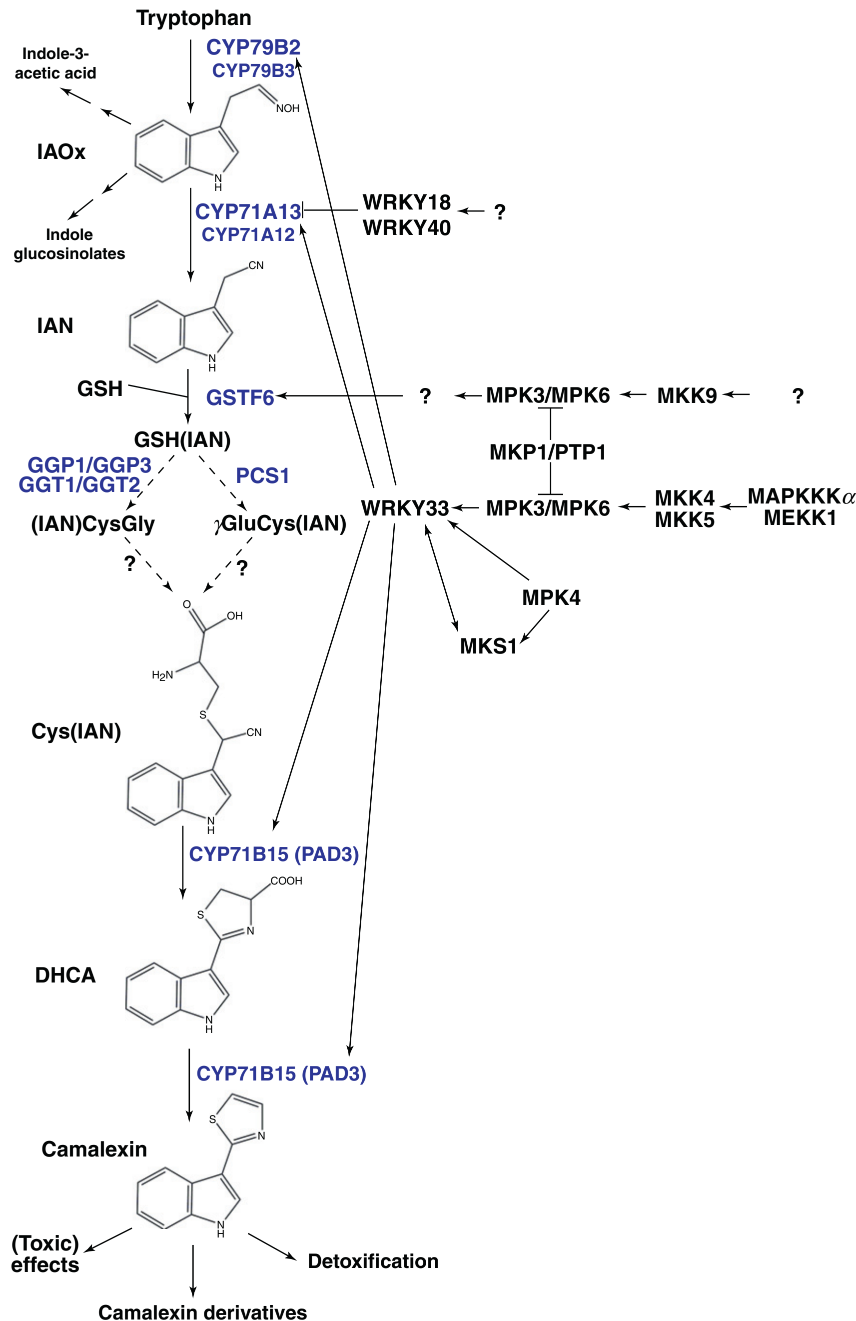


**Figure I.** Camalexin biosynthesis and regulatory mechanisms. Abbreviations: CYP, cytochrome P450; DHCA, dihydrocamalexic acid; GGP1/3, γ-glutamyl peptidases 1 and 3; GGT1/2, γ-glutamyl transpeptidase 1 and 2; GSH, glutathione; GSTF6, glutathione *S*-transferase F6; IAN, indole-3-acetonitrile; IAOx, indole-3-acetaldoxime; MEKK/MAPKKK, mitogen-activated protein (MAP) kinase kinase kinase; MPK, MAP kinase; MKK, MAP kinase kinase; MKP1, MAPK phosphatase 1; MKS1, MAP kinase substrate 1; PAD3, PHYTOALEXIN DEFICIENT 3; PCS1, phytochelatin synthase 1; PTP1, protein tyrosine phosphatase 1.

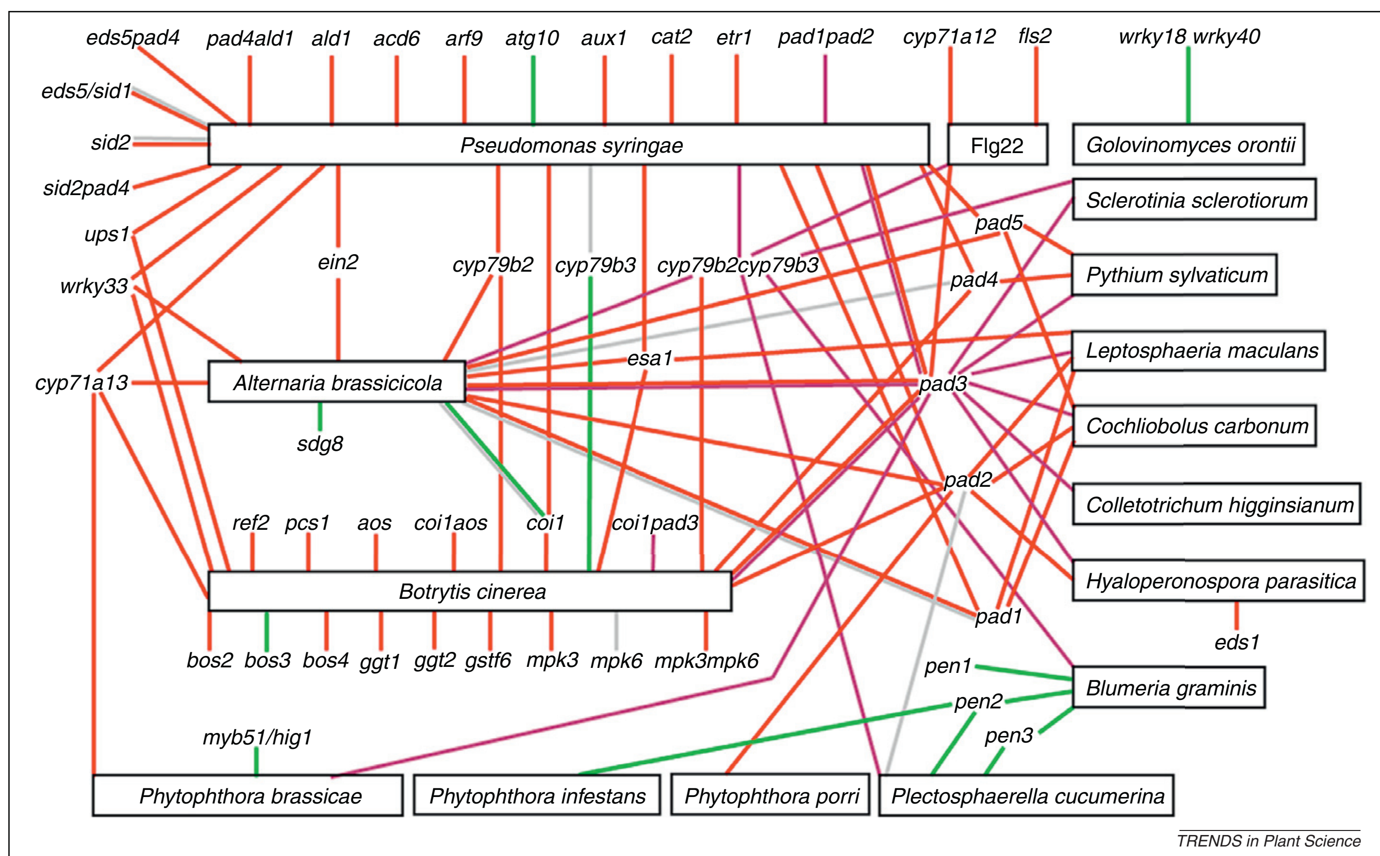


**Figure 2**. Camalexin levels in *Arabidopsis thaliana* mutants triggered upon pathogen or elicitor treatment. The effect of pathogen or elicitor treatment (boxed) on camalexin levels in a given *Arabidopsis* mutant versus wild-type plants is indicated by colored lines: a green line indicates a higher camalexin level in the mutant, whereas a red line indicates lower levels in the mutant compared with the wild type. A gray line indicates similar camalexin levels in the mutant and the wild type after treatment. A purple line indicates that no camalexin was detected in the mutant after the given treatment. Two arrows in different colors for the same pathogen–mutant pair indicate that different results were reported. More detailed information on the pathogen strains and mutant alleles represented here, as well as the references for the different studies, are given in Table S1 in the supplementary material online. Abbreviations: *acd6, accelerated cell death 6; ald1, agd2-like defense response protein 1; aos, allene oxide synthase; arf9, auxin response factor 9; aux1, auxin resistant 1; bos2/3/4, botrytis susceptible 2/3/4; cat2, catalase 2; coi1, coronatine insensitive 1; cyp79b2/b3, cyp71a12/a13, cytochrome P450s; eds1/5, enhanced disease susceptibility 1/5; ein2, ethylene insensitive 2; esa1, enhanced susceptibility to Alternaria; etr1, ethylene response 1; Flg22, flagellin 22; fls2, flagellin-sensitive 2; ggt1/2, gamma-glutamyltranspeptidase 1/2; gstf6, glutathione S-transferase F6; hig1, high indolic glucosinolate 1; mpk3/6, mitogen-activated protein kinase 3/6; myb51, myb domain protein 51; pad1/2/3/4/5, phytoalexin deficient 1/2/3/4/5; pcs1, phytochelatin synthase 1; pen1/2/3, penetration 1/2/3; ref2, reduced epidermal fluorescence; sdg8, SET domain group 8; sid1/2/4, salicylic acid induction-deficient 1/2/4; ups1, underinducer after pathogen and stress 1; wrky18/33/40; wrky transcription factor 18/33/40.*

Table 1, and Table S2 in the supplementary material online.

### Phytoalexins in Fabaceae

The Fabaceae or Leguminosae comprise many edible legumes, such as soybean (*Glycine max*), pea (*Pisum sativum*), chickpea (*Cicer arietinum*), alfalfa (*Medicago sativa*), barrel medic (*Medicago truncatula*), peanut (*Arachis hypogaea*) and lupine (*Lupinus angustifolius*) [79]. Upon biotic stress, plants of this family produce phytoalexins belonging mainly to the different classes of isoflavone aglycones (Figure 1, Tables 1, 2, and Table S2 in the supplementary material online) [80,81]. In the *Medicago* species alfalfa and barrel medic, the pterocarpan compounds medicarpin, vestitol, vestitone and sativan are synthesized in response to fungal or bacterial infection or metal ion elicitation [80,82,83]. In chickpea, medicarpin and maackiain are the major phytoalexins, whereas in pea, pisatin is the main phytoalexin [84]. Pisatin, a 6*a*-hydroxyl-pterocarpan, is relatively unique among naturally occurring pterocarpans by virtue of the (+) stereochemistry of its 6*a*–11*a* C–C bond [85]. Pisatin is believed to be synthesized via two chiral intermediates, (−)-7,2′-dihydroxy-4′,5′-methylenedioxyisoflavanone [(−)-sophorol] and (−)-7,2′-dihydroxy-4′,5′-methylenedioxyisoflavanol [(−)-DMDI]; both have an opposite C-3 absolute configuration to that found at C-6a in (+)-pisatin [96]. The phytoalexins in soybean are prenylated pterocarpans (the glyceollins), whereas in lupine these are wighteone and luteone [80,81,86,87]. After infection by a microbial pathogen, the peanut plant produces a characteristic set of stilbene-derived compounds that are considered phytoalexins [88]. Several stilbenic phytoalexins from peanuts have been reported [89–91]. In peanut, resveratrol is considered as the starting building block for the synthesis of more hydrophobic stilbenoids with higher biological activities [92,93]. The phytoalexin-based studies that have been carried out on crop plants of Fabaceae in recent years cover several aspects of the biosynthesis and production of phytoalexins and differ to some extent with their aims and consequences, as detailed below.

Alfalfa seedlings after challenge with *Colletotrichum trifolii* showed an increase in expression of genes involved in flavonoid biosynthesis, and in production of the phytoalexins medicarpin and sativan [82]. The study showed that alfalfa plants respond to avirulent fungal inoculation by developing an induced resistance that enhances gene expression in flavonoid metabolism, increases enzymatic activity of phenyl ammonia lyase (PAL) and levels of

**Table 1. Examples of phytoalexins induced in crop plants in response to pathogens and elicitors[a]**

| Plants | Phytoalexins | Pathogen infection | Elicitors | Refs |
|---|---|---|---|---|
| Brassicaceae: oilseed rape, canola and mustard (*Brassica rapa* and *Brassica juncea*) | | | | |
| *B. rapa*, *B. juncea* | Brassinin, spirobrassinin, cyclobrassinin, rutalexin, rapalexin A and brassilexin | *Albugo candida* and *Alternaria brassicola* | | [153,154] |
| Fabaceae (Leguminosae): alfalfa (*Medicago sativa*), barrel medic (*Medicago truncatula*), chickpea (*Cicer arietinum*), lupine (*Lupinus angustifolius*), pea (*Pisum sativum*), peanut (*Arachis hypogaea*) and soybean (*Glycine max*) | | | | |
| *M. sativa* | Medicarpin and sativan | *Colletotrichum trifolii* | | [82] |
| *M. truncatula* | Medicarpin and its isoflavone precursors | *Phoma medicaginis* | | [80] |
| | Medicarpin | | YE, MeJA | [83,94] |
| *C. arietinum* | Maackiain and medicarpin | | κ-Carrageenan of *Hypnea musciformis* (red algae) | [84] |
| *L. angustifolius* | Luteone and wighteone | *Colletotrichum lupini* | | [87] |
| *P. sativum* | Pisatin | *Nectria haematococca* | | [7,95,155] |
| | Pisatin | | $CuCl_2$ | [85,95,96] |
| | Pisatin and maackiain | | κ-Carrageenan | [84] |
| *A. hypogaea* | Resveratrol, arachidin-1, arachidin-2, arachidin-3, isopentadienyl-4,3′,5′-trihydroxystilbene, SB-1, arahypin-1, arahypin-2, arahypin-3, arahypin-4, arahypin-5, arahypin-6, arahypin-7, aracarpene-1 and aracarpene-2 | *Aspergillus* species: *A. caelatus*, *A. flavus*, *A. parasiticus* and *A. niger* | | [89,91,92] |
| | Resveratrol, arachidin-1, -3 and pterostilbene | | Sodium acetate | [99,100] |
| | Resveratrol and piceatannol | *Botryodiplodia theobromae* and *Ganoderma lucidum* | MeJA, SA and sucrose | [98] |
| | Resveratrol, arachidin-1, arachidin-2, arachidin-3, isopentadienyl-3,5,4′-trihydroxystilbene and phytoalexin derivatives | *Rhizopus oligosporus* | | [97] |
| *G. max* | Glyceollin | *Macrophomina phaseolina*, *Sclerotinia sclerotiorum* and *Phytophthora sojae* | | [156] |
| | Glyceollin and coumestrol | *Fusarium solani* f. sp. *glycines* (FSG) | FSG culture filtrate | [101] |
| | Gyceollins and glyceollidins | *Rhizopus microsporus* | | [79,86] |
| | Gyceollins | *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus awamori*, *Aspergillus sojae* and *Rhizopus oligosporus* | | [102,157] |
| | Glyceollin I and III | | YE | [105] |
| Solanaceae: pepper fruit (*Capsicum annuum*), tobacco (*Nicotiana tabacum*) and wild tobacco (*Nicotiana plumbaginifolia*) | | | | |
| *C. annum* | Capsidiol | | $H_2O_2$, MeJA, cellulase | [113,114] |
| *N. tabacum* | Scopoletin and capsidiol | *Botrytis cinerea*, *Phytophthora nicotianae* and *Phytophthora palmivora* | | [109,111] |
| *N. plumbaginifolia* | Capsidiol | *Botrytis cinerea* | Cellulase, arachidonic acid | [112] |
| Vitaceae: grapevine (*Vitis vinifera*), *Vitis riparia* × *Vitis berlandieri* and wild-growing grape (*Vitis amurensis*) | | | | |
| *V. vinifera* | Resveratrol, viniferins, piceids and pterostilbene | *Plasmopara viticola*, *Erysiphe necator* and *Botrytis cinerea* | | [117,118,158] |
| *V. vinifera*, *V. riparia* × *V. berlandieri* and *V. amurensis* | Piceids and resveratrol | | Jasmonic acid, MeJA, MeJA + sucrose, cyclodextrin, cyclodextrin + MeJA cyclodextrins + MeJA+ sucrose, Na-orthovanadate, DIMEB, methyl-β-cyclodextrin (MBCD) | [116,121–126, 128,159] |
| *V. amurensis* | Resveratrol | *Agrobacterium rhizogenes* | | [119] |
| Poaceae: maize (*Zea mays*), oat (*Avena sativa*), rice (*Oryza sativa*) and sorghum (*Sorghum bicolor*) | | | | |
| *Z. mays* | Kauralexins and zealexins | *Rhizopus microsporus*, *Colletotrichum graminicola*, *Fusarium graminearum*, *Cochliobolus heterostrophus* and *Aspergillus flavus* | | [4,5] |

Table 1 (*Continued*)

| Plants | Phytoalexins | Pathogen infection | Elicitors | Refs |
|---|---|---|---|---|
| *A. sativa* | Avenanthramides | *Puccinia coronata* | | [138] |
| | Avenanthramides | | (GlcNAc)$_5$, chitin, vctorin, VicBSA, benzothiadiazole | [133,135–138] |
| *O. sativa* | Momilactone A, momilactone B, phytocassane A – phytocassane E, sakuranetin and oryzalexin E | *Pyricularia oryzae, Magnaporthe grisea* and *Magnaporthe oryzae* | | [146,147,160] |
| | Momilactones and phytocassanes | | *N*-Acetylchitooctaose, cholic acid, (GlcNAc)$_8$, fungal cerebroside, xylanase | [140,142–144, 148,149] |
| *S. bicolor* | Luteolin, apigenin and 3-deoxyanthocyanidins | *Colletotrichum sublineolum* and *Cochliobolus heterostrophus* | | [150–152] |
| | 3-Deoxyanthocyanidins | | MeJA | [150] |

[a]Abbreviations: DIMEB, heptakis(2,6-di-*O*-methyl)-β cyclodextrin; $CuCl_2$, copper chloride; MBCD, methyl-β-cyclodextrin; $H_2O_2$, hydrogen peroxide; (GlcNAc)$_5$, penta-*N*-acetylchitopentaose.

medicarpin. In another study, the application of *Phoma medicaginis* spores to barrel medic plants led to an increase in medicarpin and its precursors [80], suggesting that the relative rate of their synthesis is tightly coupled to the infection process. Profiles of isoflavones were found to be altered in the leaves of narrow-leafed lupine plants after application of a *Colletotrichum lupini* spore suspension [87]. The synthesis of luteone was enhanced in the youngest leaves, whereas wighteone synthesis was induced mainly in older leaves, suggesting that some stress responses are dependent on leaf localization and age.

Cell suspensions of barrel medic accumulated medicarpin in response to yeast extract (YE) or methyl jasmonate (MeJA), accompanied by decreased levels of isoflavone glycosides in MeJA-treated cells [83]. Induction of early (iso)flavonoid pathway gene transcripts was observed in response to YE, but not MeJA. The YE-mediated induction of biosynthetic pathway genes for medicarpin formation, but induction of genes only for downstream conversion of formononetin in response to MeJA, indicated the significance of the MeJA-induced depletion of preformed glucose conjugates of formononetin. These observations implicated β-glucosidases in the mobilization of stored isoflavone glucosides during MeJA-induced medicarpin biosynthesis. A similar study showed accumulation of medicarpin with both elicitors, although coordinated increases in isoflavonoid precursors were observed only for YE- and not MeJA-treated cells [94]. However, MeJA treatment resulted in a correlated decline in isoflavone glucosides and did not induce the secretion of metabolites into the culture medium. These two studies indicated the metabolic flexibility within the isoflavonoid pathway and that the responses underlying accumulation of medicarpin depend on the nature of elicitation, and that MeJA acts as a signal for rapid deployment of pre-existing intermediates into phytoalexin biosynthesis during wound responses [83,94].

The pathogen- or elicitor-based studies on pea focused on studying the pisatin biosynthesis pathway by using transgenic hairy root lines, as stated. The hairy root tissue of transgenic pea downregulated in enzymes considered to be involved in different steps of pisatin biosynthesis produced less pisatin after inoculation with *Nectria haematococca* and showed reduced resistance to the fungus [95]. Transgenic pea (hairy roots) impaired in the expression of pisatin biosynthetic enzymes showed a reduction in or no accumulation of pisatin after elicitation with $CuCl_2$ [95,96]. Some hairy root lines containing RNAi constructs of isoflavone reductase (IFR) and sophorol reductase (SOR) accumulated 7,2′-dihydroxy-4′5′-methylenedioxyisoflavone (DMD) and (–)-sophorol, respectively, and were deficient in (+)-pisatin biosynthesis, supporting the involvement of chiral intermediates with a configuration opposite to that found in (+)-pisatin in the biosynthesis of (+)-pisatin. Hairy roots containing RNAi constructs of (+)6*a*-hydroxymaackiain 3-*O*-methyltransferase (HMM) also were deficient in (+)-pisatin biosynthesis, and did not accumulate (+)-6*a*-hydroxymaackiain, the proposed precursor of (+)-pisatin. Instead, 2,7,4′-trihydroxyisoflavanone (TIF), daidzein, isoformononetin and liquiritigenin accumulated. The accumulation of these four compounds was consistent with blockage of the synthesis of (+)-pisatin at the hydroxyisoflavanone-4′-O-methyltransferase (HI4′OMT)-catalyzed step, resulting in the accumulation of liquiritigenin and TIF and diversion of the pathway to produce daidzein and isoformononetin, compounds not normally made by pea. This study, with support from previous studies, showed the involvement of two similar methyl transferases (hydroxymaackiain-3-*O*-methyltransferase and hydroxyisoflavanone-4′-*O*-methyltransferase) and chiral intermediates in pisatin synthesis [85,95,96].

Germinated peanuts have been shown to produce phytoalexins, such as resveratrol, arachidins and isopentadienyl-3,5,4′-trihydroxystilbene, and up to 45 stilbenoid phytoalexin derivatives after inoculation with the food-grade fungus *Rhizopus oligosporus* [97]. Analysis of phytoalexins produced at different distances from the site of infection of peanut kernels with different *Aspergillus* fungal strains revealed temporal, spatial and strain-specific differences in phytoalexin profiles. Higher concentrations of phytoalexin accumulated with longer incubation, and the composition of phytoalexins varied significantly by layer [92]. Challenge of peanut seeds with an *Aspergillus caelatus* strain produced known stilbenes as well as new stilbenoids (arahypin-1, arahypin-2, arahypin-3, arahypin-4, arahypin-5, arahypin-6 and arahypin-7) and pterocarpenes (aracarpene-1 and aracarpene-2), which have a defensive role against pathogenic organisms [89–91]. Again in peanut, a comparison of fungi and chemicals on the induction of

**Table 2. Examples of pathogens and elicitors that mediate production of phytoalexins in crop plants**[a]

| Plants | Pathogens or elicitors | Biosynthesis pathways, signaling components and other defense responses | Gene, protein, or enzyme[b] | Phytoalexins | Refs |
|---|---|---|---|---|---|
| *Medicago sativa* | *Colletotrichum trifolii* | Flavonoid biosynthesis | *PAL*, *CA4H*, *IFR* and *PAL* activity | Medicarpin and sativan | [82] |
| *Medicago truncatula* | YE, MeJA | Medicarpin biosynthesis | Early (iso)flavonoid gene transcripts, ABC transporters, transcription factors, β-glucosidases and genes encoding enzymes for conversion of formononetin to medicarpin | Medicarpin | [83] |
| *Pisum sativum* | *Nectria haematococca* and *Mycosphaerella pinodes* | Pisatin biosynthesis, Pisatin tolerance | *IFR*, *HMM*, HMM activity, *NhABC1* and *PDA1* | Pisatin | [7,95,161] |
| | $CuCl_2$ | Pisatin biosynthesis | *IFR*, *HMM*, IFR, HMM and SOR | Pisatin | [85,95,96] |
| *Glycine max* | *Fusarium solani* and *Aspergillus niger*, *Aspergillus oryzae* and *Rhizopus oligosporus* | Phenylpropanoid pathway,oxooctadecadienoic acids (KODEs, including 13-*Z,E*-KODE, 13-*E,E*-KODE, 9-*E,Z*-KODE, and 9-*E,E*-KODE), superoxide, ROS, $H_2O_2$ | PAL activity | Glyceollins | [101,102] |
| | β-glucan, *Phytophthora sojae* cell wall glucan elicitor, YE, *Gm*Pep914: smallest defense peptide signal | MAPK signaling, glyceollin biosynthesis and production, HR cell death | *Gm*MPK3, *Gm*MPK6, *Gm*MKK1, *CHR*, *IFS*, *PR-2*, *G4DT*, *IFS1*, *IFS2*, *HID*, *P6aH*, *CYP93A1*, *Chib1-1* and *Gmachs1* | Glyceollins | [103–106] |
| *Capsicum annum* | Cellulase, $H_2O_2$ and MeJA | ROS, $H_2O_2$, $Ca^{2+}$, superoxide anion radical, lipid peroxidation, G proteins | Ascorbate oxidase and phospholipase $A_2$ activities | Capsidiol | [113,114] |
| *Nicotiana tabacum* | *Botrytis cinerea* and *Phytophthora nicotianae* | Superoxide release, HR cell death | PR proteins | Scopoletin and capsidiol | [109,111] |
| *Nicotiana plumbaginifolia* | *Botrytis cinerea* / cellulase and arachidonic acid | Capsidiol synthesis, ABA | *EAS*, *EAH* and *ABAH* | Capsidiol | [112] |
| *Vitis vinifera* and *Vitis riparia* × *Vitis berlandieri* | MeJA + sucrose, jasmonic acid, MeJA, CD, DIMEB, MBCD and Na-orthovanadate | Stilbene pathway, systemic acquired resistance, cell wall metabolism, phenylpropanoid metabolism | *PAL1*, *CHS3*, *STS*, UFGT, *PIN*, *CHIT4c*, *GLU*, *C4H*, *4CL*, peroxidase activity and isoenzymes, PR-protein type 10, PR-proteins, chitinases, proteins encoding stilbene synthase and glutathione-S-transferases | Resveratrol and piceids | [121,123,124, 127,128] |
| *Vitis amurensis* | *Agrobacterium* rhizogenes | Tyrosine phosphorylation, cell death | *rolB* | Resveratrol | [119] |
| *Zea mays* | *Rhizopus microsporus*, *Colletotrichum graminicola*, *Fusarium graminearum*, *Cochliobolus heterostrophus* and *Aspergillus flavus* | Kauralexin synthesis and jasmonic acid–ethylene synergy | *An2*[c], *Tps6*, *Tps11*, chitinases and PR proteins | Kauralexins and zealexins | [4,5] |
| *Avena sativa* | *Puccinia coronata* | Avenanthramide biosynthesis | *AsHHT1*, *AsCCoAOMT* | Avenanthramides | [138] |
| | Victorin and VicBSA | Avenanthramide biosynthesis, $Ca^{2+}$, nitric oxide, protein kinases, extracellular alkalization, NAD(P)H oxidation, ROS and programmed cell death | *AsHHT1*, *AsCCoAOMT*, *Vb/Pc-2*, GDC and HHT activity | Avenanthramides | [133,136–138] |
| *Oryza sativa* | *Magnaporthe oryzae* | Phytocassanes, momilactones and oryzalexin synthesis, and HR-associated phytoalexin biosynthesis | *OsCPS2*, *OsKSL7 OsCPS4*, *OsKSL4*, *OsKSL10* and *OsKLS8* | Momilactone A and momilactone B, phytocassane A–phytocassane E and sakuranein | [147] |

**Table 2** (*Continued*)

| Plants | Pathogens or elicitors | Biosynthesis pathways, signaling components and other defense responses | Gene, protein, or enzyme[b] | Phytoalexins | Refs |
|---|---|---|---|---|---|
| | Chitin oligosaccharides, xylanase, cholic acid and fungal cerebroside | Momilactone and phytocassane biosynthesis, MEP pathway, HR cell death, lignin, MAPK cascades, glycolysis, chitin signaling, ROS, MAMPs, $Ca^{2+}$, mitochondrial dysfunction, PR protein synthesis, ion leakage | *OsTGAP1*, *OsCPS2*, *OsCPS4*, *OsKSL4*, *CYP99A2*, *CYP99A3*, *OsMAS*[c], *OsKSL7*, *OsDXS3*, *AK103462*, *Os*MPK3, *Os*MPK6, *Os*MKK4, *Os*MKK4$^{DD}$, *Os*CERK1, *PAL*, *β-Glu*, *HIP*, *OsCIPK14*, *OsCIPK15*, OsCBL4, *PBZ1*, *CHT-1*, *OsDXS*[c], *OsDXR*[c], *OsCMS*[c], *OsMCS*[c], *OsHDS*[c], *OsHDR*[c] and *OsCMK*[c] | Momilactones and phytocassanes | [140–145, 148,149] |
| *Sorghum bicolor* | *Colletotrichum sublineolum* and *Cochliobolus heterostrophus* | Flavone biosynthesis from flavanones? $H_2O_2$ accumulation, papilla formation, callose deposition, HRGP-cross linking, cell death | *SbCHS 1*, *SbCHS 7* and *SbDFR3* | Luteolin, apigenin and 3-deoxyanthocyanidins | [150–152] |
| | MeJA, MeJA + SA | 3-Deoxyanthocyanidin biosynthesis pathway | *SbDFR3* | 3-Deoxyanthocyanidin | [150] |

[a]Abbreviations: ABA, abscisic acid; $Ca^{2+}$, calcium; CD, cyclodextrin; DIMEB, heptakis(2,6-di-*O*-methyl)-β-cyclodextrin; $CuCl_2$, copper chloride; $H_2O_2$, hydrogen peroxide; HR, hypersensitive reaction; HRGP, hydroxyproline-rich glycoprotein; MBCD, methyl-β-cyclodextrin; MEP, methylerythritol phosphate; PR, pathogenesis-related.

[b]Gene, protein or enzyme abbreviations: *ABAH*, ABA 8′-hydroxylase; *An2*, an *ent*-copalyl diphosphate synthase; *AK103462*, a putative dehydrogenase; *AsCCoAOMT, S*-adenosyl-L-methionine:*trans*-caffeoyl-CoA 3-*O*-methyltransferase; *AsHHT1*, hydroxycinnamoyl-CoA:hydroxyanthranilate *N*-hydroxycinnamoyltransferase; *β-Glu / GLU*, β-glucanase; *4CL*, 4-coumarate:coenzyme A ligase; *Chib1-1*, chitin elicitor binding protein; *C4H*, cinnamate-4-hydroxylase; *CA4H*, cinnamic acid 4-hydroxylase; *Chib1-1,* a chitinase; *CHR*, chalcone reductase; *CHS*, chalcone synthase; *CYP93A1,* 3,9-dihydroxypterocarpan 6*a*-monooxygenase; *CYP99A2* and *CYP99A3*, cytochrome P450 monooxygenase (P450) genes; *DFR*, dihydroflavonol 4-reductase; *EAH,* 5-*epi*-aristolochene hydroxylase; *EAS,* 5-*epi*-aristolochene synthase; *G4DT*, (6*a*S, 11*a*S)-3,9,6*a*-trihydroxypterocarpan [(2)-glycinol] 4-dimethylallyltransferase; GDC, glycine decarboxylase complex; *Gmachs1,* soybean chalcone synthase; GmMPK3 and GmMPK6, soybean mitogen-activated protein kinases; HHT, hydroxycinnamoyl CoA: hydroxyanthranilate N-hydroxycinnamoyl transferase; *HID,* 2-hydroxyisoflavanone dehydratase; *HIP*, harpin-induced 1 domain-containing protein; *HMM*, (+)6*a*-hydroxymaackiain 3-*O*-methyltransferase; HRGP, hydroxyproline-rich glycoproteins; *IFR*, isoflavone reductase; *IFS*, isoflavone synthase; *NhABC1*, ATP-binding cassette (ABC) transporter; OsCBL4, similar to calcineurin B-like protein 4; OsCERK1, LysM receptor-like kinase; *OsCIPK14* and *OsCIPK15,* similar to protein kinase PK4; *OsCMK* and *OsCMS*, 4-(cyti cytidine 5′-diphospho)-2-C-methyl-D-erythritol- kinase and synthase; *OsCPS2/OsCyc2*, terpenoid synthase domain containing protein; *OsCPS4/OsCyc1*, similar to isoform 3 of Syn-copalyl diphosphate synthase; *OsDXR* and *OsDXS*, 1-deoxy-D-xylulose 5-phosphate- reductoisomerase and synthase; *OsDXS3*, deoxyxylulose phosphate synthase; *OsHDR* and *OsHDS*, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase and synthase; *OsKS4/OsKSL4*, Syn-pimara-7,15-diene synthase; *OsKSL7/OsDTC1*, similar to *Ent*-kaurene synthase 1A; *OsKSL8*, similar to isoform 2 of stemar-13-ene synthase; *OsKSL10*, similar to *Ent*-kaurene synthase 1A; *OsMAS*, similar to stem secoisolariciresinol dehydrogenase; *OsMCS*, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; *Os*MKK4, rice MAPK kinase; *Os*MKK4$^{DD}$, active form of OsMKK4; OsMPK3 and *Os*MPK6, MAP kinases; *OsTGAP1*, a chitin oligosaacharide elicitor-inducible basic leucine zipper transcription factor; *P6aH*, pterocarpan 6*a*-hydroxylase; *PAL*, phenylalanine ammonia lyase; *PBZ1/PR10a*, probenazole-inducible gene1; *PDA1*, encodes pisatin demethylase; *PIN*, proteinase inhibitor; *PR-2*, elicitor-releasing endoglucanase; *rolB*, a protein possessing a tyrosine phosphatase activity; *SbCHS1- SbCHS7*, expected to encode functional chalcone synthase enzymes; *SbDFR3*, a pathogen-inducible DFR gene; SOR, sophorol reductase; *STS*, stilbene synthase; *TPS6* and *TPS11,* terpene synthases; *UFGT*, UDP glucose-flavonoid 3-*O*-glucosyl transferase; *Vb/ Pc-2, Vb* for sensitivity of oats to victorin C and *Pc* for resistance to certain races of the crown rust fungus.

[c]Putative,

*trans*-resveratrol and *trans*-piceatannol found fungi to be the most effective [98]. *Ganoderma lucidum* mycelium-treated peanut callus was proposed to be a good source of bioactive components.

A new peanut hairy root line that produces resveratrol and arachidin-1 and arachidin-3 upon sodium acetate-mediated elicitation was generated [99]. Sodium acetate elicitation resulted in 60-fold induction and secretion of *trans*-resveratrol and, to a lesser extent, of other stilbenes, including *trans*-pterostilbene, into the medium of peanut hairy root cultures [100]. These studies demonstrated the benefits of hairy root culture systems in studies of the biosynthesis of stilbenoids, and their use as an effective bioprocessing system for valued nutraceuticals, such as resveratrol and its derivatives [99,100].

In a study of soybean hairy roots, the glyceollin content after inoculation with *Fusarium solani* was observed to be higher for a partially resistant cultivar than for a susceptible cultivar [101], suggesting that the ability to produce sufficient amounts of glyceollin rapidly in response to *Fusarium solani* infection is important in providing partial resistance to this fungus [101]. The inoculation of germinating soybean seeds elicited glyceollins, although the amount of glyceollin formed was dependent on the type of microbe used [102]. Of the microbes *Aspergillus niger*, *Aspergillus oryzae* and *Rhizopus oligosporus*, *Rhizopus oligosporus* yielded a maximum amount of glyceollins. Large-scale challenge of germinated soybean seeds with the food-grade fungus *Rhizopus microsporus* and application of malting technology caused the accumulation of compounds that have been tentatively assigned as glyceollins and glyceollidins [79]. Following this approach, the more than tenfold increase in bioactive compounds seems promising for the production of more novel higher-potency nutraceuticals.

GmMPK3 and GmMPK6 were activated by β-glucan elicitors of *Phytophthora sojae* under conditions that favor induction of phytoalexin production in cell cultures and other tissues of soybean [103]. The *Phytophthora sojae* cell-wall glucan elicitor triggered a cell death response in roots that was suppressed by silencing of genes involved in the biosynthesis of 5-deoxyisoflavonoids [104]. Moreover, silencing of the elicitor-releasing endoglucanase *PR-2* led to loss of hypersensitive response (HR) cell death and race-specific resistance to *Phytophthora sojae*, and of isoflavone and cell death responses to the cell-wall glucan elicitor. A cDNA encoding G4DT, a pterocarpan 4-dimethylallyltransferase yielding the direct precursor of glyceollin I, was identified and characterized [105]. Treatment of cultured soybean cells with YE led to coordinated transcriptional upregulation of enzymes of the glyceollin pathway and glyceollin I accumulation. The soybean peptide elicitor *Gm*Pep914, induced the expression of *CYP93A1*, *Chib1-1* and *Gmachs1*, which are all involved in phytoalexin synthesis [106]. These studies suggested a role of an MAPK cascade in mediating β-glucan signal transduction [103] and that the *in situ* release of active fragments from a general resistance elicitor (MAMP) is important for HR-related cell death in soybean roots [104]. Moreover, the first identification of G4DT provides new insights into reactions involved in the disease resistance mechanism of soybean [105], and the novel peptide *Gm*Pep914 has importance in activating defense-related genes and phytoalexin production [106].

### Phytoalexins in Solanaceae

The crop plants tobacco (*Nicotiana tabacum*) and pepper fruit (*Capsicum annuum*) belong to the Solanaceae. Capsidiol is the major phytoalexin produced by inoculation of pepper fruit and tobacco with pathogenic fungi [107,108] (PMN http://www.plantcyc.org/tools/tools_overview.faces; accessed November 20, 2011). Capsidiol is a bicyclic sesquiterpene that prevents the germination and growth of several fungal species, and has been isolated from many Solanaceae species. Scopoletin, a major phytoalexin of tobacco plants, is a hydroxycoumarin [107,109] (PMN http://www.plantcyc.org/tools/tools_overview.faces; accessed November 20, 2011). Its accumulation in tobacco was shown to correlate strongly with tobacco mosaic virus-induced localized acquired resistance [110].

Challenge of suspension cell cultures of *Nicotiana tabacum* with zoospores of incompatible isolates of *Phytophthora nicotianae* elicited a biphasic burst of superoxide release [111]. Given that the accumulation of the terpenoid capsidiol and HR-related cell death occur in both incompatible and non-host interactions, this suggests that these responses are regulated by pathways that diverge downstream of superoxide release. These assays showed that superoxide release is necessary for phytoalexin accumulation in tobacco during the expression of cultivar-race and non-host resistance towards *Phytophthora* spp. Resistance of tobacco to *Botrytis cinerea* was cultivar-specific, correlated with accumulation of scopoletin and PR proteins, and occurred whether the cultivar was challenged with fungal spores or mycelium. These findings are important for understanding the strategy used by *Botrytis cinerea* to establish disease on tobacco plants [109]. Given that mycelium, but not spores, has the capacity to metabolize scopoletin and suppress the accumulation of PR proteins, the tobacco was more resistant to infection by *Botrytis cinerea* spores than by mycelium. Wild tobacco (*Nicotiana plumbaginifolia)* mutants deficient in abscisic acid (ABA) synthesis exhibited a twofold higher level of capsidiol than did wild-type plants when elicited with either cellulase or arachidonic acid or when infected by *Botrytis cinerea* [112]. Expression of the capsidiol biosynthetic genes *5-epi-aristolochene synthase* (*EAS*) and *5-epi-aristolochene hydroxylase* (*EAH*) followed the same trend. ABA has been proposed to play an essential role in fine-tuning the amplification of capsidiol synthesis in challenged wild tobacco plants.

In pepper fruit, elicitation by arachidonic acid caused an initial burst of ROS, and maximum $H_2O_2$ production was reached within 6 h, whereas exogenous $H_2O_2$ treatment induced capsidiol accumulation [113]. Ascorbate peroxidase activity decreased after arachidonic acid treatment, suggesting its importance in regulating $H_2O_2$ accumulation in pepper. The treatment of growth-phase cell suspension cultures of pepper fruit with cellulase or mastoparan, a G protein activator, increased capsidiol production, which is likely to be mediated by phospholipase A2 ($PLA_2$) and G protein activities [114]. These studies show

that capsidiol production is induced by arachidonic acid dependent on an oxidative burst and by cellulase through $PLA_2$ activation [113,114].

### Phytoalexins in Vitaceae

Phytoalexins of grapevine (*Vitis vinifera*) belong mainly to the stilbene family (Tables 1, 2 and Table S2 in the supplementary material online), the skeleton of which is based on *trans*-resveratrol [115,116]. Resveratrol represents a parent compound of a family of molecules, such as resveratrol glucosides (piceid), methylated derivatives (pterostilbene) and oligomers (α-viniferin and ε-viniferin), with some expressing higher fungicide toxicity compared with resveratrol [107,115] (PMN http://www.plantcyc.org/tools/tools_overview.faces; accessed November 20, 2011).

Resistant grapevine cultivars have been shown to react rapidly to *Plasmopara viticola* infections by producing high concentrations of stilbenes at the site of infection, confirming their crucial role and effectiveness in grapevine resistance to downy mildew [117]. Analysis of stilbenes in *Erysiphe necator*-infected leaf discs from grapevine indicated that stilbene synthesis is confined to infected cells. The rapid production of resveratrol, as well as its transformation into viniferins, appears to enhance powdery mildew resistance in grapevine cultivars [118]. The highest concentrations of viniferins on resistant cultivars coincided with the observed inhibition of pathogen growth. Transformation of *Vitis amurensis* V2 with the *Agrobacterium rhizogenes rolB* gene (encoding a protein possessing tyrosine phosphatase activity) increased resveratrol production in transformed calli by more than 100-fold [119]. The biosynthesis of resveratrol was observed to be tightly correlated with the abundance of *rolB* mRNA transcripts. Given that resveratrol has been shown to be a potent anti-inflammatory, anticancer and chemoprotective agent, enhancing resveratrol production in this way might provide dietary benefits [10,119,120].

Given that phytoalexins from Vitaceae are important in disease resistance and possess therapeutic properties, especially resveratrol, research during the past few years has focused on the use of different elicitors to enhance the production of resveratrol. The elicitors MeJA, JA, cyclodextrins (in standard or in modified form) and Na-orthovanadate, when used individually or in combination on plant cell cultures, have activated plant defenses and induced or enhanced the production of stilbene phytoalexins. Additionally, the use of transcriptomic and proteomic approaches in elicitor-based studies has identified defense genes and proteins involved in the production of these compounds. For example, in grapevine, MeJA in combination with sucrose stimulated defense gene expression and accumulation of *trans*-resveratrol and piceids (resveratrol glucosides) [121]. A recent study also showed that the highest productivity of *trans*-resveratrol is dependent on levels of sucrose in the elicitation medium and the combined action of MeJA and cyclodextrins [122]. Furthermore, both MeJA and cyclodextrin transiently induced the expression of stilbene biosynthetic genes, but only cyclodextrin induced the production of resveratrol. However, when cells were simultaneously elicited with cyclodextrin and MeJA, a synergistic effect on the accumulation of resveratrol was observed [123]. MeJA and Na-orthovanadate have also been shown to enhance accumulation of resveratrol; MeJA was particularly effective [124]. In grapevine cell cultures, treatment with heptakis(2,6-di-*O*-methyl)-β-cyclodextrin and methyl-β-cyclodextrin caused extracellular accumulation of resveratrol [116,125]. This elicitation also led to induced levels of defense and stress-related proteins, such as chitinases, β-1,3-glucanase and secretory peroxidases, in the extracellular proteome of grapevine [125,126]. Moreover, transcriptomic analysis of *Vitis riparia* × *Vitis berlandieri* grapevine cells in response to methyl-β-cyclodextrin led to identification of a specific set of induced genes belonging to phenylpropanoid metabolism, including stilbenes and hydroxycinnamates, and defense proteins, such as PR proteins and chitinases [127]. In addition, a study investigating the role of various partners involved in MeJA-stimulated defense responses showed that cytosolic calcium due to calcium influx through the plasma membrane appears to be essential for MeJA-induced stilbene accumulation [128]. It was also observed that MeJA-elicited responses are mediated by ROS in a process in which superoxide anions play a greater role than does $H_2O_2$.

Knowledge gained from such cell culture treatments could prove helpful in developing efficient disease control strategies for protecting grapevine berries in vineyards, and in other biotechnological applications [121,123–125]. For instance, because it is a naturally occurring phytoalexin and antioxidant, resveratrol has attracted much research interest, and enhancing its levels through cell culture treatments is a significant achievement. The engineering of resveratrol has been accomplished with some success in plants, microbes and mammals [129,130]. Expression of the stilbene synthase-encoding gene (*STS*; necessary for the production of resveratrol) in plants such as tobacco, tomato (*Solanum lycopersicum*), rice (*Oryza sativa*), wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*), oilseed rape (*Brassica napus*) and hop (*Humulus lupulus*) has thus provided increased resistance against pathogens [129,131].

### Phytoalexins in Poaceae

Maize (*Zea mays*), oat (*Avena sativa*), rice and sorghum are Poaceae crop plants. The phytoalexins that accumulate in these plants in response to pathogen attack are kauralexins and zealexins (maize), avenanthramides (oat), diterpenoids and the flavonoid sakuranetin (rice) and 3-deoxyanthocyanidins (sorghum), as detailed below and in Tables 1, 2 and Table S2 in the supplementary material online.

It has recently been shown that maize stem attack by fungi (*Rhizopus microsporus* and *Colletotrichum graminicola*) induces the accumulation of six *ent*-kaurane-related diterpenoids, collectively termed kauralexins [4]. Notably, physiologically relevant concentrations of kauralexins inhibited the growth of these pathogens. Accumulation of the fungal-induced kaurene synthase 2 (*An2*) transcript preceded highly localized kauralexin production, and a combination of JA and ethylene application demonstrated their synergistic role in kauralexin regulation. Other maize phytoalexins, termed zealexins, have also been

recently discovered, following attack by *Fusarium graminearum* [5]. Microarray analysis of *Fusarium graminearum*-infected maize stem tissue revealed that the terpene synthase-encoding genes *Tps6* and *Tps11* were among the most highly upregulated genes, as was *An2* [4,5]. Characterization of these recently discovered kauralexins and zealexins should help in elucidating the roles of nonvolatile terpenoid phytoalexins in maize disease resistance [4,5].

A group of phenolic antioxidants termed avenanthramides [132–135], have been well characterized as phytoalexins in oat. Avenanthramide accumulation is triggered by elicitors or activators, such as chitin, penta-*N*-acetylchitopentaose, victorin and benzothiadiazole [133–137]. They also accumulate in oat leaves in incompatible interactions with crown rust fungus (*Puccinia coronata*) and are considered important in defense against pathogens. Inoculation of oat leaves with *Puccinia coronata* increased expression of hydroxyanthranilate hydroxycinnamoyltransferase (*AsHHT1*) and caffeoyl-CoA 3-*O*-methyltransferase (*AsCCoAOMT*) in both incompatible and compatible interactions [138]. However, significant accumulation of avenanthramides was observed only in incompatible interactions. *AsHHT1* and *AsCCoAOMT* are thought to be involved in the biosynthesis of avenanthramide phytoalexins. Transformation of oat plants with these genes might allow closer evaluation of avenanthramides and provide new strategies for disease control.

Transcripts of *AsHHT1* and *AsCCoAOMT* also showed simultaneous increases with phytoalexin accumulation after treatment with victorin, a toxin produced by *Cochliobolus victoriae* [138]. As with native victorin, a bovine serum albumin–fluorescein derivative of victorin (VicBSA) elicited apoptosis-like cell death, production of avenanthramide A, extracellular alkalization, generation of nitric oxide and production of ROS in oat [136]. These studies support the idea that victorin functions as a specific elicitor of resistance expression in *Vb*/*Pc-2* oats, and a model has been proposed in which victorin kills the host cell by activating an HR-like response [136,138]. The model shows that victorin may interact with an extracellular mediator(s) in *Vb*/*Pc-2* oats and stimulate ion fluxes across the plasma membrane, followed by the activation of defense responses and rapid cell death.

Mesophyll cells of oat treated with penta-*N*-acetylchitopentaose could be classified into three cell phases, which occurred serially over time [137]. These studies indicated that avenanthramide biosynthesis and HR occur in identical cells; therefore, avenanthramide production may be one of the sequential events programmed in HR leading to cell death. Upregulation of avenanthramide biosynthesis in leaf tissue could also be achieved by treatment of roots with the SA analog benzothiadiazole [133]. However, avenanthramide increases in roots exhibited slower dynamics and lower levels than in leaves, suggesting that avenanthramides are transported from the leaves.

Rice produces many diterpenoid phytoalexins in response to infection by pathogens such as rice blast fungus (*Magnaporthe oryzae*) or through the perception of elicitors [139,140]. Elicitors commonly used to trigger phytoalexin synthesis in rice are chitin oligosaccharides, fungal cerebroside and cholic acid (a primary bile acid in animals) [141–143]. As well as the diterpenoid phytoalexins, the flavonoid sakuranetin is a major phytoalexin in rice [144–146]. The diterpenoid phytoalexins have been classified into four structurally distinct types of polycyclic diterpene based on the structures of their diterpene hydrocarbon precursors: phytocassanes A–E, oryzalexins A–F, momilactones A and B, and oryzalexin S [139]. Their importance in HR-mediated disease resistance has been demonstrated in a study that showed that after inoculation with *Magnaporthe oryzae*, phytoalexins accumulated more quickly and to a higher extent in resistant rice than in susceptible rice, inducing severe restriction of fungal growth [147]. These findings could be of importance in understanding the dynamic host–parasite battle for survival on phytoalexins through their biosynthesis.

In recent years, elicitor treatments have been used as an approach in many studies aimed at elucidating the biosynthesis pathways of rice phytoalexins. This has generated insightful information with respect to genes involved in biosynthetic pathways, biosynthetic gene clusters, and genes regulating the production of diterpenoid phytoalexins and related defense responses. Knock out of the basic leucine zipper transcription factor *OsTGAP1*, a key regulator of the coordinated transcription of genes involved in inductive diterpenoid phytoalexin production in rice, led to a loss of momilactone production upon chitin oligosaccharide treatment [141]. Furthermore, simultaneous knockdown of *CYP99A2* and *CYP99A3* specifically suppressed the elicitor-inducible production of momilactones, suggesting their involvement in momilactone biosynthesis [144]. Chitin activated two rice MAPKs (OsMPK3 and OsMPK6) and one MAPK kinase (OsMKK4). The OsMKK4–OsMPK6 cascade plays a crucial role in reprogramming plant metabolism during MAMP-triggered defense responses [148]. OsMPK6 has been shown to be essential for chitin elicitor-induced biosynthesis of diterpenoid phytoalexins. OsMKK4$^{DD}$-induced cell death and expression of diterpenoid phytoalexin pathway genes were dependent on OsMPK6. The chitin elicitor receptor kinase 1 (OsCERK1) was also reported to be necessary for chitin signaling in rice because *OsCERK1* knockdown cell lines lost the ability to generate ROS, to induce expression of defense genes and to synthesize phytoalexins in response to a chitin oligosaccharide elicitor [142]. Other rice genes, namely CBL-interacting protein kinases 14 and 15 (*OsCIPK14* and *OsCIPK15*), were also rapidly induced by MAMPs, including chitooligosaccharides and xylanase [149]. Functional characterization of these CIPKs suggested their involvement in various xylanase-induced layers of defense responses, including HR-related cell death, phytoalexin biosynthesis and PR gene expression. Treatment of rice leaves with cholic acid induced the accumulation of phytoalexins, HR-related cell death, PR protein synthesis and increased resistance to infection by virulent pathogens [143]. Cholic acid induced these defense responses more rapidly than did the sphingolipid elicitor fungal cerebroside. Furthermore, cholic acid preferentially induced the formation of phytocassanes in suspension-cultured rice cells, whereas fungal cerebroside and a fungal chitin oligosaccharide elicitor induced both phytocassanes and

momilactones, suggesting that cholic acid is recognized specifically by rice [140,143]. *N*-Acetylchitooctaose treatment on suspension-cultured rice cells revealed two types of early-induced expression (EIE-1 and EIE-2) nodes and a late-induced expression (LIE) node, which included phytoalexin biosynthesis [145]. The LIE node contains genes that might be responsible for the methylerythritol phosphate (MEP) pathway, a plastidic biosynthetic pathway for isopentenyl diphosphate, an early precursor of phytoalexins. Activation of the MEP pathway is required to supply sufficient terpenoid precursors for the production of phytoalexins in infected rice plants.

Sorghum synthesizes a unique group of phytoalexins called 3-deoxyanthocyanidins (orange–red coloration) in response to fungal infection [150]. 3-Deoxyanthocyanidins are a rare class of plant pigments with chemical properties that are very different from those of their anthocyanin analogs [13]. 3-Deoxyanthocyanidins can also be induced in sorghum roots by MeJA, but its stimulation effect is antagonized by SA treatment [150]. Following inoculation with *Colletotrichum sublineolum*, luteolin showed more rapid and elevated accumulation in seedlings of a resistant sorghum cultivar compared with a susceptible cultivar [151], and apigenin was the major flavone detected in infected susceptible seedlings. Luteolin inhibited *Colletotrichum sublineolum* spore germination more strongly than did apigenin. Inducible defense responses in resistant genotypes of sorghum to challenge by *Colletotrichum sublineolum* included accumulation of $H_2O_2$, hydroxyproline-rich glycoproteins and 3-deoxyanthocyanidins [152]. A significant correlation between $H_2O_2$, papilla formation and cell wall cross-linking can be exploited for host resistance in sorghum. Infection of sorghum with *Cochliobolus heterostrophus* has also been shown to induce 3-deoxyanthocyanidin accumulation. Further work is needed to dissect the remaining enzymatic steps in the pathogen-inducible 3-deoxyanthocyanidin biosynthesis pathway and the molecular regulatory network [150].

## Conclusions and future directions

Recent studies on the phytoalexins in *Arabidopsis* and some crop plants from the Brassicaceae, Fabaceae, Solanaceae, Vitaceae and Poaceae have generated information on basic aspects of plant defenses, including ideas on how to improve disease control. Most of the steps involved in camalexin biosynthesis in *Arabidopsis* have been identified, but some intermediate steps and its further conversion remain to be elucidated. More importantly, efforts put into studying the regulation of camalexin biosynthesis have led to the recent identification of possible signaling pathways. However, the roles of each of these pathways under specific inducing conditions, as well as their interactions, are far from well understood and require further investigation. Furthermore, the ways in which camalexin acts upon pathogens to contribute to plant defense and the mechanisms that some pathogens have developed to detoxify camalexin are also still poorly understood.

Phytoalexin research has focused not only on dicot species (e.g. *Arabidopsis*, peanut and grapevine) but also on monocots (e.g. rice, maize and sorghum), which has increased our understanding of plant resistance mechanisms. The most novel findings are the identification of kauralexins and zealexins in maize and the biosynthesis and regulation mechanisms for rice phytoalexins. Studies to determine the mechanisms regulating phytoalexins in these species as well as other crop plants should have great potential in developing strategies to manipulate and improve disease resistance in such plants.

Although phytoalexins are considered important for plant resistance against pathogens, the phytoalexins of most species and cultivars have yet to be characterized. Novel approaches, such as genome-wide analyses, should open the door for studies of the regulatory networks controlling the metabolism of phytoalexins and provide for a better understanding of the role of phytoalexins in defense against pathogens. Better knowledge of the mode of action of phytoalexins and the mechanisms used by pathogens to bypass this line of defense should reveal new possibilities for the directed control of phytoalexin production in specific tissues and at specific developmental stages.

### Acknowledgements

We gratefully acknowledge financial support from the Norwegian Research Council, grants 185173 and 184146. We thank Tore Brembu and Per Winge for critically reading the manuscript and Paddy Mahony (Cambridge Academy of English, Cambridge) for revising the English language. We also thank two anonymous reviewers for their comments and helpful suggestions. We acknowledge the copyright permissions given by Shigehiko Kanaya (NAIST Comparative Genomics Laboratory) to use images from the KNApSAcK database (http://kanaya.naist.jp/KNApSAcK/) [3], and publishers of journals *PNAS* (National Academy of Sciences, USA) and *Plant Physiology* (American Society of Plant Biologists, USA) to reproduce figures from [4] and [5], respectively, for Figure 1. We apologize to authors whose work could not be cited because of space constraints.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tplants.2011.11.002.

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