




Biosynthesis and biological function of secondary metabolites of the rice blast fungus *Pyricularia oryzae*

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Abstract: Filamentous fungi have many secondary metabolism genes and produce a wide variety of secondary metabolites with complex and unique structures. However, the role of most secondary metabolites remains unclear. Moreover, most fungal secondary metabolism genes are silent or poorly expressed under laboratory conditions and are difficult to utilize. *Pyricularia oryzae*, the causal pathogen of rice blast disease, is a well-characterized plant pathogenic fungus. *P. oryzae* also has a large number of secondary metabolism genes and appears to be a suitable organism for analyzing secondary metabolites. However, in case of this fungus, biosynthetic genes for only four groups of secondary metabolites have been well characterized. Among two of the four groups of secondary metabolites, biosynthetic genes were identified by activating secondary metabolism. These secondary metabolites include melanin, a polyketide compound required for rice infection; tenuazonic acid, a well-known mycotoxin produced by various plant pathogenic fungi and biosynthesized by a unique nonribosomal peptide synthetase–polyketide synthase hybrid enzyme; nectriapyrones, antibacterial polyketide compounds produced mainly by symbiotic fungi, including plant pathogens and endophytes, and pyriculols, phytotoxic polyketide compounds. This review mainly focuses on the biosynthesis and biological functions of the four groups of *P. oryzae* secondary metabolites.

Keywords: Rice blast fungus *Magnaporthe oryzae*, Plant pathogenic fungus, Secondary metabolite biosynthetic gene cluster, Biological function

Introduction

Filamentous fungi, including plant pathogenic fungi, produce a wide variety of secondary metabolites with complex and unique structures. However, the biological role of secondary metabolites remains unclear. Filamentous fungi have been a rich source of secondary metabolites for medicines and agrochemicals. Whole-genome sequencing analyses have indicated that filamentous fungi have many more secondary metabolism genes than expected, suggesting that most secondary metabolism genes are silent or poorly expressed under laboratory conditions. To utilize the ability of fungi to produce secondary metabolites, secondary metabolism has been activated through various approaches, including overexpression of pathway-specific transcription factors, manipulation of global regulators, epigenetic control, ribosome engineering, heterologous expression of secondary metabolite biosynthetic gene clusters (BGCs), coculture, and chemical induction (Macheleidt et al., 2016; Netzker et al., 2015; Ochi & Hosaka, 2013; Rutledge & Challis, 2015). A recent study showed that rice extracts can induce secondary metabolite gene expression in the filamentous fungus *Aspergillus nidulans* (Lacriola et al., 2020), suggesting the possible involvement of fungal secondary metabolites in plant–fungus interactions.

The major fungal secondary metabolites, polyketides and nonribosomal peptides, are biosynthesized by polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs), respectively. Fungal PKSs can be categorized into three types. The first type is iterative type I PKS that consists of multiple

catalytic domains, including ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains, along with several optional β -keto modifying domains, such as β -ketoacyl reductase (KR), dehydratase (DH), and transacting enoyl reductase domains (Fischbach & Walsh, 2006). The second type is type III PKS, which consists of only homodimeric KS (Hashimoto et al., 2014). The third type is a fungal PKS–NRPS hybrid enzyme, which consists of an iterative type I PKS followed by a single-module NRPS (Boettger & Hertweck, 2013; Böhnert et al., 2004; Eley et al., 2007; Fisch, 2013; Song et al., 2004). In fungal PKS–NRPS, the PKS part has KS, AT, and ACP domains, along with several modifying domains including KR, DH, and methyltransferase (MT) domains. The NRPS part has adenylation (A), peptidyl carrier protein (PCP), condensation (C), and terminal release or cyclization (R, reductase or DKC, Dieckmann cyclization) domains (Boettger & Hertweck, 2013).

Pyricularia oryzae (syn. *Magnaporthe oryzae*) (Fig. 1) is the causal pathogen of rice blast disease and is a well-characterized plant pathogen. *P. oryzae* infects rice plants through an infection-specific organ appressorium and proliferates inside rice plants via filamentous growth and causes rice blast disease (Howard & Valent, 1996). *P. oryzae* is also rich in secondary metabolism genes (Collemare et al., 2008b; Dean et al., 2005). An antiSMASH analysis predicted the presence of 23 iterative type I PKSs, 2 type III PKSs, 6 fungal PKS–NRPS hybrid enzymes, and 9 NRPSs (unpublished data). Secondary metabolites of *P. oryzae* may be involved in rice infection (Fig. 1). The biosynthetic genes of only four groups of secondary metabolites (melanin, tenuazonic acid, nectriapyrones,

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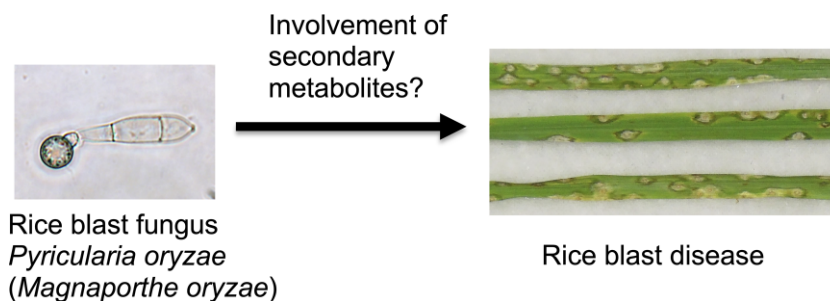


Fig. 1. Are secondary metabolites of the rice blast fungus *Pyricularia oryzae* involved in rice infection?

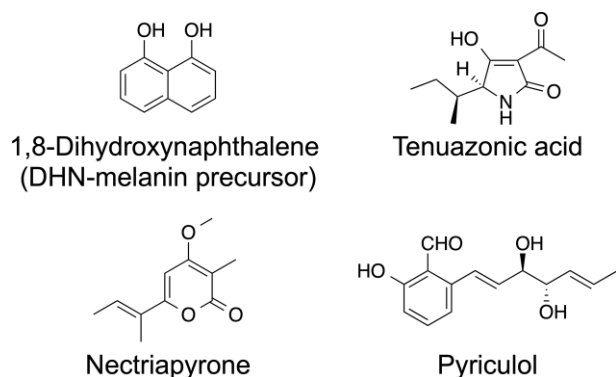


Fig. 2. Chemical structures of the secondary metabolites of *Pyricularia oryzae*.

and pyriculols) have been well characterized in *P. oryzae* (Fig. 2). Two (tenuazonic acid and nectriapyrones) of the four groups of secondary metabolites were identified by activating secondary metabolism.

Here, we mainly focused on the four groups of secondary metabolites, as shown in Fig. 2. We describe the biosynthesis and biological roles of secondary metabolites in the rice blast fungus *P. oryzae*.

Melanin: A Polyketide Compound Required for the Fungal Infection to Rice

P. oryzae synthesizes the black pigment melanin (Fig. 2), which is required for rice infection (Howard & Ferrari, 1989). Melanin does not work as a toxin, but this secondary metabolite is required for rice infection by the mechanism described below. *P. oryzae* forms an infection-specific organ, appressorium, and infects rice plants using this organ. Appressorium formation and appressorium melanization are essential for rice infection. Rice plant invasion is achieved by an infection peg that is formed at the base of an appressorium, which attaches tightly to the host surface. The mechanical force produced by appressoria is necessary to successfully penetrate the infection peg (Howard & Ferrari, 1989). An appressorial melanin layer between the cell membrane and the cell wall is essential for mechanical force generation. Turgor pressure is concentrated toward the cuticular surfaces of the rice plant, and the pressure inside the appressoria has been assessed to be as high as 8 MPa (Howard et al., 1991; Money & Howard, 1996). This pressure can be generated by 3.2 M glycerol accumulated inside the appressorium (de Jong et al., 1997). Melanin is proposed to function as a semipermeable membrane that passes water, but not glycerol, and as a structural support for this extremely high pressure.

Melanin is a well-known natural black pigment. *P. oryzae* produces dihydroxynaphthalene (DHN)-melanin, which is a type of fungal melanin biosynthesized by polymerizing the polyketide compound 1,8-dihydroxynaphthalene (1,8-DHN) (Bell & Wheeler, 1986; Butler & Day, 1998). In *P. oryzae*, melanin biosynthetic genes have been identified, and their biosynthetic pathways have been elucidated (Fig. 3) (Eliahu et al., 2007; Lundqvist et al., 1994; Thompson et al., 2000; Vidal-Cros et al., 1994). The iterative type I PKS enzyme ALB1/MGG_07219 catalyzes the synthesis of the backbone compound 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN). Melanin was originally shown to be a pentaketide compound. However, analysis of an ALB1 homolog in the closely related fungus *Colletotrichum lagenarium* showed that melanin is a hexaketide compound and the backbone (1,3,6,8-THN) is biosynthesized using acetyl-CoA and five malonyl-CoA (Vagstad et al., 2012). Then, 1,3,6,8-THN is converted to 1,8-DHN using three enzymes: 1,3,6,8-THN reductase (4HNR), scytalone dehydratase (SDH1/RSY1), and 1,3,8-trihydroxynaphthalene (1,3,8-THN) reductase (3HNR/BUF1). Finally, 1,8-DHN is oxidatively polymerized to form DHN-melanin. Melanin biosynthesis can be induced by genetic modification of the factors involved in epigenetic control (Maeda et al., 2017) and signal transduction (Motoyama et al., 2008).

Biosynthetic enzymes of DHN-melanin have been agrochemical development targets, and three types of commercial melanin biosynthesis inhibitors (MBIs) have been successfully developed (Fig. 3). MBIs are classified into three types: MBI-R (tricyclazole, phthalide, and pyroquilon), MBI-D (carpropamid, fenoxanil, and diclocymet), and MBI-P (tolprocarb) (Banba et al., 2017; Hagiwara et al., 2019; Motoyama & Yamaguchi, 2003). The targets of MBI-R, MBI-D, and MBI-P are 1,3,8-THN reductase (3HNR/BUF1), scytalone dehydratase (SDH1/RSY1), and PKS (ALB1), respectively (Fig. 3). MBIs are known to be environmentally friendly agrochemicals because they can inhibit fungal infection without inhibiting fungal growth. As MBIs have no growth inhibitory activity, it has long been believed that no resistant strain will be created. However, the occurrence of resistant strains for MBI-D has been reported (Kaku et al., 2003; Sawada et al., 2004; Yamada et al., 2004), suggesting that resistant strains can become dominant depending on the target enzymes and application conditions.

Tenuazonic Acid: A Well-Known Mycotoxin Biosynthesized by a Unique NRPS-PKS Enzyme

The tetramic acid derivative tenuazonic acid (TeA, Fig. 2) is a well-known mycotoxin that was first isolated from *Alternaria tenuis* culture broth in 1957 (Rosett et al., 1957). *P. oryzae* and other plant pathogenic fungus *Phoma sorghina* (sorghum pathogen) are

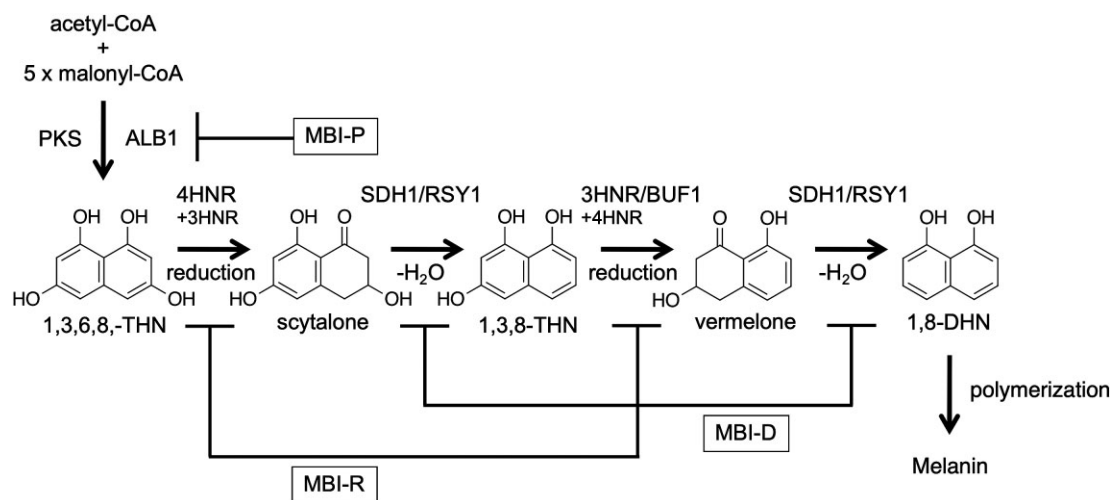


Fig. 3. Melanin biosynthetic pathway of *Pyricularia oryzae*. Inhibition points of the three groups of melanin biosynthesis inhibitors (MBIs) are shown. 1,3,6,8-THN, 1,3,6,8-tetrahydroxynaphthalene; 1,3,8-THN, 1,3,8-trihydroxynaphthalene; 1,8-DHN, 1,8-dihydroxynaphthalene.

also known as TeA producers (Steyn & Rabie, 1976; Umetsu et al., 1972). *Alternaria* is a ubiquitous plant pathogenic fungus that causes spoilage of various food crops and fruits in the field and postharvest decay (Ostry, 2008). TeA has been detected in many *Alternaria*-contaminated vegetables, fruits, and crops (Gross et al., 2011; Lohrey et al., 2013; Siegel et al., 2009). A TeA analog from valine is also produced by *P. oryzae* as a minor product (Lebrun et al., 1990). Among the *Alternaria* toxins, TeA showed the highest toxicity, with acute toxicity in mammals. The oral median lethal doses for male and female mice were 182 or 225 mg kg⁻¹ and 81 mg kg⁻¹ body weight, respectively (Miller et al., 1963; Smith et al., 1968). The European Food Safety Authority determined the threshold of toxicological concern of TeA to be 1500 ng kg⁻¹ body weight (Asam & Rychlik, 2013).

TeA inhibits protein synthesis by preventing the release of polypeptides from the ribosome (Shigeura & Gordon, 1963). TeA also shows antiviral, antibacterial, antitumor, phytotoxic, and plant disease-controlling activities (Aver'yanov et al., 2007; Gitterman, 1965; Lebrun et al., 1988; Miller et al., 1963). TeA can inhibit photosynthesis (Chen et al., 2015, 2007, 2010) and the potential use of TeA as a herbicide that targets photosystem II (PSII) has been proposed (Chen & Qiang, 2017). A recent study indicated that TeA can inhibit plant plasma membrane (PM) H⁺-ATPase at micromolar concentrations (Bjørk et al., 2020). PM H⁺-ATPase inhibition results in depolarization of the membrane potential and finally necrosis. Currently, it remains unknown whether TeA is required for plant infection. We induced the production of TeA in *P. oryzae* and identified the biosynthetic gene *TAS1*, which encodes the first reported fungal NRPS-PKS hybrid enzyme (Motoyama & Osada, 2016; Yun et al., 2015). We have shown the biosynthetic and induction mechanisms of TeA (Yun et al., 2017, 2020). In the following section, we present the data on the biosynthesis and induction of TeA.

TeA has been shown to be biosynthesized using an isoleucine and two acetates (Stickings & Townsend, 1961). TeA was also predicted to be a product of a PKS-NRPS hybrid enzyme because it has a tetramic acid-containing structure (Collemare et al., 2008a). We successfully induced TeA production by 1% dimethyl sulfoxide (DMSO) treatment and disruption of the *OSM1* gene, which encodes an osmosensory mitogen-activated protein kinase (MAPK) that works downstream of the two-component signal transduction system involved in environmental responses. We found that

the TeA biosynthetic gene *TAS1/MGG_07803* was upregulated under TeA-inducing conditions (Motoyama & Osada, 2016; Yun et al., 2015). Unexpectedly, the biosynthetic enzyme *TAS1* (tenuazonic acid synthetase 1) was not a PKS-NRPS hybrid enzyme but an NRPS-PKS hybrid enzyme. PKS-NRPS hybrid enzymes have also been found in bacteria (Blodgett et al., 2010; Yu et al., 2007). Moreover, a different type of hybrid enzymes, NRPS-PKS hybrid enzymes (which begin with an NRPS module), have been found in bacteria (Du et al., 2001; Gerc et al., 2012; Müller et al., 2014; Silakowski et al., 1999; Simunovic et al., 2006; Tang et al., 2004). We reported that *TAS1* is the first fungal NRPS-PKS hybrid enzyme, which consists of an NRPS module of domains C-A-PCP and a terminal PKS KS domain (Fig. 4A). *TAS1* is a new type of NRPS-PKS hybrid enzyme that starts with an NRPS module (C-A-PCP). The domain structure is quite different from that of conventional fungal PKS-NRPS enzymes that start with a PKS module (Fig. 4A). The PKS part of *TAS1* only has a KS domain, in contrast to other NRPS-PKS hybrid enzymes. This KS domain has a unique sequence and is essential for *TAS1* activity. Phylogenetic analysis revealed that this KS domain formed an independent clade close to the type I PKS KS clade. We elucidated that *TAS1* biosynthesizes TeA from isoleucine and acetoacetyl-CoA (diketide) (Fig. 4B). This unique KS domain catalyzes the final Dieckmann cyclization step for tetramic acid ring formation and TeA release, although previous studies have suggested the involvement of this KS domain in diketide biosynthesis (Stickings & Townsend, 1961). In contrast, fungal NRPSs use the terminal condensation-like domain for substrate cyclization (Gao et al., 2012) and bacterial NRPSs use the terminal thioesterase domain for substrate cyclization (Trauger et al., 2000). Moreover, fungal PKS-NRPSs use the terminal reductase-like cyclization (DKC) domain for substrate cyclization (Boettger & Hertweck, 2013). This information shows that *TAS1* is a unique type of biosynthetic enzyme that may be used for the production of various compounds.

The PKS KS domains usually conduct the decarboxylative Claisen condensation of acyl and malonyl blocks to extend the polyketide chain (Hertweck, 2009). In contrast, the terminal KS domain of *P. oryzae* *TAS1* catalyzes substrate cyclization (Yun et al., 2015). Interestingly, a KS domain with a cyclization and not ketide extension role has only been reported in *TAS1*. We analyzed the unique features of the KS domain of *TAS1* (Yun et al., 2020). We revealed that the KS domain of *TAS1* is unexpectedly

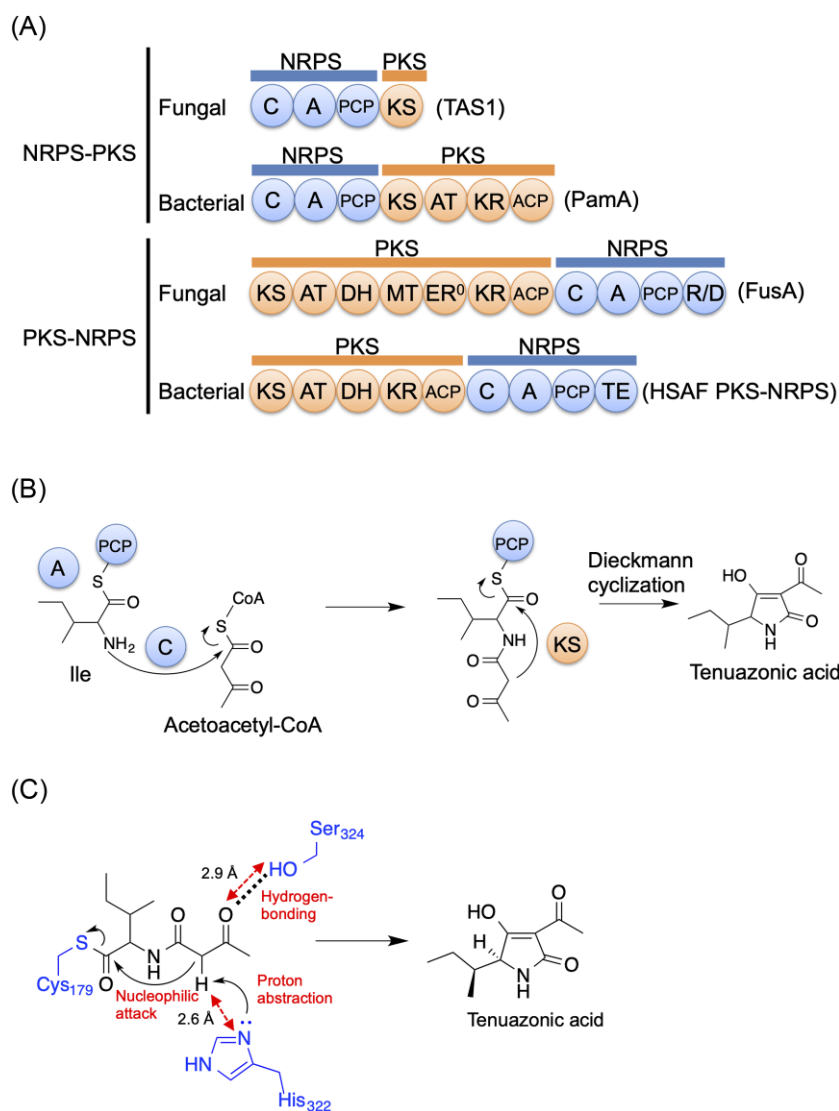


Fig. 4. Tenuazonic acid biosynthesis. (A) Comparison of the domain structures of TAS1 and other enzymes. (B) Biosynthetic pathway of tenuazonic acid. (C) Proposed cyclization mechanism for tenuazonic acid. KS, ketosynthase; NRPS, nonribosomal peptide synthetase; PKS, polyketide synthase; TAS1, tenuazonic acid synthetase 1.

monomeric such as NRPSs (Tanovic et al., 2008) although KSs are normally dimeric (Dutta et al., 2014; Kao et al., 1996; Weissman, 2015). The 1.68 Å resolution crystal structure of the TAS1 KS domain suggested that substrate cyclization is initiated by proton abstraction from the active methylene moiety in the substrate by the catalytic residue H322 (Fig. 4C). We also revealed that TAS1 KS shows broad substrate specificity and promiscuously accepts aminoacyl substrates. Moreover, this behavior could be increased by a single amino acid change in the substrate-binding pocket. Our data provided insight into the substrate cyclization mechanism catalyzed by the KS domain in TeA biosynthesis and how the KS domain in the NRPS-PKS hybrid enzyme TAS1 receives large amino acid-containing substrates.

The expression of fungal secondary metabolism genes should be properly regulated in response to various environmental signals. To understand the interaction between fungi and their environments, it is important to elucidate the regulatory mechanisms of these secondary metabolism genes. For example, clarification of the regulatory mechanism of mycotoxin biosynthesis is important to protect human and animal health by inhibiting mycotoxin pro-

duction. As explained previously, TeA is biosynthesized by TAS1 in *P. oryzae*, and its production is induced by 1% DMSO treatment or osmosensory MAPK gene (*OSM1*) deletion. However, the detailed regulatory mechanism of TeA production remains unknown. We identified two positive regulators of TeA production (Fig. 5) (Yun et al., 2017). Most fungal secondary metabolites are produced using BGCs. Some gene clusters have a cluster-specific DNA-binding binuclear Zn(II)₂Cys₆-type transcription factor, which is unique to fungi and stimulates the transcription of the clustered genes to generate a secondary metabolite (Keller et al., 2005). Transcription factor genes include *Fusarium sporotrichioides* *tri6* for trichothecene biosynthesis (Proctor et al., 1995), *A. nidulans* *afIR* for aflatoxin biosynthesis (Yu et al., 1996), *Aspergillus fumigatus* *gliZ* for gliotoxin biosynthesis (Bok et al., 2006), and *Monascus purpureus* *ctnA* for citrinin biosynthesis (Shimizu et al., 2007). We identified TAS2 (MGG_07800), a Zn(II)₂Cys₆-type transcription factor that regulates the production of TeA. TAS2 is located in the upstream region of TAS1 (Fig. 5). In fungi, the secondary metabolite production is regulated by upper-level regulators. These regulators are called global regulators, which are transacting positive or

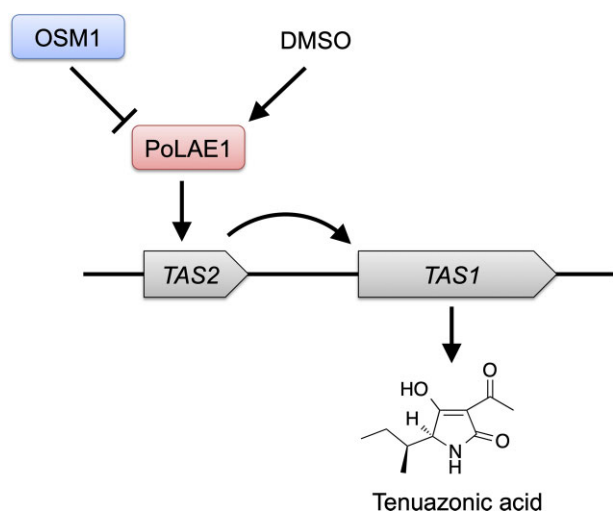


Fig. 5. Regulation of tenuazonic acid production in *Pyricularia oryzae*.

negative transcription factors of secondary metabolite BGCs. Loss of *afIR* expression (LaeA) is a well-characterized global regulator of secondary metabolism that was originally identified in *Aspergillus* spp. (Bayram et al., 2008; Brakhage, 2013; Perrin et al., 2007; Yin & Keller, 2011). LaeA orthologs have been identified in other fungi, including *Fusarium* spp., *Cochliobolus heterostrophus*, and *Monascus pilosus* (Bok & Keller, 2016). We identified the LaeA ortholog PoLAE1 (MGG_01233) from *P. oryzae*. Analysis of PoLAE1 knockout and overexpression strains indicated that PoLAE1 positively controlled the production of TeA. We also found that the two TeA-inducing signals, 1% DMSO treatment and OSM1 deletion, were transmitted through PoLAE1. These results showed that TeA production was regulated by two specific transcriptional regulators, TAS2 and PoLAE1, in *P. oryzae* (Fig. 5). Recently, it has been shown that TeA production can be induced by mycoviruses via upregulation of TAS2 (Ninomiya et al., 2020).

Nectriapyrones: Antibacterial Polyketide Compounds

Nectriapyrone (Fig. 2) is a polyketide compound, which is produced by various fungi (Andolfi et al., 2015; Avent et al., 1992; Claydon et al., 1985; Evidente et al., 2011; Gong et al., 2015; Guimaraes et al., 2008; He et al., 2016; Meister et al., 2007; Nair & Carey, 1975; Thines et al., 1998; Weber et al., 2005; Xi et al., 2012; Zhu et al., 2013). Interestingly, nectriapyrone producers are mainly symbiotic fungi, including plant pathogens (Andolfi et al., 2015; Evidente et al., 2011), endophytes (Claydon et al., 1985; Guimaraes et al., 2008; Lu et al., 2009; Meister et al., 2007; Weber et al., 2005; Xi et al., 2012), and sponge-associated fungi (Gong et al., 2015; He et al., 2016; Zhu et al., 2013). We recently revealed that nectriapyrone production can be induced in *P. oryzae* by disturbing the two-component signal transduction system (Motoyama et al., 2019). We succeeded in identifying the nectriapyrone BGC and analyzed its physiological functions, as described below.

The production of secondary metabolites is predicted to be strictly regulated under specific environmental conditions. Thus, we expected that secondary metabolite production might be activated by manipulating the signal transduction pathways involved in environmental responses. The two-component system (TCS) is a signal transduction system that controls various cellular functions in response to environmental signals and is found in various

organisms, including bacteria, archaea, plants, slime molds, and fungi (Catlett et al., 2003; West & Stock, 2001). The *P. oryzae* TCS was disturbed by deletion of OSM1 and PoYPD1, which encode HOG MAP kinase and His-containing phosphotransfer protein, respectively. This genetic manipulation induced the production of two polyketide compounds, nectriapyrone and its oxidized analog.

We found a nectriapyrone BGC containing an iterative type I PKS gene (NEC1/MGG_00806) and an O-MT gene (NEC2/MGG_14657) (Fig. 6A). Overexpression of these two genes induced overproduction of nectriapyrone and five analogs, including a new derivative, zaepyron (Fig. 6A) (Abramson & Wormser, 1981; Burkhardt & Dickschat, 2018; Cai et al., 2017; Hamerschmidt et al., 2014; Motoyama et al., 2019). Nectriapyrone is similar to gibepyrone (Fig. 6B) from *Fusarium* spp. Gibepyrone lacks a methoxy group found in nectriapyrone, and the O-MT gene is absent from the gibepyrone BGC (Janevska et al., 2016). Nectriapyrone also shows similarity to the *Streptomyces* metabolite germicidins (Aoki et al., 2011; Petersen et al., 1993; Xu et al., 2011) (Fig. 6B). A type III PKS is known to biosynthesize the germicidin backbone (Song et al., 2006). In contrast, in case of nectriapyrone, a type I PKS (NEC1) biosynthesizes the nectriapyrone backbone.

Nectriapyrones are α -pyrone compounds that have a wide range of biological activities (McGlacken & Fairlamb, 2005; Schaberle, 2016). For example, germicidins, produced by *Streptomyces* strains, act as autoregulators of spore germination (Aoki et al., 2011; Petersen et al., 1993). Photopyrones are bacterial signaling molecules that control cell clumping (Brachmann et al., 2013). The biological activities of nectriapyrone have been reported, although the functions of nectriapyrone in its producers are unknown. Nectriapyrone is toxic to bacteria, plants, and tumor cells (Evidente et al., 2011; Guimaraes et al., 2008; Nair & Carey, 1975; Turkkan et al., 2011). Nectriapyrone stimulates the formation of DOPA (3,4-dihydroxyphenylalanine) melanin in B16-F1 melanoma cells (Thines et al., 1998). In addition, it has been reported to inhibit monoamine oxidase in the mouse brain (Lee et al., 1999).

Identification of the nectriapyrone BGC enabled us to analyze the biological roles of nectriapyrones in the fungus that produce them (Motoyama et al., 2019). Although many nectriapyrone producers have been identified from plant pathogens, we have revealed that nectriapyrones are not required for rice infection and have different roles. The chemical structure of nectriapyrone is similar to that of germicidins produced by *Streptomyces* spp. Our data showed that nectriapyrones can control growth and pigment formation in *Streptomyces griseus* and have a growth-promoting effect on *P. oryzae* in interactions with *S. griseus*. Therefore, nectriapyrones may be involved in microbe–microbe interactions with other environmental bacteria, such as endophytic *Streptomyces* strains. To identify the active nectriapyrone analogs in this interaction, we tested the bioactivity of each nectriapyrone analog (Fig. 6A) and showed that nectriapyrone was the only active analog, suggesting that other nectriapyrone analogs may be detoxified compounds of nectriapyrone.

Pyriculols: Phytotoxic Polyketide Compounds

Pyriculol (Fig. 2) is a major secondary metabolite of *P. oryzae* and is a phytotoxin (Iwasaki et al., 1973). Several pyriculol analogs have also been reported. The major analogs are pyriculariol (Nukina et al., 1981), dihydropyriculol (Kono et al., 1991), and dihydropyriculol (Tanaka et al., 2011b) (Fig. 7A). It is also reported that other pyriculol analogs, griseaketides, are produced by a rice blast

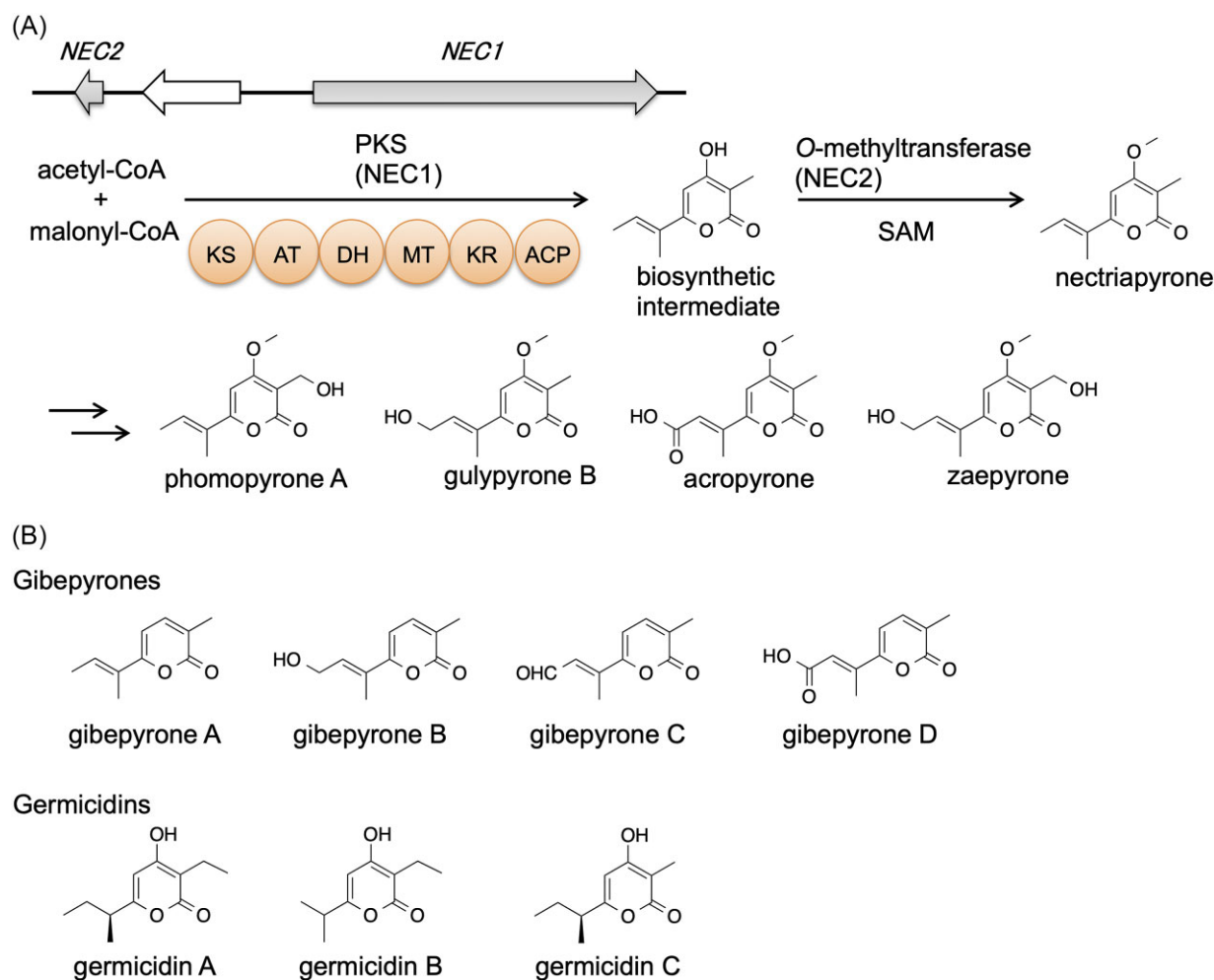


Fig. 6. Nectriapyrones and related compounds. (A) Biosynthesis of nectriapyrones. (B) Related compounds of nectriapyrones.

fungus isolate (Yang et al., 2019). Pyriculols are classified into two groups: aldehyde-type (pyriculol and pyriculariol) and alcohol-type (dihydropyriculol and dihydropyriculariol). It has been shown that aldehyde-type analogs induce lesion-like necrosis in rice leaves, whereas alcohol-type analogs are inactive (Jacob et al., 2017; Kono et al., 1991; Tanaka et al., 2011b). Four analogs are produced simultaneously (Jacob et al., 2017), and interconversion between oxidized aldehyde-type analogs and reduced alcohol-type analogs is expected. It is important to know why and how *P. oryzae* produces both alcohol and aldehyde analogs. Identification of the genes responsible for this oxidoreductive conversion will help to answer this question. Here, we describe the identification of the pyriculol BGC and analyze the pyriculol function using the biosynthetic genes.

Pyriculols are polyketide compounds, and their BGC have been reported (Furuyama et al., 2021a; Jacob et al., 2017). This gene cluster has an iterative type I PKS gene (*MGG_10912/MoPKS19/PYC1*) and other genes predicted to be responsible for the biosynthesis of pyriculols (Furuyama et al., 2021a; Jacob et al., 2017). It has been suggested that aldehyde-type analogs are biosynthesized first and converted to alcohol-type analogs by a reduction reaction (Tanaka et al., 2011b). The gene (*MGG_10961/MoC19OXR1/PYC10*) responsible for the oxidation of alcohol-type analogs to aldehyde-type analogs has been reported (Jacob et al., 2017), although the gene catalyzing the reverse reductive reaction has not yet been identified. We recently identified the gene (*MGG_16813/PYC7*) for this reverse reaction from aldehyde-type analogs to alcohol-type analogs (Furuyama et al., 2021a) (Fig. 8). Furthermore, previous

studies have predicted that alcohol-type analogs are biosynthesized via aldehyde-type analogs. We indicated that aldehyde-type analogs are biosynthesized via alcohol-type analogs (Fig. 8), in contrast to the previous prediction. Similarly, a recent study has shown that a pyriculol-related compound, flavoglaucin (Fig. 7B), is biosynthesized via alcohol intermediates in *Aspergillus ruber* (Nies et al., 2020). We suggest that the rice blast fungus controls the amount of pyriculol analogs using two oxidoreductases, *PYC10* and *PYC7*, thereby controlling the bioactivity of the phytotoxin.

Neurospora crassa produces sordaria (Fig. 7B), which shows high structural similarity to pyriculols. A sordarial biosynthetic mechanism has also been proposed (Zhao et al., 2019). The sordarial BGC is highly similar to the pyriculol BGC and has a *PYC10* homolog (*srdI*) and a *PYC7* homolog (*NCU02930*). *N. crassa* may also control the amount of sordarial analogs using two oxidoreductases homologous to *PYC10* and *PYC7*. In addition, it has been proposed that an aldehyde-type intermediate is released from PKS (*SrdA*) and cyclized by *SrdC/D/E*. The aldehyde-type intermediate is proposed to be converted by *SrbB* and *SrdG* to yield a sordarial. In this pathway, an alcohol-type intermediate is thought to be produced from an aldehyde-type compound by an endogenous reductase, which is expected to be a *PYC7* homolog. We assume that an alcohol-type compound is the release product from PKS (*SrdA*), and an aldehyde-type compound will be produced using the *PYC10* homolog *SrdI*.

The PKS gene (*MGG_10912/MoPKS19/PYC1*) knockout strain extract failed to induce phytotoxic lesions in rice leaves, suggesting that pyriculols are the sole lesion-inducing compounds

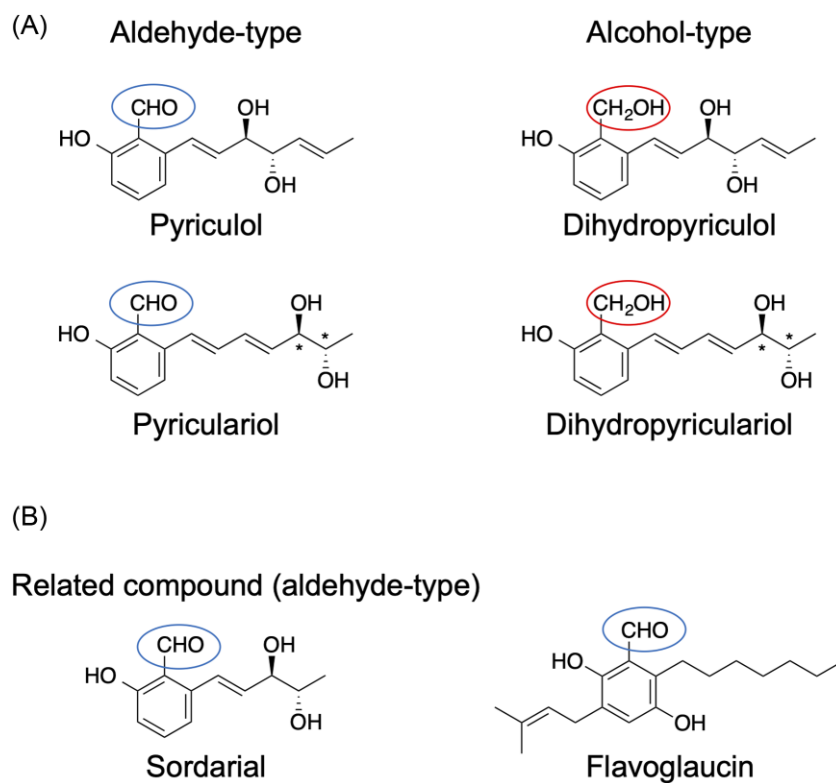


Fig. 7. Structures of the pyriculols (A) and a related compound (B).

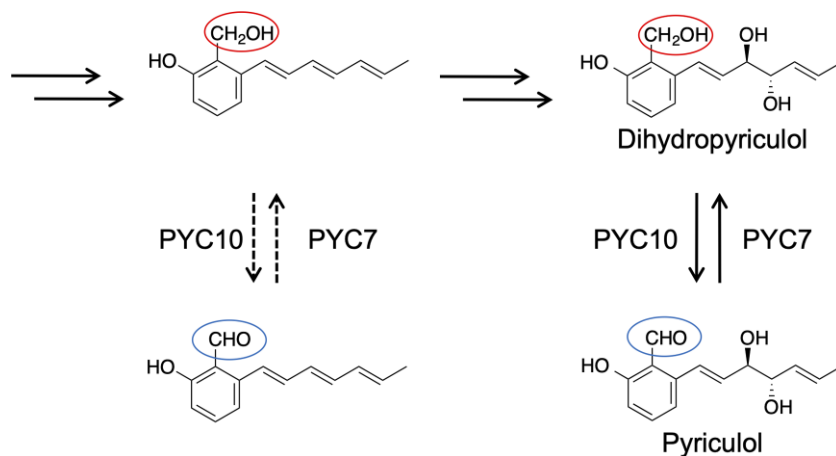


Fig. 8. Interconversion of the two types of pyriculols.

produced by the wild-type strain under the culture conditions used (Jacob et al., 2017). Importantly, the PKS gene knockout strain is as pathogenic as the wild-type strain, demonstrating that pyriculols are not needed for infection (Jacob et al., 2017). Dihydropyriculol is thought to be an inactive analog of the phytotoxin, pyriculol. We found that cycloheximide, an antibiotic with protein biosynthesis inhibitory activity, can induce the production of dihydropyriculol (Furuyama et al., 2021b). We revealed that pyriculol and dihydropyriculol have potent growth inhibitory activity against the actinomycete *S. griseus*, which is a cycloheximide producer. Pyriculol inhibits the growth of *P. oryzae*. Localization analysis of dihydropyriculol indicated that this compound can reach *S. griseus* under confrontation culture. These data suggest that dihydropyriculol can be used as

a chemical weapon against *S. griseus*. Pyriculols may be involved in microbe–microbe interactions. Each pyriculol analog may have different roles, and *P. oryzae* may control its biosynthesis to produce a suitable analog, depending on the ecological context.

Other Secondary Metabolites

Penicillin G production in *P. oryzae* has been reported recently (Saha et al., 2020). Overexpression of a *laeA* homolog (MoLAEA/MGG_07964) induced the production of penicillin G compared to the parental strain. In contrast, the knockdown strain of this gene did not produce penicillin G. The NRPS gene (MGG_14767) was predicted to be involved in penicillin G biosynthesis although it is characteristically different from the *acvA*

(*pcbAB*) gene encoding ACV synthetase. MGG_14767 was upregulated threefold in the MoLAEA-overexpression strain, whereas it was downregulated 3.8-fold in the knockdown strain. Transcriptomic analysis showed that MoLAEA regulates the expression of secondary metabolism genes. Interestingly, the *laeA* homolog (MoLAEA/MGG_07964) is different from the *laeA* homolog (PoLAE1/MGG_01233) for TeA production control. Another *laeA* homolog (PoLAE2/MGG_08161) has also been reported to affect the cAMP signaling pathway and appressorium formation (Prajanket et al., 2020). Multiple *laeA* homologs may be involved in controlling secondary metabolite production.

ACE1 (MGG_12447) is a secondary metabolism gene that encodes a PKS–NRPS hybrid enzyme (Böhnert et al., 2004). However, the chemical structure of the final product is unknown. ACE1 was identified as an avirulence gene, and the *P. oryzae* isolate carrying the ACE1 gene is specifically recognized by rice cultivars carrying the resistance gene Pi33. Defense responses are activated by this recognition in resistant plants. The secondary metabolite whose synthesis is dependent on ACE1 is predicted to be recognized by resistant rice plants. ACE1 is located in a gene cluster consisting of 15 genes that show a rice-infection-specific expression pattern (Collemare et al., 2008b). Of the 15 genes, 14 genes were predicted to be involved in secondary metabolism because they encode for proteins such as a second PKS–NRPS (SYN2), two enoyl reductases (RAP1 and RAP2), and a putative Zn(II)₂Cys₆-type transcription factor (BC2). Heterologous expression of both ACE1 and RAP1 in *Aspergillus oryzae* enables the production of an amide compound that is similar to the PKS–NRPS-derived backbone of cytochalasin (Song et al., 2015). However, bioactivity analysis indicated that the produced compound was not responsible for ACE1-mediated avirulence. These data suggest that the final active product may be a cytochalasin-like compound.

ABM (MGG_04777) is a monooxidase gene located in a putative secondary metabolite BGC with a PKS gene (MGG_04775) (Patkar et al., 2015). Although the role of Abm (antibiotic biosynthesis monooxygenase) in this gene cluster is unclear, it can oxidize endogenous free jasmonic acid (JA) into 12OH-JA in *P. oryzae*, which is secreted during infection and helps evade the defense response by inhibiting JA-signaling induction. Loss of Abm in *P. oryzae* causes accumulation of methyl JA, which induces host defense and blocks fungal infection. In addition, Abm itself is secreted after infection and is predicted to convert plant JA into 12OH-JA to facilitate host colonization.

P. oryzae also produces other plant hormones, such as abscisic acid (ABA) (Jiang et al., 2010), auxins (indole-3-acetic acid [IAA]) (Tanaka et al., 2011a), and cytokinins (CKs) (Jiang et al., 2013), and their biosynthetic genes for ABA and CKs have been described (Chanclud et al., 2016; Spence et al., 2015). Gene disruption of MoABA4/MGG_07514, a homolog of the *Botrytis cinerea* ABA4 gene responsible for ABA biosynthesis, reduces ABA levels by twofold (Spence et al., 2015). The virulence of the Δ Moaba4 strain was strongly reduced, suggesting that ABA contributes to the virulence of *P. oryzae*. CKS1/MGG_04857 encodes a putative tRNA-isopentenyl transferase required for CK biosynthesis (Chanclud et al., 2016). The interaction between the Δ csk1 strain and rice plants was analyzed. This analysis suggested that *P. oryzae*-derived CKs are required for full virulence by affecting nutrient distribution, fungal oxidative stress tolerance, and rice defenses.

Conclusions

P. oryzae has numerous secondary metabolism genes, and some secondary metabolites are expected to be required for rice

infection. In this review, we focused on the biosynthesis and biological roles of secondary metabolites in this fungus. Five groups of secondary metabolites (melanin, TeA, nectriapyrones, pyriculols, and penicillin G) have been indicated to be produced by *P. oryzae*. Biosynthetic genes for three (TeA, nectriapyrone, and penicillin G) of the five groups of secondary metabolites were identified by activating secondary metabolism. TeA and nectriapyrones were induced by genetic modification of TCS factors. Penicillin G was induced by overexpression of a *laeA* homolog. Activation of secondary metabolism is a useful method for the identification of secondary metabolites and their biosynthetic genes. DHN-melanin is a well-characterized secondary metabolite that is required for rice infection. Unexpectedly, identification and analysis of the biosynthetic genes showed that three groups of secondary metabolites (TeA, nectriapyrones, and pyriculols) are not required for rice infection. Nectriapyrones, pyriculols, and penicillin G show antibacterial activity and are predicted to be involved in the interactions with bacteria. Control of plant pathogenic fungi is important in agriculture. Elucidating the roles of secondary metabolites in plant pathogenic fungi will help in the development of agrochemicals.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability

Experimental data are provided in the manuscript. Authors agree to provide any other data if requested.

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