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Regulation of phytoalexin biosynthesis for agriculture and human health

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Abstract Phytoalexins are diverse secondary metabolites of plants that are biosynthesized transiently and in relatively low amounts in response to pathogens and certain abiotic stresses. They commonly have potent antimicrobial and medicinal activities. As such, scientists have attempted to increase their accessibility by inventing diverse in vitro and in vivo approaches. Among these approaches, bioengineering plant transcription factors that directly regulate phytoalexin biosynthesis genes may be the most promising. Recent research has identified conserved transcription factors that directly regulate distinct phytoalexin biosynthesis pathways in different plant species. The intriguing results provide new insight into how conserved defense signaling pathways in plants result in lineage-specific biochemical defenses. These recent findings also suggest that a common transcription factor network could be engineered to enhance the biosynthesis of different phytoalexins in plants. However, the picture is far from complete since one or more transcription factors required to fully activate phytoalexin biosynthesis remain unidentified, and the exact mechanism of how the conserved factors regulate diverse phytoalexin pathways remains to be clarified. Here we review the agricultural and medicinal importance of phytoalexins, recent approaches to increase their accessibility, and the mechanisms that plants employ to activate and limit their biosynthesis. This review contributes to providing a systems level understanding of the regulation of phytoalexin biosynthesis so that effective bioengineering strategies can be developed to enhance phytoalexin biosynthesis for medicine and agriculture.

Keywords Bioengineering · Transcription factor · Defense · Secondary metabolite · Signaling

Abbreviations

ABA	Abscisic acid
BLAST	Basic local alignment search tool
BLASTPs	BLAST for proteins
CHIP	Chromatin immunoprecipitation
CHIP-Seq	Chromatin immunoprecipitation next generation sequencing
COR	Cornatine
DNA	Deoxyribonucleic acid
ED	Effective dose
H22 cells	Hippocampal neuronal cell line
HR	Hypersensitive response
JA	Jasmonic acid
PCR	Polymerase chain reaction
pH	Potential hydrogen
PP2C	Protein phosphatase 2C
qRT-PCR	Quantitative real-time reverse transcription PCR

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RNA	Ribonucleic acid
RNAi	RNA interference
RNA-Seq	RNA next generation sequencing
ROS	Reactive oxygen species
RT-PCR	Reverse transcription PCR
TF	Transcription factor
UV	Ultra violet irradiation
WGE	Wall glucan elicitor
WT	Wild type

Introduction

The concept of a *phytoalexin* was first introduced by Karl O. Müller who described that potato inoculated with an incompatible race of *Phytophthora infestans* provoked the synthesis of a putative defense metabolite that provided resistance to a compatible race of the pathogen (Müller et al. 1939). Later in 1940 along with Hermann Borger, Müller defined phytoalexins as metabolites specific to plants that are produced upon contact with microbes and that show antimicrobial properties (Müller 1940). Due to their implicit role in plant defense, various aspects of phytoalexins were intensively researched over the following decades, including their chemical structures, biosynthetic genes and pathways, anti-pathogenic activities, and elicitors (Jeandet et al. 2014; Großkinsky et al. 2012; Ahuja et al. 2012; Jeandet et al. 2013).

Over time, phytoalexins that were biosynthesized in different plant species were discovered to be highly diverse in chemical structure. In fact, their chemical diversity may have caused a decrease in research intensity on phytoalexins over the past decades in favor of defense responses that are less diversified among plant species. Eventually evidence mounted that several phytoalexins had major roles in providing crop plants resistance to important microbial pathogens (Fig. 1) (Ahuja et al. 2012; Graham et al. 2007; Großkinsky et al. 2012). Yet, evidence also mounted that no single phytoalexin was effective against all microbial pathogens, despite their broad-spectrum antimicrobial activities. For example, the pen3 pdr12 double mutant of Arabidopsis that is defective in the cellular secretion of camalexin and the *pad3* mutant of that is defective in the final step of camalexin synthesis exhibit enhanced susceptibility to *Botrytis cinerea* and a number of fungal and oomycete pathogens, respectively (He et al. 2019). However, camalexin is not required for the WRKY33-dependent resistance to the hemibiotrophic fungus *Colletotrichum higginsianum* (Schmidt et al. 2020). WRKY33 is a well-characterized transcription factor (TF) that activates camalexin biosynthetic genes (described below), and may also activate the synthesis of indole carbonyl nitriles (Schmidt et al. 2020), a recently discovered group of cyanogenic phytoalexins from Arabidopsis (Rajniak et al. 2015). The fact that phytoalexins continue to be discovered even in model plants that are intensively studied highlights how our knowledge of phytoalexins is still extremely limited.

Now 80 years after their discovery, evidence has mounted that not only do phytoalexins have important roles in plant protection against microbes, many of them have potent medicinal activities such as anticancer and neuroprotective properties, that render them highly attractive for pharmaceutical development (Pham et al. 2019; Sen 2017; Cheng et al. 2020). Plant biologists remain challenged with the difficult task of seeking-out these 'hidden' metabolites, describing their diversity, medicinal and plant protective activities, and understanding how to improve their accessibility for human health and agriculture.

Importance of phytoalexins in agriculture

Müller and Borger proposed phytoalexin theory in 1939 (Mueller and Börger 1939; Müller et al. 1939). They suggested that the rapidity and magnitude of phytoalexin accumulation, rather than the magnitude alone, were critical features required for providing potato tubers resistance against *Phytophthora infestans*. Prior inoculation of tubers with an incompatible race of *P. infestans* triggered *rapid* and high level resistance against a compatible race. Presently, there is a large body of experimental evidence supporting that phytoalexins play critical roles in plant defense against microbial pathogens when they are biosynthesized *rapidly* at concentrations that can provide toxicity [for reviews see (Ahuja et al. 2012; Großkinsky et al. 2012; Kuc 1995)]. For example, to mediate incompatibility with *Magnaporthe oryzae*, gene transcripts and metabolites of the diterpenoid phytoalexins of rice had to accumulate more rapidly and to a higher level than the susceptible genotype to restrict fungal growth

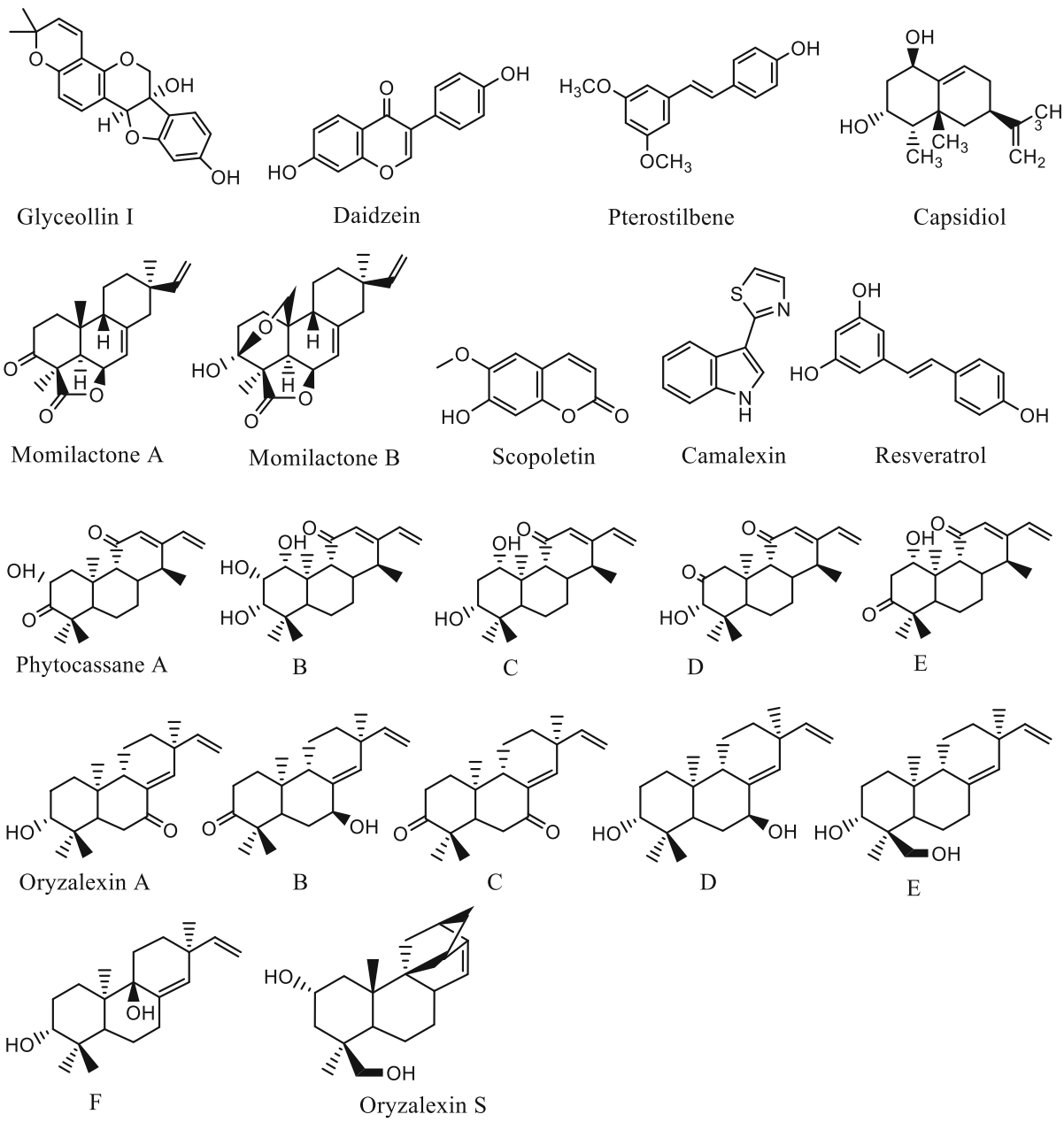

Fig. 1 Phytoalexins with well supported roles in mediating pathogen resistance in plants

prior to the HR-response (Hasegawa et al. 2010). Similarly, phytoalexin gene transcripts and metabolites accumulated ~ 100 h earlier during the incompatible interaction of a resistant common bean genotype compared to the compatible interaction of the the susceptible genotype (Lamb et al. 1992). In this example, the compatible genotype demonstrated water soaked anthracnose lesions by that time, whereas in the resistant genotype lesion spread of *Colletotrichum lindemuthianum* was arrested soon after peak phytoalexin accumulation. Similarly, the treatment of tobacco plants with cytokinins resulted in the rapid accumulation of scopoletin and capsidiol and provided resistance to *Pseudomonas syringae* (Großkinsky et al. 2012). Scopoletin and capsidiol were determined to be the cause of resistance by infiltrating them into

tobacco leaves at physiological levels just prior to bacterial inoculation. The rapidity of scopoletin and capsidiol biosynthesis, rather than their absolute levels was concluded to be the decisive factor for providing incompatibility since their absolute levels were much higher in the susceptible, mock-treated control, at later stages of infection.

The glyceollins of soybean have long served as a model for understanding how phytoalexins mediate resistance to pathogens. Yoshikowa and colleagues were the first to suggest that the rapid elicitation of glyceollins was critical for mediating resistance to a agricultural pest of soybean, namely *Phytophthora sojae*. In these early studies, glyceollin concentrations were measured in localized infection sites of hypocotyls of the resistant variety Harosoy 63 and the susceptible nearly isogenic variety Harosoy at various times after inoculation with zoospores of *P. sojae* (Yoshikawa and Masago 1982; Yoshikawa et al. 1978). The extent of mycelium growth was then compared as a proxy of infection. In the resistant genotype Harosoy 63, the levels of glyceollins exceeded the ED90 values (i.e. the effective dose required to inhibit growth of 90% of the population) at the site of infection within 8 h after inoculation and increased transiently thereafter. However, it took the susceptible variety 24 h to accumulate glyceollins to concentrations exceeding the ED90. *P. sojae* grew at similar rates in both the resistant and susceptible hypocotyls until 8 h after inoculation, but then growth was restricted in the resistant hypocotyls and no further growth occurred after 9 h. Bhattacharyya and Ward confirmed the delayed accumulation of glyceollins in susceptible Harosoy compared to resistant Harosoy 63 following treatment of hypocotyls with race 1 *P. sojae* zoospores (Bhattacharyya and Ward 1986). Plant genotypes are thought to be resistant due to their ability to rapidly increase the ratio of phytoalexins relative to the number of pathogen cells, rather than simply exceeding a specific threshold concentration (Großkinsky et al. 2012). Graham and colleagues observed that the inoculation of cotyledons of race 1-resistant variety Williams 79 resulted in glyceollin levels peaking within 24 h after elicitation but they did not peak until 48 h after the infection front had passed in the susceptible nearly-isogenic variety Williams (Graham et al. 1990). Similarly, the hypocotyls of Williams 79 exhibited delayed elicitation of glyceollins during the compatible interaction with Race 3 *P. sojae* compared to the incompatible interaction with Race 1 (Hahn et al. 1985). Purified glyceollins and several other intermediates were confirmed to be toxic to *P. sojae*, and unlike several other pathogens, *P. sojae* was found to lack genes for glyceollin degradation (Bhattacharyya and Ward 1985; Fischer et al. 1990; Lygin et al. 2010). It was Graham and colleagues that provided compelling evidence that glyceollins and other similar metabolites were responsible for mediating resistance rather than other defense responses. They silenced soybean *ISOFLAVONE SYNTHASE* genes reducing the biosynthesis of glyceollins and several 5-deoxy-isoflavonoids including daidzein, which resulted in susceptibility to *P. sojae* (Graham et al. 2007). Finally, by silencing the glyceollin TF *GmMYB29A2* in variety Williams 82 that is resistant to *Race 1 P. sojae*, and overexpressing the same gene in the susceptible variety Williams, we recently found that compatibility and incompatibility coincided with reduced and enhanced accumulations of glyceollin I specifically, and not the other glyceollins or 5-deoxyisoflavonoids (Jahan et al. 2020) Since *P. sojae* causes an annual yield loss of $1–2 billion worldwide in soybean agriculture (Lin et al. 2014; Tyler 2007), bioengineering *GmMYB29A2* expressions should be field tested as an approach to enhance food security. Various reports have hinted that glyceollins may have roles in defending crop plants against insects (Huang and Barker 1991; Hohenstein et al. 2019; Chang and Hartman 2017; Liu et al. 1992), so the approach could have broader utility. Various studies have demonstrated potent medicinal properties of some phytoalexins, such as glyceollin I. Thus, engineering phytoalexin gene regulatory networks could also be an economical tool for producing phytoalexins for human health.

Value of phytoalexins for human health

Phytoalexins may inherently have bioactivities that render them useful as medicines due to their evolution as plant defense compounds. Yet, they have not been characterized from most plant species, and hence their medicinal properties remain largely unknown. It also remains unknown how some phytoalexins show protective activities in some cell types, yet they inhibit the proliferation of others. The glyceollins are an

example of phytoalexins that are toxic to cancer cells yet promote the survival of insulted neurons. Glyceollin I exhibits potent antiestrogenic activities that are distinct from the conventional therapeutic tamoxifen (Zimmermann et al. 2010). It directly binds to estrogen receptor (ER) alpha and inhibits breast tumor progression (Salvo et al. 2006). It also hinders the invasion and migration of breast cancer cells that are resistant to letrozole and it reverses the transition of epithelial to mesenchymal cells in part by inhibiting the expression of EGFR (Epidermal growth factor receptor) (Carriere et al. 2015). A mixture of glyceollins I, II, and III inhibits the survival and tumor proliferation of triple-negative breast cancer (TNBC) by an unknown mechanism that is ER–independent (Rhodes et al. 2012). TNBC is resistant to conventional chemotherapeutics and thus is a cancer subtype that is in need of new therapies. Glyceollins were also shown to inhibit cell proliferation by blocking the synthesis and destabilizing hypoxia inducible factor 1 alpha (HIF-1α) in TNBC, non-small cell lung (NSCL) cancer, and in other chemo-recalcitrant cell types (Lee et al. 2015). In contrast, glyceollins protect primary cortical neurons derived from mice and hippocampal HT22 cells from glutamate-induced cytotoxicity (Seo et al. 2018). The medicinal properties of glyceollins and potential mechanisms have recently been reviewed (Pham et al. 2019; Bamji and Corbitt 2017).

Other phytoalexins with promising medicinal properties include the (ptero)stilbenes from grapevine and camalexin from Arabidopsis. Camalexin induces apoptosis in prostate cancer cells (Smith et al. 2014) with strong preference over non-cancerous cells (Pilatova et al. 2013). Pterostilbene inhibits invasion, metastasis, and migration of human hepatoma cells (Pan et al. 2009) and suppresses the establishment of abnormal crypt foci in a rat colon cancer model (Suh et al. 2007). Resveratrol is a stilbene that has anticancer, anti-inflammatory, and cardio protective activities (Shukla and Singh 2011; Sen 2017). Recently, resveratrol was suggested to reduce the viability and migration of melanoma cells by inhibiting the AKT/mTOR pathway (Gong and Xia 2020). Treatment with the autophagy inhibitor, 3-methyladenine, reversed the resveratrol-dependent effects (Gong and Xia 2020). Resveratrol also protects hippocampal neurons against cerebral ischemia, putatively by scavenging free radicals and elevating cerebral blood flow resulting from the release of nitric oxide (Lu et al. 2006). There has been widespread efforts to semi-synthesize methoxylated, hydroxylated, and halogenated derivatives of resveratrol that have improved bioavailability, anticancer, cardioprotective, neuroprotective or antioxidant properties (Nawaz et al. 2017). Synthetic chemistry approaches were also used to make 5-bromosubstituted derivatives of indole phytoalexins (e.g. brassinin) (Budovská et al. 2020). Some of these had greater antiproliferative activities in cancer cells than cisplatin and lower toxicity to immortalized primary mouse embryonic fibroblasts (Budovská et al. 2020). Approaches that could be used for making novel derivatives of phytoalexins that have enhanced medicinal properties include combining semi-synthesis with genetically engineered microbes and plants in succession and in different combinations to (bio)synthesize novel molecules (Gary et al. 2018).

While phytoalexins have been described from relatively few plant species, their impressive effects on human health and diverse chemical structures highlights why scientists have suggested 'mining' plants for these valuable molecules (Mead 2007). The fact that new classes of phytoalexins continue to be discovered from some of the best-characterized plant species (Huffaker et al. 2011; Rajniak et al. 2015; Ube et al. 2019) suggests that our understanding of the biochemical diversity of phytoalexins and their medicinal activities remains in its infancy. Large scale metabolomics screenings for phytoalexins could very much improve our understanding of their diversity and evolution in plants, and could uncover novel pharmaceuticals. Yet, accessibility to these molecules will likely remain a challenge since phytoalexins are biosynthesized transiently and in low amounts in plants.

Efforts to improve accessibility to phytoalexins

Since phytoalexins are biosynthesized in low amounts and only transiently upon elicitation, various research efforts worldwide have focused on enhancing their accessibility. These have mainly been focused on enhancing their elicitation in plants, to synthesizing them using chemistry methods, or when known, introducing their entire biosynthetic gene sets into microbes. For example, the traditional method to obtain glyceollins has been to elicit soybean seeds

with microbial pathogens (Boue et al. 2000). Various chemical synthesis methods have been developed, yet even the most promising approach remains highly uneconomical (Khupse et al. 2011; Luniwal et al. 2011; Malik et al. 2015). Efforts to improve the elicitation of glyceollins include fermentation of soybeans (Park et al. 2012), and treating seeds with pathogens and malting (Simons et al. 2011), pathogens and environmental stresses (Aisyah et al. 2013), and pathogens and chemicals (Farrell et al. 2017). Screening various environmental stresses for their effects on phytoalexin biosynthesis recently identified acidic growth medium (pH 3.0) as a potent elicitor of glyceollins (Jahan et al. 2019; Jahan and Kovinich 2019). Yet, bioengineering may be the most promising strategy to improve accessibility. Overexpressing the isoflavonoid synthesis gene *ISOFLAVONE REDUCTASE* (*IFR*) in soybean seedlings increased glyceollin amounts only 3-fold (Cheng et al. 2015), which notably provided greater increases in yield compared to other elicitor treatments. This example highlights that overexpressing a gene for one rate-limiting biosynthetic step indefinitely uncovers another. For this reason, it may be more effective to overexpress the transcription factors (TFs) or signaling proteins that positively regulate all genes that are required to biosynthesize a phytoalexin. We recently demonstrated that overexpressing the NAC-family TF *GmNAC42-1* in soybean hairy roots increased glyceollin II elicitation more than 10-fold (Jahan et al. 2019). Notably, bubble-type bioreactors can be used to further enhance the production of secondary metabolites from roots (Lee et al. 2020).

An alternative production method has been to transfer full biosynthetic gene sets into microbes that have modified primary metabolism. For example, an *E. coli* strain engineered to supply more L-tyrosine could produce 3.6-fold more pterostilbene than the wild-type strain (Heo et al. 2017). Yet, this approach is not feasible for biosynthetic pathways where all biosynthetic genes have not been identified, such as for the glyceollins. Thus, for most phytoalexins, the key to providing economical accessibility could be engineering phytoalexin gene regulatory networks and their feedback mechanisms in plants. Fortunately we are entering an era where non-species-specific genetic transformation methods, such as nanoparticle-based methods are making efficient genetic engineering possible in a multitude of plant species (Demirer et al. 2019; Doyle et al. 2019; Zhang et al. 2019).

Diverse phytoalexin biosynthetic pathways have common elicitors

Phytoalexin biosynthesis genes and their metabolite products are highly diverse among plant species (Ahuja et al. 2012; Jeandet et al. 2014). Species of the *Leguminosae* family biosynthesize glyceollins (soybean), medicarpin (alfalfa), pisatin (*Pisum sativum*), phaseollin (*Phaseolus vulgaris*), and Maackiain (*Maackia*, *Trifolium* and *Cicer* groups) (Ingham 1979; VanEtten et al. 1983; Strange et al. 1985). Plants of the Poacae family biosynthesize diterpenoid momilactones, phytocassanes, oryzalexins (*Oryza sativa*), kauralexins and zealexins (*Zea mays*), the phenolic alkaloid avenanthramides (oat), and the flavonoids sakuranetin (*Oryza sativa*) and 3-deoxyanthocyanidins (*Sorghum bicolor*) (Poloni and Schirawski 2014; Huffaker et al. 2011; Schmelz et al. 2014; Yamamura et al. 2015; Koga et al. 1995). The Solanaceae family is known to produce the terpenoid rishitin, and the phenylpropanoids chlorogenic acid and caffeic acid (Ohnishi et al. 1994). Grapevine (*Vitis vinifera*), from the Vitaceae family biosynthesizes stilbenes like resveratrol (Jeandet et al. 2019b; Kiselev 2011). *Eschscholzia californica* (Papaveraceae) produces benzophenanthridine alkaloids while *Catharanthus roseus* (Apocynaceae) biosynthesizes monoterpenoid indole alkaloids (Heinze et al. 2015). *Arabidopsis thaliana* (Arabidopsis) from the Brassicaceae family produces the indole alkaloid camalexin, the indoles brassinin, indole-3-carboxylic acid, and the hydroxyindole 4-hydroxy-indole-3-carbonyl nitriles (Pastorczyk et al. 2020; Stahl et al. 2016; Rajniak et al. 2015). Thus, even closely related plant species biosynthesize phytoalexins from a wide range of metabolic pathways.

Despite their biosynthetic heterogeneity, phytoalexins share common elicitors. These have been described in several reviews (Ahuja et al. 2012; Großkinsky et al. 2012; Jeandet et al. 2010). Elicitors that commonly induce phytoalexin biosynthesis in different plant species include microbial pathogens, UV irradiation, and inorganic heavy metals. Common elicitors could suggest that the diverse phytoalexin biosynthesis pathways of different plant species are

stimulated by the same conserved signaling pathway(s). Since phytoalexin biosynthesis genes are commonly regulated at the level of gene transcription (Jeandet et al. 2019a; Höll et al. 2013; Moy et al. 2004; Saga et al. 2012; Yamamura et al. 2015), it is possible that TFs that directly regulate phytoalexin biosynthesis genes are components of this conserved signaling network. Here we review the TFs and signaling proteins that regulate diverse phytoalexin biosynthesis pathways. We also highlight two conserved TFs that were recently identified to directly regulate distinct phytoalexin biosynthesis pathways in different plant species, thus providing initial evidence of the 'conserved TF network hypothesis'.

Transcription factors that activate phytoalexin biosynthesis

Out of 58 families of TFs in plants, six have been found to be involved in regulating phytoalexin biosynthesis. These families are: WRKY (WRKYGQK motif), MYB (myeloblastosis related), NAC [no apical meristem (NAM) *Arabidopsis* transcription activation factor (ATAF1/2) cup-shaped cotyledon (CUC2)], bHLH (basic helix-loop-helix), AP2/ERF (APETALA2/ethylene responsive factor), and bZIP (basic leucine zipper). The first TF identified to regulate the biosynthesis of a phytoalexin was a WRKY family protein from cotton (*Gossypium arboreum* L.). The term 'WRKY' denotes a zinc-finger TF that has a 60-70 amino acid WRKY domain containing the WRKYGQK motif (Amorim et al. 2017; Song et al. 2018; Jiang et al. 2017). *GaWRKY1* transcripts were elicited both spatially and temporally with gossypol biosynthesis genes by pathogen and inorganic elicitors (Xu et al. 2004). Further, the GaWRKY1 protein was able to directly bind the promoter of the gossypol biosynthesis gene (+)-δ-cadinene synthase (*CAD1*) in the yeast one-hybrid (Y1H) system. The second WRKY-family TF that was identified to regulate phytoalexin biosynthesis was WRKY33 for camalexin in *Arabidopsis* (Qiu et al. 2008). It directly bound to the promoter of the camalexin biosynthesis gene *PHYTOALEXIN DEFICIENT 3* (*PAD3*) by ChIP-PCR upon treatment with the fungal elicitor flg22. WRKY33 requires phosphorylation from the pathogen-responsive mitogen-activated protein kinases (MAPKs) MPK3 and MPK6 for full activity (Mao et al. 2011). Despite that WRKY33 is essential for the activation of camalexin biosynthesis, the overexpression of wild-type (WT) or a phospho-mimic forms of WRKY33 did not enhance camalexin levels in elicited tissues beyond the WT (Qiu et al. 2008). This suggests that WRKY33, while essential, was not a rate-limiting for camalexin gene regulation.

MYB-family TFs were also found to regulate phytoalexin biosynthesis. *VvMYB14* and *VvMYB15* (R2R3-type MYB TFs) from grapevine (*Vitis vinifera*) were co-induced with stilbene biosynthesis genes and could directly bind the promoter of *STILBENE SYNTHASE* (*STS*) in transient promoter-reporter assays (Höll et al. 2013). *VvMYB15* enhanced the accumulation of stilbenes 2- to 5-fold when overexpressed in grapevine hairy roots. Further, the overexpression of *MYB14* from Chinese wild grape, *Vitis quinquangularis*-Pingyi, increased stilbene biosynthesis and the expressions of *STS* genes (Luo et al. 2019; Duan et al. 2016; Luo et al. 2020). Various promoter segments of *MYB14* and *MYB15* responsible for induction by pathogens have been identified (Luo et al. 2020). Potential ROS-responsive regions of the MYB14 promoter from *Vitis labrusca* 'Concord' were also reported (Bai et al. 2019). Yet, the transacting factors that bind those promoter segments have not been reported.

Other MYBs were implicated in regulating camalexin biosynthesis in Arabidopsis. Reduced camalexin levels were observed upon elicitation of double and triple mutants of *AtMYB34*, *AtMYB51*, and *AtMYB122* (Frerigmann et al. 2015). However, promoter-reporter assays found that these MYBs do not bind camalexin biosynthesis gene promoters directly, suggesting indirect roles in camalexin gene regulation.

bHLH-type TFs were found to regulate phytoalexin synthesis in rice and *Medicago truncatula*. The elicitation of sakuranetin in rice was almost completely eliminated by RNAi silencing of the bHLH gene *OsMYC2* (Ogawa et al. 2017). OsMYC2 was involved in the direct binding of the sakuranetin biosynthesis gene promoters by promoter-reporter assays (Ogawa et al. 2017). OsDPF (DITERPENOID PHYTOALEXIN FACTOR), another bHLH-family TF from rice, directly binds the promoters of phytocassane and momilactone biosynthesis genes by promoter-reporter assays (Yamamura et al. 2015). Two other bHLH-family TFs, TSAR1 and TSAR2

(TRITERPENE SAPONIN BIOSYNTHESIS ACTIVATING REGULATOR), directly bound triterpene saponin biosynthesis genes in *Medicago truncatula* (Goossens et al. 2015).

bHLHs are often required by R2R3-type MYBs for the MYBs to bind DNA in order to regulate various branches of phenylpropanoid metabolism (Zimmermann et al. 2004; Xu et al. 2015). However, the VvMYB14 and VvMYB15 from grapevine did not require a bHLH to bind the *STS* gene promoter (Höll et al. 2013). Likewise, in *Arabidopsis* no bHLH-family TF was found to be involved in regulating camalexin biosynthesis. In contrast, loss-of-function mutants of the NAC-family TF ANAC042 revealed that it was required for WT levels of camalexin elicitation by pathogens and heavy metals (Saga et al. 2012). Chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) found that ANAC042 (a.k.a. JUNGBRUNNEN1) directly binds key genes involved in gibberellin (GA) and brassinosteroid (BR) biosynthesis to suppress plant growth (Shahnejat-Bushehri et al. 2016). It remains unclear whether ANAC042 directly binds camalexin gene promoters in the presence of an elicitor.

Importantly, no phytoalexin TFs were found to be homologous among plant species until recently. Technically, reciprocal BLASTPs of each phytoalexin TF did not identify another phytoalexin TF from other plant species in the top 10 most similar proteins. This has led to the concept that phytoalexin TFs are as diverse as the biosynthetic pathways that they regulate. Yet, in two recent studies we reported two TFs that regulate glyceollin biosynthesis in soybean that are orthologous to TFs that regulate different phytoalexin biosynthesis pathways in other plant species. By screening a panel of abiotic stresses, we identified acidity stress to be a novel elicitor and dehydration stress to be a suppressor of glyceollin biosynthesis, respectively (Jahan et al. 2019). RNA-seq of seedlings treated with those stresses found several TF genes that were up- and down-regulated with glyceollin biosynthesis gene transcripts and metabolites. *GmNAC42-1*, the soybean ortholog of the camalexin regulator *AtANAC042* from Arabidopsis (Saga et al. 2012) was among those TF genes. Silencing *GmNAC42-1* in soybean hairy roots elicited with *P. sojae* WGE decreased the expressions of glyceollin gene transcripts and metabolites, demonstrating that it was essential for the elicitation of glyceollin biosynthesis (Jahan et al. 2019). The GmNAC42-1 protein interacted with the promoters of two glyceollin biosynthesis genes, namely *ISOFLAVONE SYNTHASE 2* (*IFS2*) and *GLYCINOL 4-DIMETHYLALLYLTRANSFERASE* (*G4DT*) in the yeast one hybrid system, suggesting that GmNAC42-1 is a direct regulator of glyceollin biosynthesis. Overexpressing *GmNAC42-1* increased the expressions of glyceollin gene transcripts and metabolites in soybean hairy roots treated with WGE. However, overexpression was not sufficient to upregulate all glyceollin biosynthesis genes in the absence of an elicitor. Thus *GmNAC42-1* was not sufficient on its own to activate the entire glyceollin biosynthesis pathway. Since *GmNAC42-1* was the soybean ortholog of *AtANAC042* this strongly challenged the concept that phytoalexin TFs are pathway/lineage-specific. The results also provide a major focal point to address the long-standing question of how plants have evolved lineage-specific biochemical defenses that are regulated by conserved signaling networks. Yet, it remains unknown exactly how ANAC042/GmNAC42-1 TFs have evolved to activate different biosynthetic pathways.

In a second study aimed at identifying glyceollin TFs, we conducted transcriptomics on two soybean varieties responding to *P. sojae* WGE and identified two soybean R2R3-type MYB TFs that had contrasting roles in regulating glyceollin biosynthesis (Jahan et al. 2020). GmMYB29A2 was essential for eliciting glyceollin biosynthesis upon WGE treatment. Silencing *GmMYB29A2* in hairy roots decreased the expressions of *GmNAC42-1*, glyceollin biosynthesis gene transcripts and metabolites, demonstrating that it was essential for elicitation. Overexpressing *GmMYB29A2* increased the levels of those gene expressions and metabolites in the presence of WGE, but it failed to fully activate all glyceollin biosynthesis genes in the absence of an elicitor treatment, similar to *GmNAC42-1* (Jahan et al. 2019). The GmMYB29A2 protein interacted with the promoters of two glyceollin biosynthesis genes in yeast one-hybrid and electrophoretic mobility shift assays (EMSAs), demonstrating that GmMYB29A2 was a direct regulator of glyceollin biosynthesis genes. By contrast, overexpressing the second R2R3-type MYB, namely *GmMYB29A1*, reduced glyceollin accumulation despite that it encoded a protein that has only 10 amino acid differences compared to GmMYB29A2 (Jahan et al. 2020). Overexpressing GmMYB29A1

had little effect on the expression of glyceollin biosynthesis genes, suggesting that it reduced glyceollin levels by activating glyceollin turnover and/or competing biosynthetic genes.

Again supporting the concept that there exists a conserved network of phytoalexin TFs, we found that *GmMYB29A2* is the soybean ortholog of the stilbene biosynthesis regulator from grapevine *VvMYB14* (Höll et al. 2013; Bai et al. 2019; Duan et al. 2016; Luo et al. 2020). Again, how evolution resulted in conserved TFs regulating different phytoalexin biosynthetic pathways remains unclear. Perhaps the most parsimonious explanation is that, under evolutionary pressure, these TFs co-opted different biosynthetic genes through the evolution of recognition elements in their promoters. Figure 2 demonstrates our very limited knowledge of the lineage-specific roles of the conserved ANAC042/GmNAC42-1 and GmMYB29A2/VvMYB14 networks.

Recognition elements of phytoalexin transcription factors

The recognition elements of conserved phytoalexin TFs could inform on how those TFs regulate diverse biosynthetic genes. The *cis*-elements of the WRKY DNA binding domain (Brand et al. 2013) have a consensus sequence of TTGACT/C that is called the W-box (Rushton et al. 1996). WRKY33 binds to

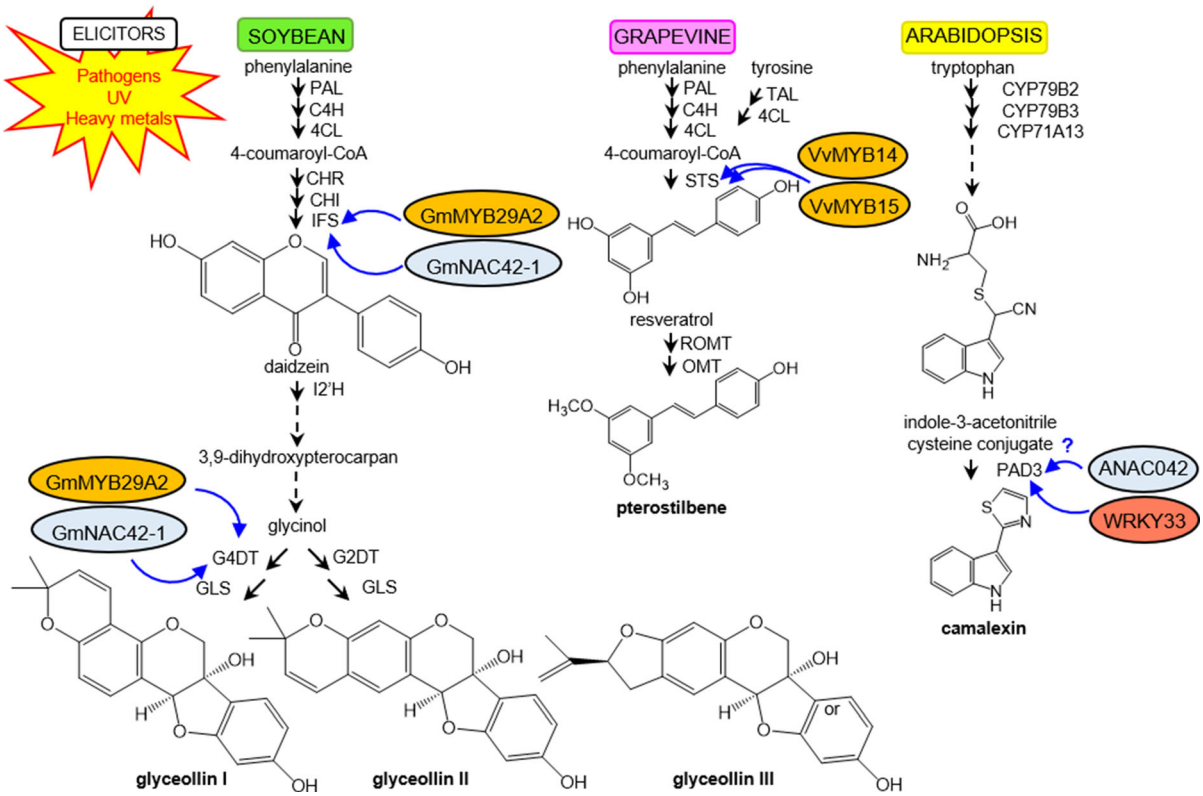

Fig. 2 Conserved transcription factors regulate diverse phytoalexin biosynthesis pathways. The grapevine (TF) VvMYB14 and its soybean ortholog GmMYB29A2 directly regulate stilbene- and glyceollin-specific genes, respectively. The Arabidopsis TF ANAC042 and its soybean ortholog GmNAC42-1 regulate camalexin and glyceollin biosynthesis pathways, respectively. Blue arrows indicate direct binding of a TF to the promoter of a biosynthesis gene. Most biosynthesis gene promoters have not been tested for binding by their putative TFs. ANAC042 positively regulates camalexin biosynthesis; however it is not known whether it directly binds biosynthesis gene promoters. Solid and broken arrows indicate single and multiple enzymatic steps, respectively. CHR, chalcone reductase; CHI, chalcone isomerase; IFS, isoflavone synthase; I2′H, isoflavone 2′-hydroxylase; G4DT, glycinol 4-dimethylallyl transferase; G2DT, glycinol 2-dimethylallyl transferase; GLS, glyceollin synthase; TAL, tyrosine ammonia lyase; STS, stilbene synthase; ROMT, resveratrol O-methyltransferase; OMT, O-methyltransferase; CYP, cytochrome P450; PAD3, phytoalexin deficient 3 (a.k.a. CYP71B15)

W-box elements in the promoters of *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE 2* (*ACS2*)/*ACS6* to activate ethylene biosynthesis in response to *B. cinerea* (Li et al. 2012). The *Zea mays* WRKY ZmWRKY79 directly binds two W-box elements in the promoter of *ANTHER EAR 2* (*ZmAn2*), a gene involved in the biosynthesis of kauralexins (Fu et al. 2017). WRKY62 and WRKY45 from rice form homodimers and heterodimers and bind the W-box-like sequences TTGACC and ATGACT of diterpenoid phytoalexin biosynthetic genes and their regulator *DPF* (Fukushima et al. 2016). Also in rice, mutation analysis of the kaurene synthesis gene *OsKSL4* revealed a *cis*-acting TGACG motif that is needed for elicitation (Okada et al. 2009). Further, the basic leucine zipper (bZIP) TF *OsTGAP1* is responsible for the JA-dependent activation of *OsKSL4* possibly by binding to TGACGT sequences (Yoshida et al. 2017).

The N termini of NAC transcription factors have ~ 150 amino acids in their NAC domain with DNA binding abilities (Puranik et al. 2012). However, the recognition sequences of individual NACs are still not clear due to limited data (Lindemose et al. 2014). Many NACs bind the core CGT[G/A] sequence with differences in the flanking bases (Xu et al. 2013b). The CGT[G/A] flanking sequences may dictate the binding specificity of different NACs (Lindemose et al. 2014). In the absence of an elicitor, the camalexin TF ANAC042 [a.k.a. JUNGBRUNNEN1 (JUB1)] binds to the consensus sequence RRYGCCGT in the promoter of *DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN2A* (*DREB2A*), which is an important positive regulator of dehydration responses (Wu et al. 2012). The fact that dehydration and camalexin biosynthesis are opposing responses (Mewis et al. 2012), similar to dehydration and glyceollin biosynthesis (Jahan et al. 2019), could suggest that the homologous TFs ANAC042 and GmNAC42-1 have distinct (possibly opposite) roles in the presence or absence of a pathogen elicitor. Thus, it should be investigated whether their recognition elements and mechanism of gene regulation differ in the presence and absence of elicitation.

The MYB TF VvMYB14 was demonstrated to bind the Box-L5 element GAGTTGGTGAGA to regulate an *STS* gene in yeast one-hybrid and promoter reporter assays (Fang et al. 2014). Whether VvMYB14 binds similar elements in other genes, in the presence of an elicitor, or whether its homolog GmMYB29A2 from soybean binds similar sequences in glyceollin genes remains to be determined.

Signaling pathways for the elicitation of phytoalexin biosynthesis

While elicitation ultimately converges on the cis-regulatory elements of phytoalexin biosynthetic genes and the TFs that regulate them, upstream signaling pathways have critical roles in activating those direct regulatory processes, and have been more intensively studied. Currently the plant immune system is viewed to consist of two branches [reviewed by (Jones and Dangl 2006)]. In the first branch, plant trans-membrane pattern recognition receptors (PRRs) recognize and trigger a response to highly conserved, slowly evolving, pathogen-associated molecular patterns (PAMPs). This activates PAMP-triggered immunity (PTI) that stimulates broadly the defense responses of plants with no pathogen-specific prioritization. PRRs include receptor-like proteins (RLPs) and receptor-like kinases (RLKs). Examples of PRRs that are known to signal phytoalexin biosynthesis include the lysine motif receptor kinases (LYKs) AtLYK4/AtLYK5/AtCERK1 from Arabidopsis (Cao et al. 2014), FLAGELLIN SENSING 2 (FLS2) from Arabidopsis (Felix et al. 1999), EF-Tu receptor (EFR) from Arabidopsis (Kunze et al. 2004), and β-glucan-binding protein (GBP) from soybean (Fliegmann et al. 2004). Their corresponding PAMPs are chitin from fungi, the N-terminal portion of bacterial flagellin (flg22), elongation factor TU (EF-Tu) from bacteria, and WGE from oomycetes, respectively. Despite that PAMPs are highly conserved, a recent study has demonstrated that different plant species recognize different PAMPs, the example being long and short β-1,3-linked glucans (Wanke et al. 2020), raising the possibility that different PRR variants exist among plant species.

The second branch, known as effector triggered immunity (ETI), is triggered by effector proteins that are specific to certain species or races of a pathogen. ETI acts on PTI to prioritize (i.e. accelerate/increase in amplitude) particular defense responses over others. Effector proteins are numerous and encoded by variable, high-copy number genes. Effector proteins have generally evolved to suppress PTI, establishing

effector triggered susceptibility (ETS). Plant resistance (R) genes have evolved to recognize specific effector proteins and prevent ETS. R genes commonly encode nucleotide binding-leucine rich repeat (NB-LRR) proteins (Gao et al. 2005). NB-LRR proteins function to block effector activities such as binding PRRs (the Guard Hypothesis) (Dangl and Jones 2001). R genes are numerous in plant genomes and their encoded proteins recognize effector proteins with gene-for-gene specificity (Flor 1942). Yet, pathogen recognition generally involves the stimulation of a complex web of partially overlapping signaling networks stimulated by PAMPs and ETIs (Wu et al. 2018).

Following the perception of a biotic elicitor (i.e. a PAMP or effector) by plant cell receptor proteins, sequential events that typically follow include reversible phosphorylation and dephosphorylation of plasma membrane and cytosolic proteins, spiking of Ca^{2+} and proton levels in the cytosol, mitogen-activated protein kinase (MAPK) activation, reactive oxygen species (ROS) production, early defense gene expression, ethylene/JA, oxylipin, or salicylic acid (SA) biosynthesis and signaling, and finally the activation or expression of TFs for defense gene expressions such as those for the biosynthesis of phytoalexins (Zhao et al. 2005).

Specifically which hormone signaling pathways control the elicitation of phytoalexins may differ depending on the combination of PAMPs and effectors presented by the pathogen. For example, camalexin biosynthesis was elicited by either SA-dependent or -independent pathways upon recognition of *Pseudomonas syringae*, *Peronospora parasitica*, and *Phytophthora porri* (Denby et al. 2005; Nawrath and Metraux 1999; Roetschi et al. 2001). SA and JA typically act antagonistically in plant defense signaling (Ahuja et al. 2012; Takahashi et al. 2004), yet JA also had a role in inducing camalexin in response to some pathogens. For example, the Arabidopsis JA biosynthesis mutant *aos* elicited only 14% of wild-type levels of camalexin in response to *Botrytis cinerea* (Rowe et al. 2010). By contrast the JA signaling mutant *coi1* did not exhibit a reduction in camalexin biosynthesis after infection with *Alternaria brassicicola* (Thomma et al. 1999). JA belongs to the oxylipin family of biomolecules. Treatment of soybean cell cultures with a biosynthetic precursor of JA, namely 12-oxo phytodienoic acid (OPDA), dramatically induced the accumulation of glyceollins, whereas JA and methyl jasmonate (MeJA) did not (Fliegmann et al. 2003). Cytosolic Ca^{2+} signaling was also found to have a role in activating glyceollin biosynthesis in response to *P. sojae* WGE (Ebel et al. 1995; Fliegmann et al. 2003; Stäb and Ebel 1987). Ca^{2+} signaling and the calcium-dependent protein kinase CPK5 function upstream of SA biosynthesis and signaling in the Arabidopsis response to *Pseudomonas syringae* (Guerra et al. 2020).

Phytoalexins are often a major output of R gene signaling. To begin to understand the signaling responses mediated by the *RESPONSE TO PHYTOPHTHORA SOJAE* (*Rps*) genes of soybean, Lin and colleagues conducted comparative transcriptomic analyses on 10 nearly-isogenic soybean lines (NILS) that were incompatible with race 1 *P. sojae* (Lin et al. 2014). A comparison of the 10 incompatible NILs identified 369 and 770 genes that were collectively up- and down-regulated compared to the susceptible variety Williams., respectively. All resistant lines shared the upregulation of the glyceollin biosynthesis gene *PAL*, and 13 other genes from ROS, SA, brassinosteroid (BR), ethylene and MAPK pathways.

Overall, a systems-level study of the responses of a wide range of plant species to PAMP/effector combinations is needed to understand how these hormone signaling pathways function to effect phytoalexin elicitation. Since PRRs and NB-LRRs directly affect the phosphorylation of proteins and kinase signaling is broadly involved in phytoalexin signaling, it is likely that hormones affect kinase signaling.

Kinase signaling of phytoalexin biosynthesis

The elicitation of camalexin biosynthesis by ANAC042 in Arabidopsis is suppressed by K252a, which is a potent inhibitor of Ca^{2+}/calmodulin-dependent protein kinases and serine/threonine protein kinases (Saga et al. 2012). However, most research on the involvement of kinases in signaling phytoalexin biosynthesis comes from studies of mitogen-activated protein kinases (MAPKs) in Arabidopsis. MAPK signaling is universal in eukaryotes. Upon recognition of a PAMP by PRRs, the signal is thought to be transmitted through sequential phosphorylations of MAPKKKs, MAPKKs, and then MAPKs. MAPKKKs phosphorylate the two serine/threonine residues that

are in the conserved S/T-X33-5-S/T motif of specific MAPKKs, which then phosphorylate the conserved tyrosine (Y) and threonine (T) residues of MAPKs (Huang et al. 2011; Zaïdi et al. 2010). The activated MAPKs then phosphorylate specific downstream proteins, such as TFs, to trigger cellular responses. In *Arabidopsis*, camalexin biosynthesis is regulated by the *AtMKK4/AtMKK5-AtMPK3/AtMPK6* cascade (Kishi-Kaboshi et al. 2010). In the absence of a pathogen, the activation of MPK3/MPK6 by MAPKK or MAPKKK is adequate to stimulate some camalexin biosynthesis (Ren et al. 2008). The activation of MPK3/MPK6 upregulates multiple tryptophan (Trp) and camalexin biosynthesis genes, such as *PAD3* (Ren et al. 2008). A careful molecular analysis demonstrated that MPK4 phosphorylates a nuclear localized protein complex of MKS1 and the camlexin TF WRKY33, releasing WRKY33 to bind the promoter of *PAD3* (Qiu et al. 2008). Further, WRKY33's transcription is regulated by the MPK3/MPK6 cascade and its phosphorylation by MPK3/MPK6 is required for full activity (Mao et al. 2011). These kinase signaling pathways leading to the activation of camalexin biosynthesis are illustrated in Fig. 3.

Molecular mechanisms that limit phytoalexin accumulation by pathogens

In the final sections of this review, we focus on factors that limit or reduce phytoalexin accumulation. Prior to discussing mechanisms that exist in plant cells, we describe mechanisms used by microbes since (1) they are relevant to crop improvement for agriculture, and (2) potentially similar mechanisms could exist in plants. Phytoalexins are generally broadly toxic to microbial pathogens. Yet, some pathogens have evolved enzymes to catabolize, transform, or suppress phytoalexin synthesis to overcome their toxicity. The number of genes that are encoded by a pathogen to reduce the toxicity of any one phytoalexin remains unknown. Recently, using a transcriptomics approach, Wang and colleagues found that 187 genes were differentially expressed (DEGs) in *Bursaphelenchus xylophilus* in response to the terpenoid phytoalexin carvone (Wang et al. 2019). By RNAi silencing of a cathepsin protease gene, they demonstrated that it was required for virulence. This could suggest that the degradation of phytoalexin biosynthesis or signaling proteins may be a mechanism used by some pathogens to reduce toxicity.

Several fungi detoxify phytoalexins directly using enzymes that have catabolic activities (Zeilinger et al. 2015). For instance, *A. brassicicola* catabolizes brassinin using the enzyme brassinin hydrolase (Pedras et al. 2011). Fungal catabolism of phytoalexins can involve oxidation (brassinins, a dithiocarbamate detoxified by *Leptosphaeria* sp), or reduction of the oxoindole ring double bonds (wasalexin A by detoxified by *L. maculans* (Pedras and Abdoli 2017). Resveratrol from grapevine has antifungal activities against *Rhizopus stolonifer Plasmopara viticola*, and *B. cinerea* (Adrian et al. 1997). However, some races of *B. cinerea* can catabolize stilbenes by oxidation (Breuil et al. 1998; Bavaresco et al. 1997; Sbaghi et al. 1996). Sakuranetin is catabolized into sternbin and naringenin by 3′-hydroxylation and 7-*O*-demethylation activities encoded by *Pyricularia oryzae* (Katsumata et al. 2017). Similarly, the fungus *Fusarium solani* encodes the enzyme kievitone hydratase that can efficiently catabolize kievitone into a less toxic product (Li et al. 1995).

In addition to degradation, pathogens also biotransform phytoalexin molecules. The cruciferous phytoalexin rapalexin A undergoes the addition of a thiol group by *Colletotrichum higginsianum* and *Colletotrichum dematium* (Pedras and Thapa 2020) . Notably, this is the same metabolic transformation that is catalyzed by insects and mammals (Pedras and Thapa 2020). Camalexin can be bio-transformed by *Rhizoctonia solani* Kuhn to 5-hydroxycamalexin which is further hydroxylated into more polar metabolites that are less toxic (Pedras and Khan 2000). *P. sojae* was shown to lack the ability to biotransform glyceollins, as some other microbes could, which may be the reason why glyceollins are toxic to that pathogen (Lygin et al. 2013). *L. maculans* can biotransform a number of derivatives of camalexin, brassinin, and other phytoalexins, but it could not metabolize specifically camalexin and rapalexin, which were toxic to the pathogen. Similarly, camalexin could not be detoxified by *A. brassicicola* (Pedras and Abdoli 2017). Thus, introducing new phytoalexins into plants could help broaden their pathogen resistance.

The successful biotransformation of benzoxazolinone by *Fusarium pseudograminearum* correlates with infection in wheat (Kettle et al. 2015). In maize,

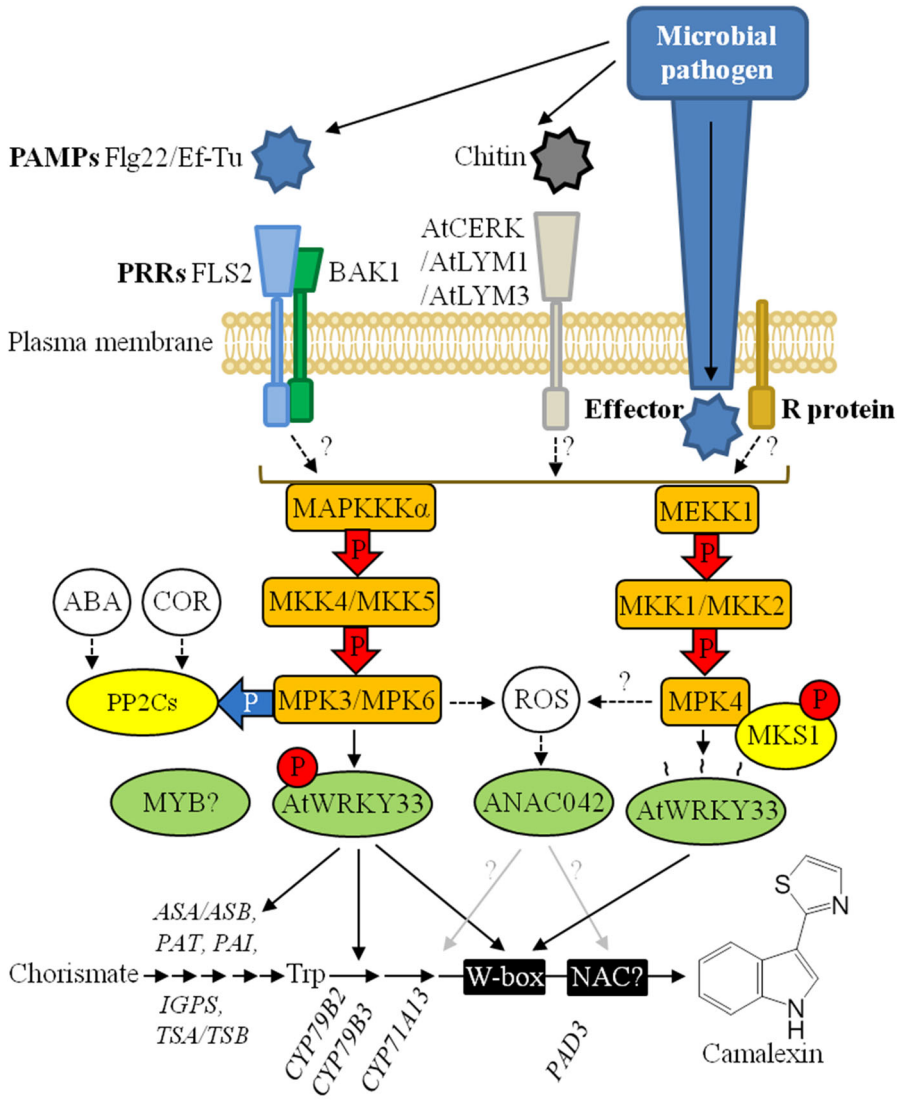

Fig. 3 Phytoalexin kinase signaling networks in Arabidopsis. Signaling cascades that elicit camalexin biosynthesis begin with the recognition of pathogen-associated molecular patterns (PAMPs) and effectors by plant pattern recognition receptors (PRRs) and resistance (R) proteins, respectively. These initiate PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI), respectively. Pathogens deliver effectors into the plant cell to suppress plant immunity, some plant genotypes encode the corresponding resistance (R) protein that recognize effectors, providing race-specific resistance. Among the earliest signaling events of PTI and ETI is mitogen-activated protein kinase (MAPK) cascades. MAPKs stimulate the activation of transcription of camalexin biosynthesis genes through the phosphorylation of WKRY33, complexed proteins (e.g. MKS1), and potentially other transcription factors (TFs). A strategy to enhance phytoalexin biosynthesis in plants includes overexpressing TFs, or phospho-mimicking mutants of TFs, to circumvent the MAPK cascade to partially (prime) or fully activate a phytoalexin biosynthesis pathway. The MYB orthologs GmMYB29A2 and VvMYB14 have conserved roles in activating glyceollin and stilbene biosynthesis pathways in soybean and grapevine, respectively. Red arrows indicate phosphorylation steps, blue arrow dephosphorylation and dotted arrows indicate multiple steps. Question marks indicate unidentified signaling components. This figure was adapted from Meng and Zhang (2013). (Color figure online)

the biotransformation of benzoxazolinone by *Fusarium verticillioides* is facilitated by an enzyme called *N*-malonyltransferase, encoded by the gene *FUSARIUM DETOXIFICATION OF BENZOXAZOLINONE 2* (*FDB2*) (Kettle et al. 2015). Exogenous application of benzoxazolinone induced the

expression of a nine gene cluster in *F. pseudograminearum.* Transgenic approaches including knocking out *FDB2* and gene complementation of mutants with homologous *FDB2* genes from *F. verticillioides* and *F. graminearum* showed that *N*-malonyltransferase is essential for benzoxazolinone detoxification (Kettle et al. 2015). The *N*-malonyltransferase enzyme from *F. verticillioides* also transforms 6-methoxy-benzoxazolin-2-one (MBOA) and benzoxazolin-2-one (BOA) (Glenn and Bacon 2009). These results raise the possibility that at least some pathogens have evolved promiscuous enzymes for the detoxification of *classes* of phytoalexins. *Sclerotinia sclerotiorum* (Lib.) encodes an inducible *N*-glucosyltransferase that converts brassinin to 1-b-D-glucopyranosylbrassinin (Pedras et al. 2003). Similarly, virulent isolates of *Leptosphaeria maculans* transform brassinin into 3-indolecarboxaldehyde, however the enzyme remains unidentified (Pedras et al. 2003).

Interestingly, some phytoalexins can induce the detoxification of others. Camalexin induced BRASSININ GLUCOSYLTRANSFERASE (BGT) expression in *S. sclerotiorum* which then glucosylated brassinin and possibly camalexin (Pedras and Ahiahonu 2002). Similarly, the rate of brassinin detoxification in *L. maculans* cultures increased significantly with the addition of camalexin, whereas spirobrassinin remained unaffected (Pedras and Ahiahonu 2005). Since the presence of one phytoalexin may induce the detoxification of another structurally similar molecule, engineering plants to biosynthesize a diversified repertoire of phytoalexin molecules could come at a cost in some instances.

Molecular mechanisms of plant cells that limit phytoalexin accumulation

In addition to signaling pathways that stimulate phytoalexin biosynthesis, there are also mechanisms in plant cells that limit the accumulation of those molecules. These include the inhibition of biosynthesis, metabolite sensing, conversion, and degradation. Phytoalexins are biosynthesized in relatively low amounts and only transiently upon elicitation. It remains unclear whether this is to limit any potential self-toxicity or to ensure sufficient cellular energy for other processes of the defense response program. In soybean, glyceollin degradation is constitutive. Supplying non-elicited seed tissues with glyceollins resulted in their rapid degradation (Farrell et al. 2017). Further, co-treatment with the heavy metal elicitor silver nitrate slowed the rate of glyceollin degradation and stimulated the hydrolysis of 6″-*O*-malonyldaidzin to daidzein, which is a biosynthetic intermediate of glyceollins. The putative enzyme or process responsible for catabolizing glyceollins remains unknown. Interestingly, the paralogue of the glyceollin activator *GmMYB29A2*, namely *GmMYB29A1*, reduced glyceollin metabolite accumulation when overexpressed in WGE-elicited soybean roots without affecting the transcript levels of most glyceollin biosynthesis genes (Jahan et al. 2020). Previous pulse-chase experiments using biosynthetic intermediates demonstrated that elicitation by *P. sojae* enhanced not only glyceollin I biosynthesis but also its turnover (Bhattacharyya and Ward 1987). Thus, knocking out the enzyme or molecular process regulated by GmMYB29A1 could be effectve strategies for enhancing glyceollin accumulation.

A TF has been identified that limits phytoalexin biosynthesis. Both stable and transient overexpression of *VvWRKY8* in grapevine reduced expression levels of *VvMYB14*, the *STS* genes *VvSTS15/21*, and resveratrol metabolite levels (Jiang et al. 2018). VvWRKY8 was found to not bind the promoters of *VvMYB14* and *VvSTS15/21* by yeast one-hybrid assays. Yet, by using yeast two hybrid (Y2H), VvWRKY8 was shown to physically interact with the *STS* regulator VvMYB14 through their N-terminal domains. This interaction putatively blocks VvMYB14 from binding the *VvSTS15/21* promoters to prevent the activation of resveratrol biosynthesis (Jiang et al. 2018). In contrast, the WRKY TF AsWRKY44 from the agarwood tree (*Aquilaria sinensis*) is released from the promoter of the *AGARWOOD SESQUITERPENE SYNTHASE 1* (*ASS1*) gene in response to wounding or JA treatment (Sun et al. 2020), demonstrating a second type of inhibition mechanism for phytoalexin biosynthesis genes.

In addition to the degradation of phytoalexin molecules and the inhibition of their biosynthesis, metabolite sensing and subsequent signaling mechanisms have been shown to limit phytoalexin biosynthesis. The membrane associated signaling protein PHOSPHOLIPASE A2 (PLA2) is involved in initiating benzophenanthridin and monoterpene indole alkaloid phytoalexin biosynthesis in *Eschscholzia*

californica and *Catharanthus roseus*, respectively (Heinze et al. 2015). However, each alkaloid inhibited the activity of their respective PLA2 only. 3D homology modeling predicted that the binding sites of each PLA2 accommodated specifically the host alkaloid. It remains to be determined whether the binding pockets can be removed or whether introducing a PLA2 gene from one plant species into another can effectively remove this feedback inhibition mechanism.

ABA is a negative regulator of phytoalexins

The effects of plant hormones on the elicitation of phytoalexins are briefly described in the section entitled *Signaling pathways for the elicitation of phytoalexin biosynthesis* and have been reviewed in detail elsewhere (Erb et al. 2012; Mauch-Mani and Mauch 2005). However, abscisic acid (ABA) may warrant additional coverage due to relatively recent advances in understanding its role as a potent negative regulator of phytoalexin biosynthesis. ABA is a plant hormone that is well known for its prominent role in signaling particular abiotic stresses, but comparatively little is known about its role in pathogen signaling (Mauch-Mani and Mauch 2005). Early studies led to the speculation that ABA may inhibit phytoalexin responses since abiotic stresses that stimulate ABA biosynthesis strongly correlated with enhanced susceptibility. For example drought stress in Arabidopsis and cold stress in rice resulted in compatibility with *P. syringae* (Mohr and Cahill 2003) and *Magnaporthe grisea* (Koga et al. 2004), respectively. Similarly, ABA signaling was shown to suppress the biosynthesis kievitone in common bean (Goossens and Vendrig 1982), and the rishitin in potato, causing compatibility with *Phytophthora infestans* (Henfling et al. 1980). More recently, ABA biosynthetic mutants exhibited enhanced resistance to bacterial and fungal pathogens (Asselbergh et al. 2007; de Torres-Zabala et al. 2009), whereas ABA biosynthesis gene overxpressors had enhanced susceptibility (Fan et al. 2009). Further, effectors secreted by *P. syringae* stimulated the expression of ABA biosynthesis and signaling genes, rendering Arabidopsis compatible (de Torres-Zabala et al. 2007).

ABA signaling during pathogen interactions was often found to be antagonistic to ethylene, salicylic acid, and/or jasmonic acid pathways that induce plant defense gene expressions. This has been reported for tomato (Audenaert et al. 2002; Thaler and Bostock 2004), Arabidopsis (de Torres-Zabala et al. 2007, 2009; Anderson et al. 2004; Hillwig et al. 2016; Mohr and Cahill 2003; Kerchev et al. 2013), rice (Xu et al. 2013a; Jiang et al. 2010; Nahar et al. 2012) and tobacco (Kusajima et al. 2010). Transient increases in ABA were observed to accumulate at infection sites of compatible, but not incompatible interactions, in potato, sugar beet, and soybean (Cahill and Ward 1989; Henfling et al. 1980; Schmidt et al. 2008). In contrast, ABA-deficient mutants of tobacco exhibited increases in the levels of capsidiol compared to wild-type plants when elicited with *B. cinerea* (Mialoundama et al. 2009). Also, expressions of the ABA degradation gene *ABA 8'-HYDROXYLASE* increased with capsidiol biosynthesis, suggesting that ABA degradation was a component of the capsidiol elicitation mechanism (Mialoundama et al. 2009).

During the interaction of incompatible soybean variety Harosoy 63 with race 1 *P. sojae*, ABA concentrations were rapidly reduced beginning 4 h after inoculation, whereas ABA concentrations were transiently increased at this time during a compatible interaction (Cahill and Ward 1989). Treatment with the ABA biosynthesis inhibitor norflurazon rendered the compatible genotype Harosoy incompatible and conversely, treatment of resistant genotype Harosoy 63 with ABA rendered it susceptible (Mohr and Cahill 2001; Ward et al. 1989; McDonald and Cahill 1999). ABA treatment was found to inhibit the expression of *PHENYLALANINE AMMONIA-LYASE* (*PAL*) gene that typically occurred early during an incompatible interaction (Ward et al. 1989). PAL isogenes are required for glyceollin and cell wall lignin biosynthesis. It was Mohr and Cahill that discovered that treating soybean with ABA affected specifically glyceollin biosynthesis and had no effect on lignin biosynthesis or HR during compatible interactions (Mohr and Cahill 2001).

Despite that ABA is an important negative regulator of phytoalexin biosynthesis in a broad range of plant species, its mechanisms of inhibiting phytoalexin signaling remain unclear. A recent study demonstrated that ABA induces the expression of the protein phosphatases 2Cs (PP2Cs), HAI1, HAI2, and HAI3, which directly dephosphorylate MPK3 and MPK6 (Mine et al. 2017). As described in the section

entitled *Kinase signaling of phytoalexin biosynthesis*, the phosphorylation of the TF WRKY33 by MPK3/MPK6 is required for WRKY33 to fully activate camalexin biosynthesis (Mao et al. 2011). Mine and colleagues also demonstrated that *P. syringae* induces the expression of *HAI1* through a cornatine (COR)-mediated pathway to promote compatibility with *P. syringae* (Mine et al. 2017). Thus, PP2C-mediated dephosphorylation of MPK3/MPK6 may be a mechanism by which ABA signaling is used by plant and exploited by pathogens to reduce phytoalexin biosynthesis (Fig. 3).

Conclusions

Phytoalexins have important roles in mediating the protection of crops against economically devastating pathogens in agriculture. Further, with increased accessibility, their potent medicinal activities could render them important pharmaceuticals for the treatment and prevention of debilitating diseases. However, more research is required to assess the bioactivities of phytoalexins and understand how they can be administered or chemically modified for particular clinical treatments. Some phytoalexins are not economical to synthesize using chemistry methods, making their biosynthesis in plants a major source for improving their accessibility. This has led to diverse efforts in understanding how to enhance their biosynthesis in plants. Recent studies have provided clues as to the existence of a conserved TF network that directly regulates diverse phytoalexin biosynthesis pathways in different plant species. Bioengineering this TF network could be the key to 'unlocking' plant metabolism to biosynthesize massive amounts of phytoalexin molecules. Recent evidence suggests that it could also be manipulated to engineer resistance to pathogens. A systems level re-engineering of plant cells, including upregulating key TFs, removing amino acid residues from feedback signaling proteins, and knocking out negative regulators could be achieved using recently discovered efficient plant transformation methods. A systems level understanding of phytoalexin gene regulation should be a major goal of modern plant science since it could lead to economical sources of pharmaceuticals and improved food security.

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