SMALL MOLECULES: FROM STRUCTURAL DIVERSITY TO SIGNALLING AND REGULATORY ROLES

Biosynthesis, elicitation and roles of monocot terpenoid phytoalexins

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Received 2 November 2013; revised 22 December 2013; accepted 10 January 2014; published online 22 January 2014.
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SUMMARY

A long-standing goal in plant research is to optimize the protective function of biochemical agents that impede pest and pathogen attack. Nearly 40 years ago, pathogen-inducible diterpenoid production was described in rice, and these compounds were shown to function as antimicrobial phytoalexins. Using rice and maize as examples, we discuss recent advances in the discovery, biosynthesis, elicitation and functional characterization of monocot terpenoid phytoalexins. The recent expansion of known terpenoid phytoalexins now includes not only the labdane-related diterpenoid superfamily but also casbane-type diterpenoids and β-macrocarpene-derived sequiterpenoids. Biochemical approaches have been used to pair pathway precursors and end products with cognate biosynthetic genes. The number of predicted terpenoid phytoalexins is expanding through advances in cereal genome annotation and terpene synthase characterization that likewise enable discoveries outside the Poaceae. At the cellular level, conclusive evidence now exists for multiple plant receptors of fungal-derived chitin elicitors, phosphorylation of membrane-associated signaling complexes, activation of mitogen-activated protein kinase, involvement of phytohormone signals, and the existence of transcription factors that mediate the expression of phytoalexin biosynthetic genes and subsequent accumulation of pathway end products. Elicited production of terpenoid phytoalexins exhibit additional biological functions, including root exudate-mediated allelopathy and insect antifeedant activity. Such findings have encouraged consideration of additional interactions that blur traditionally discrete phytoalexin classifications. The establishment of mutant collections and increasing ease of genetic transformation assists critical examination of further biological roles. Future research directions include examination of terpenoid phytoalexin precursors and end products as potential signals mediating plant physiological processes.

Keywords: momilactone, phytocassane, oryzalexin, kauralexin, zealexin, herbivory, Oryza sativa, Zea mays.

INTRODUCTION

Three monocotyledonous crop plants, namely rice (Oryza sativa), maize (Zea mays) and wheat (Triticum spp.), collectively contribute almost 60% of the world’s food supply (Tilman et al., 2002). Over 2.3 billion metric tons of these dominant harvested grains are produced annually (http://faostat.fao.org/site/339/default.aspx). Not surprisingly, they have been more closely examined at the molecular and biochemical levels than the other 69 000 known species of monocots (Joppa et al., 2011). This research focus is driven by a practical interest in pre-harvest and post-harvest resistance mechanisms that protect against losses caused by microorganisms, insects and other biotic threats. A broad class of biochemical defense compounds, termed phytoalexins, are loosely defined as any ‘low molecular weight, anti-microbial compounds that are both synthesized and accumulated in plants after exposure to micro-organisms or
abiotic agents’ (VanEtten et al., 1994). Pathogen-inducible phytoalexins have been examined for over 70 years, yet many aspects of their occurrence, synthesis and function remain poorly described (Hammerschmidt, 1999).

This review focuses on the biochemistry, genetics, synthesis, elicitation and potential signaling roles of monocot terpenoid phytoalexins. Phytoalexins in other model systems have been reviewed elsewhere recently (Ahuja et al., 2012). Of all the plant biochemicals, terpenoids are the largest and most diverse class, with over 25,000 known structures (Gershenzon and Dudareva, 2007). In dicot models, terpenoid phytoalexins exist in a variety of phylogenetically scattered species, including tobacco (Nicotiana tabacum), cotton (Gossypium hirsutum), sweet potato (Ipomoea batatas) and elm trees (Ulmus americana) (Harborne, 1999). Despite the impressive diversity of monocots, ranging from palms, orchids, gingers and lilies to onions and grasses, to date only the Poaceous genera Oryza and Zea have been unequivocally demonstrated to contain terpenoid phytoalexins. Harborne (1999) noted a potential reason for this deficiency by stating ‘One of the problems in developing phytoalexin research in monocotyledonous plants is the difficulty of inoculating leaf tissue and obtaining a necrotic reaction’. Researchers attempting to generate large amounts of plant material enriched in phytoalexins will appreciate this difficulty.

**DISCOVERY AND BIOSYNTHESIS OF RICE DITERPENOID PHYTOALEXINS**

As a staple food crop, rice has been extensively investigated for disease resistance mechanisms. Over 50 years ago, production of phytoalexins was observed in rice upon exposure to Magneportha oryzae, the causative agent of fungal blast (Uehara, 1958). Currently identified rice diterpenoid phytoalexin families include momilactones A and B (Cartwright et al., 1977, 1981), oryzalexins A–F (Akatsuka et al., 1983, 1985; Kono et al., 1984, 1985; Sekido et al., 1986; Kato et al., 1993, 1994), oryzalexin S (Kodama et al., 1992), phytocassanes A–E (Koga et al., 1995, 1997; Yajima and Mori, 2000) and ent-10-oxodepressin (Inoue et al., 2013). The above diterpenoids were isolated based on their elicited accumulation and antibiotic activity against M. oryzae (Figure 1). Rice also constitutively produces additional diterpenoids, including oryzalides, oryzalic acids and oryzadiones, termed oryzalide-related compounds, which exhibit activity against the bacterial leaf blight Xanthomonas oryzae pv. oryzae (Xoo) (Watanabe et al., 1990, 1992; Kono et al., 1991, 2004). In contrast to typical phytoalexins, accumulation of oryzalide-related compounds is only moderately induced by Xoo infection, and thus resemble pre-formed phytoanticipans (VanEtten et al., 1994; Watanabe et al., 1996).

**Production of the diterpenoid precursor (E,E,E)-geranylgeranyl diphosphate**

In plants, the isoprenoid precursors isopentenyl diphosphate and dimethylallyl diphosphate may be derived from either the mevalonate- or methylerythritol phosphate-dependent pathways. Production of (E,E,E)-geranylgeranyl diphosphate in rice is likely to occur in plastids through the methylerythritol phosphate pathway, whereas sesquiterpenes and triterpenes are generally synthesized in the cytoplasm via the mevalonate pathway (Vranova et al., 2013). Diterpenoid phytoalexins are expected to arise from (E,E,E)-geranylgeranyl diphosphate via the methylerythritol phosphate pathway, with subsequent steps predicted to occur in plastids based on targeting of N-terminal transit
peptide-like sequences present in the diterpene cyclases (Cho et al., 2004; Nemoto et al., 2004; Otomo et al., 2004a, b; Prisic et al., 2004; Xu et al., 2004). The synchronous accumulation of seven mevalonolactone diphosphate pathway gene transcripts (OsDXS3, OsDXR, OsCMS, OsCMK, OsMCS, OsHDS and OsHDR) in elicitor-induced rice cells further supports involvement of the mevalonolactone diphosphate pathway. Moreover, elicitor-induced accumulation of the diterpenoid phytoalexins is suppressed by treatment with fosmidomycin and 5-ketoclamazone, chemical inhibitors of 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) activity, but not by mevastatin, an inhibitor of the mevalonate pathway (Okada et al., 2007).

**Cyclization to the olefin precursors**

Almost all rice diterpenoid phytoalexins are members of the labdane-related superfamily, which includes the gibberellins (GAs) (Peters, 2010). This superfamily is distinguished by the initial bi-cyclization of (E,E,E)-geranylgeranyl diphosphate to copalyl diphosphate (CDP), as catalyzed by class II diterpene cyclases termed CDP synthases (CPS). CDP is also the required substrate for class I diterpene synthases, termed kaurene synthase-like (KSL) based on their close phylogenetic relationship to the ent-kaurene synthases (KSs) involved in GA biosynthesis. Sequential activity CPS and KSL produces the olefin precursors of the major families of diterpenoid phytoalexins. Early work with cell-free extracts demonstrated the production of numerous expected diterpene olefins (Wickham and West, 1992). KS (L) enzymes act on either stereochemically differentiated syn-CDP as the intermediate in formation of oryzalexones and oryzalexin S, or the ent-CDP stereoisomer, which is required for biosynthesis of GAs, oryzalexins A–F, phytocassanes and oryzalide-related diterpenoids (Figure 2) (Mohan et al., 1996; Peters, 2006; Toyomasu, 2008).

Many rice CPS and KSL sequences were first identified in a comprehensive study of the enzymatic genes involved in GA metabolism (Sakamoto et al., 2004). In parallel, three groups have worked to further identify and characterize CPSs and KSLs through multiple approaches. Using homology-based PCR with elicitor-induced suspension cells, cDNAs were cloned that encoded OsKSL7, which acted on ent-CDP to produce the phytocassane precursor ent-cassadiol, 12,15-diene, and OsKSL8, which acted on syn-CDP to yield the oryzalexin S precursor syn-stemar-13-ene (Cho et al., 2004; Nemoto et al., 2004). Via a similar approach with UV-irradiated rice leaves, cDNAs were obtained that encoded OsCPS2 and OsCPS4, producing ent-CDP and syn-CDP, respectively (Otomo et al., 2004b). To define the subsequent olefins, two additional KSLs were characterized. OsKSL4 was found to act on syn-CDP to produce the momilactone precursor syn-pimara-7,15-diene, while OsKSL10 transformed ent-CDP to the oryzalexin precursor ent-sandaracopimara-7,15-diene (Otomo et al., 2004a). OsCPS4 and OsKSL4 are in close genomic proximity on chromosome 4, and demonstrate sequential activity, yielding syn-CDP and syn-pimara-7,15-diene (Sakamoto et al., 2004; Wilderman et al., 2004). Both ent-CDP-producing OsCPS1, required for GA metabolism (Sakamoto et al., 2004), and OsCPS2, which is dedicated to production of diterpenoid phytoalexins (Prisic et al., 2004), have also been characterized. The close genetic proximity of OsCPS2 and OsKSL7 confirmed the existence of an additional gene cluster on chromosome 2 that also includes OsKSL5 and OsKSL6 (Otomo et al., 2004a; Kanno et al., 2006).

Utilizing a homology-based PCR approach, OsKSL5 was shown to produce ent-pimara-8(14),15-diene, while OsKSL6 yielded ent-kaur-15-ene, the predicted precursor to oryzalide-related diterpenoids (Kanno et al., 2006). Consistent with the largely constitutive biosynthesis of oryzalides, OsKSL6 transcript accumulation is not elicited by UV damage. Intriguingly, investigation of an indica rice sub-species demonstrated that both OsKSL5 and OsKSL6 produce ent-kaur-15-ene, suggesting continuing evolution of these synthases (Xu et al., 2007). An unexpected additional KSL, OsKSL11, was found to be closely related to OsKSL8 and also reacts with syn-CDP, but instead produces syn-stemod-13(17)-ene (Morrone et al., 2006). Further biochemical analysis has demonstrated that OsKSL10 not only reacts with ent-CDP, but also with syn-CDP, to produce syn-labda-8(14),15-diene, which is observed in planta (Morrone et al., 2011).

Characterization of each rice CPS and KSL family member has revealed the presence of not only the enzymes responsible for the expected olefin precursors but also additional unexpected activity resulting in diterpene olefins whose ultimate fate remains unclear (Figure 2). Based on these studies, there does not appear to be any redundancy in either enzymatic family. Although two CPSs produce ent-CDP, OsCPS1 is uniquely required for GA metabolism, while the inducible expression pattern of OsCPS2 is consistent with a specific role in phytoalexin biosynthesis. OsKSL transcripts involved in phytoalexin biosynthesis are similarly inducible, while those suspected to function in phytoanticipin biosynthesis are transcriptionally stable.

**Synthesis of bioactive phytoalexins**

Early work with cell-free extracts suggested the involvement of cytochrome P450s (CYPs) in the addition of oxygen required for production of diterpenoid phytoalexins. For example, ent-sandaracopimaradiene-3β,15β-ol is converted into oryzalexins D and E by a microsome fraction from UV-irradiated rice leaves in the presence of O2 and NADPH, but this conversion is blocked by CYP-specific inhibitors (Kato et al., 1995). Similarly, momilactone and phytocassane accumulation in rice cells is also suppressed by P450 inhibitors (Shimura et al., 2007). By analogy to the CYP
oxidation of ent-kaurene in GA biosynthesis (Hedden and Thomas, 2012), it was anticipated that CYPs oxidize olefin precursors to yield diterpenoid phytoalexins (Peters, 2006). Additional enzymes required include short-chain alcohol dehydrogenases. Characterization of a candidate short-chain alcohol dehydrogenase demonstrated conversion of 3β-hydroxy-9βH-pimaradiene-19,6β-olide into momilactone A in vitro, and the enzyme was thus designated momilactone A synthase (OsMAS) (Atawong et al., 2002; Shimura et al., 2007).

In addition to chromosomal clustering of OsKSL4 and OsCPS4, additional neighboring genes include OsMAS,
OsCYP99A2 and OsCYP99A3 (Sakamoto et al., 2004), all of which were found to exhibit co-elicitation suggestive of related biological function (Shimura et al., 2007). The involvement of OsCYP99A2 and OsCYP99A3 in chitin elicitor-inducible production of phytoalexins was investigated by RNAi-mediated knockdown. High sequence identity resulted in co-suppression of both CYPs, with the cell lines displaying suppressed momilactone accumulation but unaltered phytocassane elicitation, demonstrating that OsCYP99A2/A3 mediate momilactone biosynthesis (Shimura et al., 2007). OsCYP99A3 specifically catalyzes a three-step oxidation of the C19 methyl of the momilactone precursor syn-pimara-7,15-diene to syn-pimara-7,15-dien-19-oic acid (Figure 3), which is found in planta (Wang et al., 2011). Additional enzymes are required to complete momilactone biosynthesis.

The production of ent-cassa-12,15-diene by sequential activity of OsCPS2 and OsKSL7 suggests a role in phytocassane production for at least some of the six CYP genes that cluster with these diterpene synthases (i.e. OsCYP76M5–8 and OsCYP71Z6–7 on chromosome 2). In support of this hypothesis, CYP transcript levels (OsCYP71Z7, OsCYP76M5, OsCYP76M7 and OsCYP76M8) are co-regulated with OsCPS2 and OsKSL7 by chitin elicitation (Okada et al., 2007). OsCYP76M7 catalyzes the hydroxylation of ent-cassa-12,15-diene at the C11α position, consistent with the presence of a further oxidized C11 keto in phytocasanes and the corresponding 11α-hydroxy-ent-cassa-12,15-diene in planta (Swaminathan et al., 2009). RNAI silencing of OsCYP76M8 resulted in co-suppression of OsCYP76M7 and reduced phytocassane production, but no consistent effects on momilactones were observed. OsCYP76M8 also catalyzes C11α-hydroxylation of ent-cassa-12,15-diene, together with the C6β-hydroxylation of syn-pimara-7,15-diene and C7β-hydroxylation of ent-sandaracopimaradiene. OsCYP76M6 and OsCYP76M5 additionally catalyze the latter reaction. Combined, these data indicate that OsCYP76M7 and/or OsCYP76M8 play a role in phytocassane biosynthesis (Wang et al., 2012a). The promiscuity of OsCYP76M8 suggests a potential function in momilactone and/or oryzalexin production; however, empirical evidence is lacking. The ent-cassa-12,15-diene precursor is also hydroxylated at the C2 position by OsCYP71Z7, although it exhibits modest affinity for this substrate (K_M = 200 μM), suggesting a possible function in the later stages of phytocassane biosynthesis (Wu et al., 2011). Consistent with this hypothesis, the production of C2-oxygenated phytocassanes is specifically suppressed in OsCYP71Z7 knockdown lines (Okada, 2011). In addition, OsCYP71Z6 catalyzes the C2 hydroxylation of ent-kaur-15-ene, which may be relevant for biosynthesis of oryzalide-related diterpenoids based on a lack of elicitation at the transcript level (Wu et al., 2011).

Sakamoto et al. (2004) also noted that rice contains multiple paralogs of the ent-kaurene oxidase (KO) CYP701A involved in GA biosynthesis, arranged as a five-gene tandem array on chromosome 6, although only one is required for GA metabolism. OsKO2 was determined to be the Tan-Ginbozu (D35) semi-dwarf gene, while the ent-kaurene oxidase-like (KOL) transcripts OsKOL4 and OsKOL5 accumulated in response to elicitation (Itoh et al., 2004). The proximity between the C19 targeted by KO, and the C3α position that is hydroxylated in almost all diterpenoid

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**Figure 3.** Roles of known rice CYPs in diterpenoid phytoalexin biosynthesis. Shown are the reactions for which biochemical and genetic data demonstrate a role for the indicated CYPs, including the specific regiochemical locations for the assigned reactions using accepted carbon (C2, 3, 7, 9, 11 and 19) numbering.
phytoalexins, led to speculation that OsKOLs catalyze similar hydroxylations (Peters, 2006). Recently, OsKOL4/ OsCYP701A8 was shown to efficiently react with ent-sandaracopimaradiene and ent-cassa-12,15-diene to produce the corresponding C3α-hydroxylated diterpenoids, consistent with oryzalexin and phytocassane biosynthesis (Wang et al., 2012b). Although activity was not found for OsKOL5/OsCYP701A9 using the tested substrates, it remains possible that this enzyme acts in a later stage of biosynthesis.

Bioactive rice diterpenoids are commonly elaborated by addition of at least two spatially separated hydroxyl groups. For example, orzyalexin D is simply 3α,7β-dihydroxylated ent-sandaracopimaradiene, while orzyalexin E is the 3α,9β-dihydroxy derivative. Notably, production of these phytoalexins appears to proceed via the initial C3α hydroxylation of ent-sandaracopimaradiene catalyzed by OsCYP701A8, while OsCYP76M6 and OsCYP76M8 catalyze subsequent hydroxylation at C9α or C7β, leading to production of oryzalexins E or D, respectively (Figure 3). These final biosynthetic steps represent the first complete pathways in the production of rice diterpenoid phytoalexins (Wu et al., 2013).

**DISCOVERY AND BIOSYNTHESIS OF TERPENOID PHYTOALEXINS IN MAIZE**

Shortly after the discovery of rice momilactones, Mellon and West (1979) demonstrated elicited diterpene accumulation in maize. After infection with Rhizopus stolonifer, *Aspergillus niger* or *Fusarium verticillioides*, cell-free extracts demonstrated increased incorporation of [14C]-labeled (E,E,E)-geranylgeranyl diphosphate and [1-3H2]-labeled CDP into ent-pimara-8(14),15-diene, ent-kaur-16-ene and ent-kaur-15-ene. Dominant radiolabeled polar fractions possessed anti-fungal activity against *Cladosporium cucumerinum*, but the three diterpene olefins did not (Mellon and West, 1979). Over 25 years later, Harris et al. (2005) cloned the maize ent-CPS, termed Anther Ear 2 (ZmAAn2), and confirmed its enzymatic function. Using a differential display technique to enrich for transcripts that accumulate after *Fusarium graminearum* infection, ZmAAn2 was identified as highly inducible in silk tissue (Harris et al., 2005). During an investigation of maize stem responses to herbivory by larvae of the European corn borer (*Ostrinia nubilalis*), a series of six acidic ent-kaurane-related diterpenoids, termed kaurexins, were discovered (Figures 1 and 2) (Schmelz et al., 2011). Stem transcript accumulation of ZmAAn2 and kaurexins was strongly induced by *O. nubilalis* larvae and Rhizopus microsporus inoculation (Dafoe et al., 2011; Schmelz et al., 2011). Kaurexins are hypothesized to be the predominant downstream products of ZmAAn2 (GRMZM2G044481), and this is now being examined using available transposable element (Ds) insertion mutants (http://www.plantgdb.org/prj/AgDSTagging/) (Vollbrecht et al., 2010).

Significant genetic and biochemical insights also preceded the discovery of maize sesquiterpenoid phytoalexins. Early evidence came from analyses of differentially expressed maize genes following infection with smut fungus (*Ustilago maydis*). One of the most highly elicited transcripts accumulating in leaves, termed *Umi2*, was identified as terpene synthase 6 (ZmTps6) (Basse, 2005; Koellner et al., 2008b). As part of a comprehensive analysis of terpene synthase genes and products, Koellner et al. (2008b) cloned the nearly identical ZmTps6 and ZmTps11 genes, and demonstrated that heterologously expressed proteins encoded by these genes synthesized (S)-β-macrocarpene and (S)-β-bisabolene in the presence of farnesyl-diphosphate. β-macrocarpene synthesis requires ZmTps6/11-mediated re-protonation of β-bisabolene, cyclization and de-protonation to yield the final bi-cyclic product. Leaf herbivory by *Spodoptera littoralis* larvae elicited accumulation of ZmTps8/11 transcripts, but did not result in emission of β-macrocarpene, which lead to the hypothesis that the end products were non-volatile (Koellner et al., 2008b). To examine their role in *U. maydis* resistance, ZmTps8/11 were co-suppressed using both stable RNAi gene silencing and virus-induced gene silencing, each of which promoted fungal growth (van der Linde et al., 2011). During an investigation of stem responses to *F. graminearum* infection, a series of acidic sesquiterpenoids based on the β-macrocarpene carbon skeleton were identified (Figures 1 and 2) and termed zealexins (Huffaker et al., 2011). Zealexins also accumulate upon exposure to the fungi *Aspergillus flavus* and *R. microsporus*, and long-term stem attack by *O. nubilalis* larvae (Huffaker et al., 2011). Using a series of fungi with various eliciting potentials, total zealexin concentrations at 24 h showed almost perfectly positive relationships with transcript levels of both ZmTps6 and ZmTps11, providing strong indirect evidence for the predicted biosynthetic pathway (Huffaker et al., 2011).

**EVIDENCE FOR DITERPENOID PHYTOALEXINS IN OTHER CEREAL CROPS**

In addition to rice and maize, wheat also contains expanded CPS and KSL gene families with stress-inducible transcription and predictably diverse biochemical functions that are likely to include production of phytoalexins (Toyomasu et al., 2009; Wu et al., 2012; Zhou et al., 2012). Phylogenetic analyses of all identified cereal CPS and KSL genes are consistent with early evolution of specialized diterpenoid metabolism in the small-grain cereals (Figure 4). Moreover, the *Brachypodium distachyon* genome (International Brachypodium Initiative, 2010) contains a KSL and CYP gene cluster with similarities to the rice chromosome 4 momilactone biosynthetic gene cluster. Similarly, the maize genome additionally contains CPS genes that are closely related to OsCPS4, with nearby CYP99A subfamily members. Although neither maize nor
**Brachypodium** contain the full gene complement present in the rice momilactone biosynthetic cluster, these patterns suggest that related gene assemblages arose early in the small-grain cereals and other members of the Bambusoideae/Ehrhartoideae/Pooideae (BEP) clade of the Poaceae family (Grass Phylogeny Working Group II, 2012).

**ACTIVATION OF BIOSYNTHESIS OF TERPENOID PHYTOALEXINS IN MONOCOTS**

Role of elicitors and microbe-associated molecular patterns

Of primary interest to the understanding of defense activation are elicitors inherent to microbes and herbivores, often termed microbe-associated molecular patterns (MAMPs) and herbivore-associated molecular patterns (Howe and Jander, 2008; Boller and Felix, 2009). Chitin, a linear polymer of N-acetylglucosamine (GlcNAc) consisting of \(\beta-(1-4)\) linkages, constitutes a significant percentage of both fungal cell walls and insect exuviae (Merzendorfer and Zimoch, 2003; Bowman and Free, 2006). Structural polysaccharides of fungal cell walls are further dominated by branched \(\beta-1,3\) glucans cross-linked with chitin. The generation of elicitor fragments is probably facilitated by the inducible accumulation of plant transcripts encoding chitinases and \(\beta-1,3\)-glucanases after pathogen and insect attack (Ji et al., 2000; Rodriguez et al., 2012). Using rice suspension cells, Yamada et al. (1993) demonstrated that...
N-acetyl-chitooligosaccharides [(GlcNac)ₙ where n = 6 or more degrees of polymerization], exhibit potent elicitation of momilactone A accumulation over a concentration range of 1–1000 ng ml⁻¹. In contrast, deactylated chitin (β-1,4-linked glucosamine), termed chitosan, and shorter chain N-acetyl-chitooligosaccharides exhibited little or no activity (Yamada et al., 1993). However, high concentrations of chitosan (0.1%) have been found to elicit momilactone A production in rice leaves (Agrawal et al., 2002).

Biochemical investigation of soluble polysaccharides derived from degraded M. oryzae cell walls also yielded a potent β-(1,3/1,6)-derived glucan, termed tetraglucosyl glucitol (Yamaguchi et al., 2000). Tetraglucosyl glucitol elicits momilactone A production in rice cells but is inactive at triggering phytoalexin accumulation in soybean (Glycine max). Conversely, hexa-(1,3/1,6)-β-glucoside, which has established activity in soybean, did not elicit diterpenoid phytoalexin production in rice. The species-specific activity of β-glucans is consistent with the existence of parallel yet separate perception pathways for fungal cell-wall elicitors (Yamaguchi et al., 2000, 2002). To avoid detection, the fungal pathogen Colletotrichum graminicola exhibits reduced β-1,3-glucan synthase (GLS1) transcription and glucan production during biotrophic growth in maize (Oliveira-Garcia and Deising, 2013). Consistent with a role in elicitation, transgenic over-expression of GLS1 in C. graminicola biotrophic hyphae results in activation of maize defenses and reduced pathogen spread. In this modifed interaction, inducible genes including ZmTps2, ZmTps3, ZmTps7 and ZmTps10 were hypothesized to play a role in production of unidentified diterpenoid phytoalexins. Similar to rice, evidence in maize strongly supports a role for β-glucan elicitors and perception systems in regulating immunity.

Additional MAMPs in rice include glycosphingolipid derivatives originating from the plasma membrane of M. oryzae. Specifically, cerebrosides A, B and C trigger enhanced cell death together with production of phytoalexins A-D and momilactones. Cerebroside elicitation promotes increases in β-glucanase-, chitinase- and peroxidase-encoding transcripts when applied at concentrations ranging from 10 to 100 µg ml⁻¹ (Koga et al., 1998; Umemura et al., 2000). Structurally, cerebrosides consist of d-glucose, a sphingoid base and either a C16 or C18 fatty acid. The presence of a C9 methyl group and 4E double bond on the sphingoid base are essential features for biological activity (Koga et al., 1998).

Lytic enzymes common in pathogenic fungi include endo-β-1,4-xylanases that degrade hemicellulose by cleaving xylose from β-1,4-xylan-rich polysaccharides (Dean et al., 1989; Bailey et al., 1990). Elicitor activity present in a Tricoderma viride-derived xylanase, termed TvX, has been demonstrated to be independent of enzymatic action and to require specific plant receptors (Furman-Matarasso et al., 1999; Ron and Avni, 2004). In rice cells, 50–600 µg ml⁻¹ of TvX elicits increased cytosolic Ca²⁺ within minutes and production of phytocassanes A-E and momilactones 24 h later (Kurusu et al., 2010; Hamada et al., 2012). Acting indirectly, fungal-derived pectinases (i.e. polygalacturonases) degrade polygalacturonan, weaken the pectin network of plant cell walls, and release oligogalacturonides that function as elicitors (Bruce and West, 1982; Davis et al., 1986). In maize, treatment of wounded stems with a Rhizopus-derived polygalacturonase elicits significant kauralexin accumulation within 24 h (Schmelz et al., 2011).

In contrast to fungi, Gram-negative bacteria are covered with complex glycolipids termed lipopolysaccharides (King et al., 2009). Treatment of rice cells with 100 µg ml⁻¹ lipo-polysaccharides from Pseudomonas aeruginosa elicits increased cell death and transcript accumulation of a β-1,3-glucanase, a class 1 chitinase and OsDTC2/OsKSL8 (Desaki et al., 2006; Nemoto et al., 2004). Although transcriptional activation of OsKSL8 was demonstrated, the relationship between lipopolysaccharide elicitation and oryzalexin S accumulation remains to be determined (Desaki et al., 2006; Erbs and Newman, 2012). Gram-negative bacterial pathogens, including Erwinia amylovora and Xoo, also have the capacity to produce acidic 44 kDa protein elicitors, termed harpins (Wei et al., 1992; Li et al., 2012). In rice, transgenic expression of the Xoo harpin-encoding gene hrf1 increases Xoo resistance and promotes the accumulation of transcripts encoding diterpenoid phytoalexin biosynthetic enzymes and end products (Li et al., 2012). Pseudomonas syringae pv. tomato DC3000 and P. syringae pv. oryzae also harbor biosynthetic genes for elicitors such as the polyketide phytotoxin coronatine (Baltrus et al., 2011; Xin and He, 2013). As a conjugate of coronamic acid and coronafacic acid, coronatine is a structural mimic of jasmonic acid (JA) conjugated with isoleucine (JA-Ile), which binds the jasmonate receptor COI1 (Coronatine Insensitive 1) (Yan et al., 2009). Application of 100 µm droplets of coronatine to wounded rice leaves triggers increased accumulation of momilactone A (Tamogami and Kodama, 2000). Although the role of coronatine production by Pseudomonas spp. in rice remains to be examined, the elicitation activity is consistent with an interactive role for jasmonate signals in terpenoid phytoalexin production (Huffaker et al., 2011; Schmelz et al., 2011; Riemann et al., 2013; Shimizu et al., 2013).

Biotic elicitors of phytoalexins also include mammalian products such as cholic acid, a triterpenoid bile acid (Koga et al., 2006). Over a concentration range of 20–200 µM, cholic acid significantly triggered the production of phytoalexins and cell death in rice leaf discs (Koga et al., 2006). Similarly, leaf and root applications of cholic acid suppressed M. oryzae lesion development. In contrast to most elicitors, cholic acid specifically triggered the accumulation
of phytocassanes but not momilactones, suggesting specificity in pathway regulation (Koga et al., 2006). Numerous additional biochemicals and herbicides that promote cell death have been shown to elicit production of rice terpenoid phytoalexins, including 7 mM methionine, 0.5 mM cantharidin, 1 mM pretioclar and butachlor (Tamogami et al., 1995; Nakazato et al., 2000; Rakwal et al., 2001). Not surprisingly, many stress factors and inputs have been shown to influence phytoalexin production (Ahuja et al., 2012).

Endogenous signaling mediates phytoalexin biosynthesis

N-acetylglucosamisaccharide elicitation in rice requires two types of lysin motif-containing plasma membrane glycoproteins, namely OsCEBiP (chitin elicitor binding protein), which lacks functional intracellular signaling domains, and OsCERK1 (chitin elicitor receptor kinase) (Figure 5) (Kaku et al., 2006; Shimizu et al., 2010). OsCEBiP was biochemically isolated based on its affinity for (GlcNAc)_8, and RNAi lines lacking detectable OsCEBiP protein exhibited an 85% reduction in elicited reactive oxygen species (ROS) (Kaku et al., 2006). RNAi silencing of OsCEBiP also resulted in a significantly greater number of M. oryzae appressoria achieving multicellular invasions (Kishimoto et al., 2010). OsCERK1 is part of a larger ‘defensome’ complex at the plasma membrane that includes OsHsp90 (heat shock protein 90), OsHsp70, OsHop/Sti1 (Hsp70/Hsp90 organizing protein/stress-induced protein 1), OsSGT1 (suppressor of G/two allele of Skp1) and OsRAR1 (required for Mla12 resistance) as molecular chaperone proteins and co-chaperone-like proteins (Shirasu and Schulze-Lefert, 2003; Thao et al., 2007; Seo et al., 2008; Chen et al., 2010).

Figure 5. Simplified model of signal transduction mediating monocot terpenoid phytoalexin biosynthesis. Solid arrows represent established relationships, while dotted arrows indicate gaps in knowledge. Chitin fragments [(GlcNAc)_8] bind the plasma membrane receptor OsCEBiP, which complexes with OsCERK1, associated defensome complex proteins (OsHsp90, OsHop and OsRacGEF1) and the small GTPase OsRac1 (Akamatsu et al., 2013). OsRacGEF1 is phosphorylated (P) by OsCERK1, and promotes activation of OsRac1 and OsRboh (Wong et al., 2007). Constitutive expression of OsRac1 promotes momilactone A accumulation (Ono et al., 2001). MAPK cascades involving OsMKK4 and OsMPK3/6 amplify signal transduction following chitin elicitation (Kishi-Kaboshi et al., 2010). The transcription factors OsTGAP1 and OsWRKY76 have positive and negative regulatory roles, respectively (Okada et al., 2009; Yokotani et al., 2013). TVX elicitation is mediated in part by the voltage-gated cation channel OsTPC1, increases in cytosolic Ca^2+; potential binding by calcineurin B-like (CBL) proteins and activation of OsCIPK14/15 (Kurusu et al., 2010; Hamada et al., 2012). Jasmonate-mediated production of phytoalexins is dependent upon OsAOC and OsJAR1 but independent pathways also exist (Riemann et al., 2013; Shimizu et al., 2013). Ethylene production by OsACS2 increases resistance to M. oryzae and interacts with phytoalexins remain unknown (Helliwell et al., 2013). β-glucans exist as potent elicitors in rice and probably maize (Yamaguchi et al., 2000; Oliveira-Garcia and Deising, 2013). Attack by fungi and O. nubilalis initiates accumulation of kauralexin and zealexin in maize, as does the synergistic activity of JA and ET in developing node tissues (Huffaker et al., 2011; Schmelz et al., 2011).
key defensesome components are the small plant-specific Rho-type GTPase OsRac1 and its associated guanine nucleotide exchange factor OsRacGEF1, which are activated within minutes of chitin perception (Figure 5) (Chen et al., 2010; Akamatsu et al., 2013). OsRac1 has been shown to directly interact with OsRbohB (respiratory burst oxidase homolog B) in a Ca2+-dependent manner (Wong et al., 2007). Transient co-expression of OsRbohB and OsRac1 in Nicotiana benthamiana induced production of ROS as indicated by 3,3′-diaminobenzidine staining (Wong et al., 2007). In rice, constitutive expression of OsRac1 leads to increased H2O2 production, cell death, OsCPS2 transcript accumulation, increased momilactone A levels and improved resistance to M. oryzae (Ono et al., 2001).

As predicted downstream signals of OsRac1, mitogen-activated protein kinase (MAPK) cascades play a critical role in responding to MAMPs (Lieberherr et al., 2005). In response to stimuli, MAPK signal amplification cascades generally consist of three consecutive phosphorylation events mediated by MAP kinase kinase (MAPKKK), MAP kinase kinase (MAPKK) and MAPK (Ichimura et al., 2002). The earliest step in MAPK signaling that has been shown to mediate diterpenoid phytoalexin production is the MAPKKK OsACDR1 (accelerated cell death and resistance 1) (Kim et al., 2009). OsACDR1-over-expressing lines display spontaneous lesions, increased accumulation of momilactone A, and strongly increased resistance to a compatible race of M. oryzae. Based on immunoprecipitation assays, elicitation of rice with GlcNAC7 results in rapid activation of OsMPK3, OsMPK4 and OsMPK6 (Kishi-Kaboshi et al., 2010). Investigation of upstream kinase interactions demonstrated that OsMKK4 promotes activation of OsMPK3 and OsMPK6 but not ROS production. Conditional expression of a constitutively active form of OsMKK4 (OsMKK4DD) induced activation of OsMPK3 and OsMPK6 and promoted accumulation of momilactones and phyto- cassanes (Kishi-Kaboshi et al., 2010). OsMKK4DD-mediated expression of numerous diterpenoid biosynthetic genes was strongly suppressed in an Osmpk6 mutant background, demonstrating a requirement for OsMPK6 signaling (Figure 5) (Kishi-Kaboshi et al., 2010). The rice receptor-like kinase OsRLCK185 may also provide a link between OsCERK1-mediated chitin perception and MAPK activation. In response to GlcNAC6, OsRLCK185 is directly phosphorylated by OsCERK1 (Yamaguchi et al., 2013). RNAi silencing of OsRLCK185 suppresses (GlcNAC6)-induced activation of OsMPK3 and OsMPK6, ROS production and expression of the defense marker transcripts OsPAL1 (phenylalanine ammonia-lyase 1) and OsPBZ1 (probenazole inducible gene 1) (Yamaguchi et al., 2013). Although these results are suggestive, direct links between OsRLCK185 signaling and phytoalexins have yet to be identified.

Other rice responses triggered by N-acetylchitoooligosaccharides include cytoplasmic acidification/extracellular alkalization and bi-phasic generation of ROS such as H2O2 (Kuchitsu et al., 1997; Yamaguchi et al., 2005). Secondary H2O2 increases are linked in part to increases in phospholipase D and associated release of phosphatidic acid (from membrane phospholipids (Yamaguchi et al., 2005). The phospholipase D inhibitor 1-butanol suppresses (GlcNAC7)-induced H2O2 and momilactone A accumulation. Although inactive as a lone treatment, phosphatidic acid synergizes (GlcNAC7)-elicited production of momilactone A (Yamaguchi et al., 2005). Among the early plant responses to MAMP perception are increases in the cytosolic Ca2+ concentration (Dodd et al., 2010). In rice, cytosolic Ca2+ elicitation by TvX is partly mediated by the plasma membrane putative voltage-gated cation channel OsTPC1 (two-pore channel 1) (Hamada et al., 2012). Ostpc1 mutants showed reductions in TvX elicitation of cytosolic Ca2+ at 3 min, accumulation of OsCPS2, OsCPS4, OsKSL4 and OsKSL7 transcripts at 6 h, and subsequent diterpenoid phytoalexin production. Importantly, TvX-induced responses were complemented in transgenic Ostpc1 lines expressing wild-type OsTPC1 (Hamada et al., 2012). Candidate targets of TvX-induced signaling are the Ca2+-sensing calcineurin B-like (CBL) proteins and CBL-interacting protein kinases (CIPKs). The OsCIPK14/15 transcripts, which show 95% nucleotide sequence identity, accumulate after TvX and (GlcNAC7), elicitation (Kurusu et al., 2010). Both OsCIPK14 and OsCIPK15 displayed significant interactions with OsCBL4, which may function as a Ca2+ sensor. RNAi-silenced OsCIPK14/15 lines with 10-30% expression remaining showed reductions in TvX-elicited responses including cell death, phytoalexin gene expression and accumulation of end products, while OsCIPK15-over-expressing lines showed enhanced TvX-elicitation of accumulation of momilactones and phytocassanes (Kurusu et al., 2010).

Comprehensive rice microarray analyses demonstrate that many genes such as those encoding diterpene synthases (OsCPS2, OsCPS4, OsKSL4 and OsKSL7), P450s (OsCYP99A2, OsCYP99A3, OsCYP71Z7, OsCYP76M5, OsCYP76M6, OsCYP76M7 and OsCYP78M8) and a dehydrogenase (OsMAS) are coordinately expressed after elicitation (Okada et al., 2007). To assess the potential role of promoters and shared regulatory elements, Okada et al. (2009) examined cis-acting elements responsive to (GlcNAC7) within a 2 kb region upstream of OsKSL4 via a luciferase reporter assay. Deletion and mutation analysis of the OsKSL4 promoter identified an elicitor-responsive cis-acting TGACG motif 1040 bp upstream of the translation start site, suggesting involvement of a basic leucine zipper (bZIP) TGA transcription factor. Of more than 100 transcription factors with bZIP features, only three proteins were TGA-type bZIP transcription factors with elicitor-responsive transcription profiles (Okada et al., 2009).
To assess transcription factor activity, rice Tos17 insertion mutants of AK073715/Os04g0637000 were elicited with [GlcNAc]₈, and displayed significantly lower momilactone accumulation than control lines but no suppression of phytocassane production (Miyao et al., 2007; Okada et al., 2009). AK073715/Os04g0637000 was thus designated as OsTGA1 (TGA factor for phytoalexin production 1) and over-expression lines further confirmed enhanced accumulation of both momilactones and phytocassanes after (GlcNAc)₈ elicitation. Levels of accumulated phytoalexins correlated with the expression of OsTGA1 protein, and with expression of key transcripts including OsKSL4, OsKSL7 and OsDXS3. Expression of five clustered momilactone biosynthetic genes was also strongly induced in OsTGA1-over-expressing cells after elicitation. Furthermore, compared to all other rice tissues, expression of OsTGA1 is constitutively elevated in roots that are known to continuously secrete momilactones (Kato-Noguchi and Ino, 2003; Fujita et al., 2010). There is significant evidence to show that OsTGA1 influences the methylerythritol phosphate pathway, multiple transcripts encoding elicitor-inducible diterpenoid biosynthetic enzymes, and production of at least two phytoalexin classes. In addition to TGA transcription factors, the plant-specific zinc finger WRKY family of transcription factors has also been shown to play significant roles in rice defense against M. oryzae (Wang et al., 2007). Recently, Yokotani et al. (2013) characterized the group IIa WRKY transcription factor OsWRKY76 and demonstrated significant accumulation of transcripts following wounding and benzothiadiazole elicitation. Surprisingly, OsWRKY76-over-expressing lines exhibited suppression of M. oryzae-elicited genes involved in phytoalexin biosynthesis, reduced diterpenoid accumulation and increased pathogen susceptibility, consistent with a transcriptional repressor role opposite to that of OsTGA1 (Yokotani et al., 2013).

Involvement of phytohormones

Jasmonates. A ubiquitous event in plants following damage is peroxidation of free linolenic acid by 13-lipoxygenases, followed by allene oxide synthase-mediated epoxide formation, cyclization by allene oxide cyclase (AOC), and three rounds of β-oxidation resulting in jasmonic acid (JA) (Wasternack and Hause, 2013). JA is further metabolized into an amino acid conjugate, (-)-JA-isoleucine (JA-Ile), by JAR1 (Jasmonate Resistant 1). Under induced conditions, JA-Ile mediates recruitment of JAZ (jasmonate ZIM domain) repressor proteins to the F-box protein CO1 (part of the Skp1/Cullin/F-box SCF²⁰¹ ubiquitin E3 ligase complex), promoting ubiquitinylation and degradation by the 26S proteasome. Upon degradation of JAZ proteins, the transcription factor MYC2 activates transcription of early JA-responsive genes (Pauwels and Goossens, 2011; Wasternack and Hause, 2013). JA and its derivatives, termed jasmonates, are widely established as key regulators of defense metabolism against many pests and pathogens (Howe and Jander, 2008; Pieterse et al., 2009).

Consistent with a role in phytoalexin production, elicitation of rice cells using either (GlcNAc)₈ or CuCl₂ results in a rapid and transient increase in endogenous JA, followed by momilactone A accumulation (Nojiri et al., 1996; Rakwal et al., 1996). Exogenous JA application also promotes momilactone A production; however, JA treatments are less active than (GlcNAc)₈ elicitation. To examine the role of jasmonates, Riemann et al. (2013) isolated two OsAOC loss-of-function mutants. Constitutive and wound-inducible expression of JA and JA-Ile was almost undetectable in Osaco rice plants, which also displayed greater levels of multiple cell infection following M. oryzae inoculation. Despite higher proportions of diseased tissue, JA-deficient mutants produced lower levels of momilactones compared to M. oryzae-infected wild-type plants (Riemann et al., 2013). In contrast, fungal-induced accumulation of phytocassanes did not differ. In the context of JA-Ile signaling, the rice Tos17 JAR1 mutant Osjar1-2 showed a significant decrease in production of the flavonoid phytoalexin sakuranetin, but not accumulation of diterpenoid phytoalexins in response to heavy metal stress and M. oryzae infection (Shimizu et al., 2013). However, the accumulation of momilactones and phytocassanes in response to exogenous JA is completely suppressed in Osjar1-2 plants, but treatment with JA-Ile restores the response. Thus, multiple lines of evidence demonstrate the existence of jasmonate-dependent and -independent pathways in the mediation of rice diterpenoid phytoalexin biosynthesis.

Jasmonate-ethylene interactions. In wounded maize stems, JA fails to elicit accumulation of terpenoid phytoalexins; however, in the presence of the ethylene (ET) (as ethephon: 2-chloroethylphosphonic acid), significant synergy was detected in terms of production of both zealexins and kauralexins (Huffaker et al., 2011; Schmelz et al., 2011). Most biotic stresses and elicitors promote ET production in plants, which requires conversion of S-adenosyl-L-methionine to 1-aminoacyclopropane-1-carboxylic acid (ACC), and the sequential activity of ACC synthase (ACS) and ACC oxidase, which exist as multi-gene families (Bleecker and Kende, 2000). In rice, M. oryzae infection promotes ET production within 24 h, and this response is particularly strong in rich rice harboring the Pi-i resistance gene (Iwai et al., 2006). In Arabidopsis, AtMPK3 and AtMPK6 are established positive regulators of AtACS2 and AtACS6 via phosphorylation, which increase enzyme stability, activity and ultimately ET biosynthesis (Liu and Zhang, 2004; Han et al., 2010). As OsMPK6 activation mediates inducible diterpenoid phytoalexin production (Kishi-Kaboshi et al., 2010) and AtMPK3/OsMPK3 and AtMPK6/OsMPK6 are orthologous, an interactive role for ET signaling is likely to exist (Ding et al., 2009). To examine the possible
significance of enhanced ET production, Helliwell et al. (2013) created OsACS2-over-expressing lines in which OsACS2 expression is under the control of the pathogen-inducible OsPBZ1 promoter. OsACS2-over-expressing plants displayed modest constitutive increases in ET production and accumulation of defense transcripts (OsPR1b and OsPR5); however, ET emission was greatly increased 48 h after M. oryzae inoculation. Encouragingly, OsACS2-over-expressing lines also exhibited increased resistance to multiple isolates of M. oryzae and Rhizoctonia solani, but phytoalexin levels were not examined (Helliwell et al., 2013). As a precursor to ET, methionine applications in rice trigger production of momilactone A (Nakazato et al., 2000). Based on study of ethephon, S-adenosyl-l-methionine and inhibitors it was concluded that methionine activity was more closely linked to ROS signaling than ET production (Nakazato et al., 2000). During the final liberation of ET from ACC, equivalent amounts of cyanide are also produced (Bleecker and Kende, 2000). Cyanide has been strongly implicated in mediating rice resistance (R) gene interactions with M. oryzae (Seo et al., 2011). Although synergistic phytohormone interactions between JA and ET have been shown to induce production of maize terpenes and terpenoids, rapidly advancing molecular resources in rice will enable critical examination of the role for ET biosynthesis and signaling in biosynthesis of diterpenoid phytoalexins (Schmelz et al., 2003, 2011).

Additional phytohormone interactions. Jasmonates, ET and salicylic acid (SA) are the hormones whose interactive roles in defense signaling are most often considered, but almost all phytohormones, including abscisic acid, auxins, cytokinins, brassinosteroids and GAs, have been shown to influence monotoc responses to biotic attack (De Vleschauwer et al., 2013). Similar to jasmonates, exogenous application of natural and synthetic cytokinins to rice cells and leaf disks induced accumulation of diterpenoid phytoalexins and transcripts encoding the respective biosynthetic enzymes (Ko et al., 2010). During M. oryzae infection, increased levels of endogenous cytokinins are also detectable, together with transcription of cytokinin-responsive genes sharing the OsRR6 (response regulator 6) promoter (Jiang et al., 2013). Co-treatment of rice leaves with cytokinins and SA promoted accumulation of OsPR1b and OsPBZ1 transcripts in a manner that is partially dependent on OsNPRI (Non-expression of Pathogenesis-Related Genes 1) (Jiang et al., 2013). Although our understanding of defense signaling roles for cytokinins is still evolving, SA has a clear function in death signaling during the hypersensitive response following challenge with incompatible biotrophic pathogens (Glazebrook, 2005). However, the extent to which SA influences rice phytoalexin production is unclear due to constitutively high SA levels and a comparatively stable SA levels following pathogen attack (Silverman et al., 1995). Over-expression of AtNPR1 in rice results in spontaneous lesions, broad-spectrum pathogen resistance and more rapid defense gene transcription after M. oryzae inoculation (Quilis et al., 2008). Exogenous root applications of SA promote accumulation of oryzalexins and momilactone A in leaves; however, the endogenous role of SA in diterpenoid phytoalexin production remains to be examined (Daw et al., 2008). Numerous plant hormones are likely to be partially involved in monocot terpenoid phytoalexin production via complex positive and negative interactions.

**BIOLOGICAL FUNCTIONS**

**Antimicrobial activity**

The primary metric of a functional phytoalexin is the corresponding level of antibiotic potency against a given attacking organism; however, while often examined separately, terpenoid phytoalexins typically occur in complex mixtures at levels that approach 1 mg g⁻¹ fresh weight in areas localized to necrotic tissues (Figure 6) (Umemura et al., 2003; Huffaker et al., 2011). Momilactones A and B exhibit potent inhibitory activity on germ tube elongation of M. oryzae, with an ED₅₀ median effective dose as low as 1-5 µg ml⁻¹ (Cartwright et al., 1977). By contrast, phytocassanes A-E inhibit M. oryzae spore germination, and have ED₅₀ values ranging from 4 to 25 µg ml⁻¹ (Koga et al., 1995, 1997). On average, oryzalexins A-D are slightly less active for inhibition of spore germination, with ED₅₀ values between 23 and 136 µg ml⁻¹ (Sekido et al., 1987). Inhibition of spore germination may have particular relevance to roots that constitutively release diterpenoid phytoalexins and are potential entry points for M. oryzae (Kato-Noguchi and Ino, 2003; Sesma and Osbourn, 2004; Toyomasu et al., 2008). In contrast, foliar phytoalexins are rarely present in advance of spore germination, and greater concentrations are required to inhibit fungal growth after infection. For example, oryzalexin D has an ED₅₀ of 23 µg ml⁻¹ for inhibition of M. oryzae spore germination, but an ED₅₀ of 230 µg ml⁻¹ for suppression of mycelial growth (Sekido and Akatsuka, 1987). To assess endogenous roles of momilactones, OsCPS4 mutants and OsKSL4 knockout plants were recently examined (Xu et al., 2012; Toyomasu et al., 2014). In one study, the momilactone-deficient lines were no more susceptible to M. oryzae infection than the corresponding parental wild-type lines (Xu et al., 2012). In contrast, a Tos17 OsCPS4 mutant line exhibited increased susceptibility to M. oryzae when challenged with low levels of inoculum (Toyomasu et al., 2014). Given the contribution of multiple biosynthetic pathways, it is not surprising that mutants that are deficient in only a portion of the phytoalexin blend yield subtle phenotypes. As a result of their comparatively recent discovery, less information exists on the activity of maize phytoalexins. In
In liquid cultures, kauralexin B3 significantly inhibits the growth of *R. microsporus* and *C. graminicola* at concentrations as low as 10 μg ml⁻¹ (Schmelz et al., 2011). Similarly, zealexin A1 concentrations of 25 μg ml⁻¹ suppressed the growth of *Aspergillus flavus* and *F. graminearum* (Schmelz et al., 2011). In contrast, zealexin A2 showed no antimicrobial activity at any concentration. Supportive of an endogenous role for the zealexin class, RNAi and virus-induced gene silencing-based suppression of *ZmTps6/11* transcription significantly enhanced the growth of *U. maydis* tumors (van der Linde et al., 2011). With evidence for the existence of at least 20 maize terpenoid phytoalexins, considerable work remains in order to understand the antimicrobial activities of pure compounds and their combinations (Huffaker et al., 2011; Schmelz et al., 2011).

**Allelopathy**

Momilactones A and B were originally isolated from rice seed husks based on their phytotoxic activity (Kato et al., 1977). While early discoveries suggested a role in allelopathic growth suppression of neighboring plants, root secretion of momilactone B was described many years later (Kato-Noguchi and Ino, 2003). Phytocassanes A–E are also constitutively exuded by rice roots; however, these are not viewed as phytotoxic (Toyomasu et al., 2008). Rice plants constitutively release momilactones into the rhizosphere, a process that is further elicited by UV, JA, CuCl₂ and root exudates from common weeds such as barnyard grass (*Echinochloa crus-galli*) (Kato-Noguchi and Peters, 2013). Across cultivars, momilactone B exudation rates closely correspond with growth inhibition of barnyard grass seedlings (Kato-Noguchi et al., 2010). Momilactone B is preferentially secreted from rice roots and has greater allelopathic activity, while momilactone A accumulates to higher levels in the plant upon infection (Kato-Noguchi and Peters, 2013).

To assess the allelopathic role of momilactones, biosynthetic pathway mutants in OsCPS4 and OsKSL4 have recently been investigated (Xu et al., 2012; Toyomasu et al., 2014). *Oscps4* T-DNA insertion mutants display a complete lack of momilactones and a reduced negative allelopathic effect compared to wild-type plants, namely increased root and hypocotyl lengths in neighboring lettuce (*Lactuca sativa*) seedlings (Xu et al., 2012). Similarly, *Oskls4* T-DNA mutants in which production of momilactone but not oryzalexin S is blocked also exhibited relaxed allelopathic effects on the root growth of both barnyard grass and lettuce (Xu et al., 2012). Using more natural growing conditions (rice paddy soil), *OsCPS4* Tos17 mutants supported improved growth of multiple species of lowland weeds, including *Monochoria vaginalis* and *Elatine triandra* (Toyomasu et al., 2014). In this system, basal *OsCPS4* transcript levels were reduced approximately sixfold in roots and shoots. *M. oryzae* infection of leaves also resulted in lower levels of induced momilactone accumulation and root exudation (Toyomasu et al., 2014). Importantly, modest reductions in momilactones corresponded to significant changes in allelopathic capacity (Figure 6).

**Interactions with insects and pathogens**

Compared to pathogen and allelopathic interactions, fewer studies have assessed the roles of terpenoid phytoalexins in plant–insect interactions. In *rice*, attack for 24 h by the white-backed planthopper (*Sogatella furcifera*) is sufficient to dramatically reduce the spread of lesions after subsequent inoculation with *M. oryzae* and *Xoo* (Kanno and Fujita, 2003; Gomi et al., 2010). Short-term challenge with *S. furcifera* results in accumulation of at least 17 *PR
transcripts that are not significantly altered by the brown plant hopper (Nilaparvata lugens) (Gomi et al., 2010). Similar to induced pathogen defense, S. furcifera attack also results in increased levels of JA, SA and monomilactone A within 24 h, all of which continue to increase over a seven-day infestation period (Kanno et al., 2012). Insect-elicited increases in monomilactone A are likely to play a positive role in the subsequent suppression of pathogens; however, a direct defense role against S. furcifera has yet to be examined (Kanno et al., 2012).

Maize diterpenoid phytoalexins were first detected in the context of induced responses to insect attack (Schmelz et al., 2011). ZmAn2 transcripts and total kauralexins significantly accumulate following O. nubilalis stem attack compared to wounding alone (Dafoe et al., 2011). Within 48 h of O. nubilalis stem herbivory, microarray analyses revealed highly induced (≥200-fold) levels of transcripts encoding chitinases (endochitinase A), pathogenesis-related proteins (ZmPr5 and ZmPr10), peroxidases, 1,3-glucanase and wound-induced proteinase inhibitor 1 (ZmWip1) (Dafoe et al., 2013). Many of these genes correspond with the most highly induced transcripts present in stems during F. graminearum infection (Huffaker et al., 2011). Prolonged O. nubilalis stem herbivory and F. graminearum infection both result in continued accumulation of kauralexins (Huffaker et al., 2011; Schmelz et al., 2011). After 8 days of herbivory, total stem kauralexin levels approach 40 µg g⁻¹ fresh weight, and, when exogenously applied to stems at similar concentrations, kauralexins resulted in O. nubilalis antifeedant activity in paired-choice assays (Figure 6) (Schmelz et al., 2011). In contrast, short-term no-choice assays with kauralexins in artificial diets did not result in significant suppression of insect growth. Longer-term assays using kauralexins in the context of plant-based diets are required to examine potential direct effects on growth of O. nubilalis.

The expression of the predicted zealexin biosynthetic genes ZmTpsβ11 is induced in both roots and shoots within 12 h of S. littoralis leaf herbivory, and promotes systemic doubling of root β-macrocarpene pools (Koellner et al., 2008b). ZmTpsβ11 transcript accumulation is also induced in stem tissues following 48 h of O. nubilalis attack (Dafoe et al., 2013). Correspondingly, zealexins accumulate in O. nubilalis-infested maize stems, but total levels are modest and have yet to be examined in the context of anti-insect defenses (Huffaker et al., 2011). In general, phytoalexins have the potential to negatively affect any biotic attacker with limited mobility or those requiring establishment of long-term feeding sites. Candidate insects include gall-formers, leaf miners, root herbivores, stem borers, aphids and midges. Given the typical 1-2 day time lag for maximal production, it is unlikely that mobile leaf feeding herbivores strongly elicit or are subjected to significant levels of non-volatile phytoalexins.

Herbivore-induced increases in transcripts encoding phytoalexin biosynthetic enzymes may serve as a preparatory step, priming the plant for more rapid responses to pathogens (Conrath et al., 2006). In the context of stem tunneling, there are well-established relationships between insect attack and pathogen infection (Keller et al., 1986; Gatch and Munkvold, 2002). Fusarium verticillioides is an almost ubiquitous asymptomatic endophyte in healthy maize plants. Stress imposed by insect stalk damage is predicted to change the relationship and promote F. verticillioides pathogenesis (Bacon et al., 2008). In an analysis of O. nubilalis damage to commercial maize lines, a significantly increased incidence of F. verticillioides stalk rot was found in insect-damaged plants (Gatch and Munkvold, 2002). Given the mechanical disruption of core xylem/phloem function and the creation of a humid contaminated microenvironment, the induction of maize phytoalexins following stem tunneling is not surprising (Dafoe et al., 2011; Schmelz et al., 2011). This situation is in contrast with the responses of exposed modular leaves, in which lepidopteran herbivory has not yet been associated with terpenoid phytoalexins. Conclusive evidence exists for insect-induced increases in monocot terpenoid phytoalexins; however, many questions remain, including the mode of elicitation, potential involvement of microorganisms, the role in protecting plants against subsequent infection, and the existence of direct effects on insects.

**TERPENOID PHYTOALEXINS AS POTENTIAL SIGNALS**

An emerging theme in plant defense research is that biochemical intermediates and end products also play regulatory roles. As largely pre-formed phytoanticipins, glucosinolates are the predominant chemical defenses of many brassicaceous plants. In Arabidopsis, pathogen-induced production of the indolic glucosinolate 4-methoxy-indol-3-ylmethylglucosinolate and hydrolysis by the myrosinase AtPEN2 (Penetration 2) are required for resistance against powdery mildew (Bednarek et al., 2009). Treatment of plants with the flagellin-derived peptide flg22 (a bacterial MAMP) triggers callose deposition. Mutations in the glucosinolate biosynthetic genes AtCYP81F2 or AtPEN2 block flg22-induced callose deposition. Application of 4-methoxy-indol-3-ylmethylglucosinolate restores callose deposition in cyp81f2 mutants but not pen2 plants, highlighting the importance of specific glucosinolate breakdown products to yield an active signal in response to flg22 (Clay et al., 2009). In a conceptually analogous system, glucosides of maize benzoazinoids such as 2,4-dihydroxy-7-methoxy-2H-1,4-benzoazin-3(4H)-one (DIMBOA) are liberated upon cell lysis by the action of β-glucosidases, and protect against both insects and pathogens (Ahmad et al., 2011; Glauser et al., 2011). Mutants in indole-3-glycerol phosphate lyase (Benzoazincless1) have impaired production of the biosynthetic precursor indole, exhibit constitutively
Defense-related terpenoid phytoalexins in monocots

Reduced DIMBOA glucoside levels, and deposit significantly less callose when infiltrated with chitosan (Ahmad et al., 2011). Leaf infiltration with chitosan is associated with apoplastic release of free DIMBOA within 24 h. Similarly, leaf treatment with 20 μg ml⁻¹ DIMBOA alone promotes callose deposition equivalent to elicitation with chitosan (Ahmad et al., 2011). Of greater relevance to diterpenoid signaling, (11E,13E)-labda-11,13-diene-8α,15-diol (WIPK-Activating Factor1; WAF-1), cis-abienol and sclereol have been identified in tobacco as endogenous regulators of plant defense (Seo et al., 2003, 2012). Guided by a wound-induced protein kinase (WIPK) activity assay, WAF-1 was biochemically purified from 15 kg of tobacco mosaic virus-infected leaves and found to be a potent regulator of SA-induced protein kinase and an activator of PR gene transcription (Seo et al., 2003). Pre-treatment of tobacco leaves with WAF-1 reduces tobacco mosaic virus necrotic lesions and accumulation of viral proteins. Dehydroabietinal has also recently been shown to function as a phloem mobile signal in Arabidopsis that triggers systemic acquired resistance against Pseudomonas syringae pv. maculicola (Chaturvedi et al., 2012). With significant activity at 1 μM, dehydroabietinal induces SA expression in distal leaves, requires NPR1 signaling and also functions in solanaceous plants. The above examples highlight the role of defense-related metabolites in further mediation of dynamic plant responses.

Proof of signaling roles for terpenoid phytoalexins is currently lacking; however, numerous examples suggest that this is a viable research direction. In the simplest scenario, intermediates may be supplied to phytohormone biosynthetic pathways. For example, the ent-CPs ZmAn1 is involved in production of ent-kaurene for GA biosynthesis. Plants harboring Zman1 mutations lack detectable ZmAn1 protein but display only semi-dwarf phenotypes, unlike severe dwarfs with other mutations in GA biosynthesis (Bensen et al., 1995). Zman1 mutants retained 20% of the wild-type levels of ent-kaurene (an estimate of GA biosynthetic capacity), demonstrating the presence of additional active ent-CPs proteins (Bensen et al., 1995). The highly pathogen-inducible ent-CPs ZmAn2 has confirmed activity, is a predicted node in kaurene biosynthesis, and is likely to partially compensate for ent-CPD deficits created by the Zman1 mutation (Bensen et al., 1995; Harris et al., 2005; Schmelz et al., 2011). In another example, rice mutations in OsCPS4 and OsKLS4 both result in a lack of momilactone production; however, only OsKLS4 mutants show a 50% reduction in seed germination rates (Xu et al., 2012). It is possible that syn-CPD products accumulate in OsKLS4 plants causing the unexpected physiological changes. It will be interesting to explore whether this is due to increased seed dormancy or reduced viability. Given their strong association with biotic stress and disease, non-volatile terpenoid phytoalexins present in otherwise healthy tissue may serve as signals that further mediate defense gene expression.

The volatile hydrocarbons ent-kaur-15-ene and β-macrocarpene, which are predicted maize phytoalexin precursors, are detectable in challenged maize tissues (Mellon and West, 1979; Koellner et al., 2008b; Huffaker et al., 2011). Conceptually, plant signaling and ecological roles for phytoalexin precursor volatiles may parallel the complexity of those established for plant-insect interactions. Herbivore-induced plant volatiles attract predators, parasitoids and entomopathogenic nematodes, thereby functioning as indirect plant defenses (Turlings et al., 1990; Degenhardt et al., 2009). Herbivore-induced plant volatiles have also been shown to promote within-plant and between-plant signaling, resulting in promotion of extra floral nectar production in lima bean (Phaseolus lunatus) (Heil and Silva Bueno, 2007), and to prime plants for greater subsequent responses to attack (Engelberth et al., 2004; Frost et al., 2007; Ton et al., 2007). Given such diverse findings, it is conceivable, if not probable, that volatile precursors of terpenoid phytoalexins also serve as signals that alter plant physiology and defense (Figure 6).

In maize, herbivore-induced plant volatiles also meet many of the general criteria associated with phytoalexins. While systemic production is detectable, synthesis is largely localized to the site of damage (Turlings and Tumlinson, 1992; Koellner et al., 2013). The most abundant S. littoralis-induced sesquiterpenes in maize are the ZmTps10 products (E)-γ-bergamotene/(E)-β-farnesene and ZmTps23-derived (E)-β-caryophyllene (Schnee et al., 2006; Koellner et al., 2008a). Following elicitation, neither transcripts nor metabolites spread across the mid-rib or propagate basally through the leaf (Koellner et al., 2013). Given their rapid biosynthesis (Turlings et al., 1998), there is reasonable potential for a role of inducible terpene volatiles as antimicrobial agents at the wound site. Importantly, this maize response is not limited to insect damage, as leaf infection by Fusarium spp. also strongly triggers (E)-β-caryophyllene emission (Piesik et al., 2011). To assess the antimicrobial activity of (E)-β-caryophyllene in planta, two AtTps21 null mutants were examined (Huang et al., 2012). After challenge with the bacterial pathogen Pseudomonas syringae pv. tomato DC3000 (Pst DC3000), Arabidopsis flowers lacking (E)-β-caryophyllene produced lighter and mis-shape seeds. Likewise, over-expression of AtTps21 in leaves resulted in elevated (E)-β-caryophyllene emission, reduced Pst DC3000 lesions and lower bacterial growth compared to wild-type plants (Huang et al., 2012). Moreover, modest levels of exogenous (E)-β-caryophyllene also reduced Pst DC3000 proliferation. Although there is no direct proof that terpene hydrocarbons fulfill the definition of phytoalexins, current results support their ability to be localized production, elicitation by microorganisms and function as antibiotics.
SUMMARY

Rice and maize, which are among the most dominant planted crops on Earth, are protected in part by complex mixtures of inducible terpenoid phytoalexins. Significant progress is being made in both identifying additional family members and defining essential enzymes in the corresponding biosynthetic pathways. Our understanding of endogenous signal cascades that mediate elicitor-induced terpenoid phytoalexin accumulation is also rapidly expanding, and is certain to far exceed the complexity of the biosynthetic pathways. Collectively, the development of null mutants and transgenic over-expression lines is enabling critical examination of biological functions that already extend well outside the confines of traditional phytoalexin definitions.

ACKNOWLEDGMENTS

This work was supported by US Department of Agriculture Agricultural Research Service Projects 6615-21000-010-00 and 6615-22000-027-00, and by National Science Foundation Division of Integrative Organismal Systems Competitive Award 1139329.

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