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Controlling the production of phytotoxin pyriculol in *Pyricularia oryzae* by aldehyde reductase

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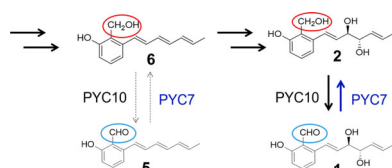
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ABSTRACT

Pyricularia oryzae is one of the most devastating plant pathogens in the world. This fungus produces several secondary metabolites including the phytotoxin pyriculols, which are classified into 2 types: aldehyde form (pyriculol and pyriculariol) and alcohol form (dihydropyriculol and dihydropyriculariol). Although interconversion between the aldehyde form and alcohol form has been predicted, and the *PYC10* gene for the oxidation of alcohol form to aldehyde is known, the gene responsible for the reduction of aldehyde to alcohol form is unknown. Furthermore, previous studies have predicted that alcohol analogs are biosynthesized via aldehyde analogs. Herein, we demonstrated that an aldo/keto reductase *PYC7* is responsible for the reduction of aldehyde to alcohol congeners. The results indicate that aldehyde analogs are biosynthesized via alcohol analogs, contradicting the previous prediction. The results suggest that *P. oryzae* controls the amount of pyriculol analogs using two oxidoreductases, *PYC7* and *PYC10*, thereby controlling the bioactivity of the phytotoxin.

Graphical Abstract



We found that *PYC7* is involved in the reduction of pyriculols. In addition, we revealed that alcohol 2 is the precursor of an aldehyde 1, contrary to the previous prediction.

Keywords: biosynthesis, natural products, *Pyricularia oryzae*, pyriculol

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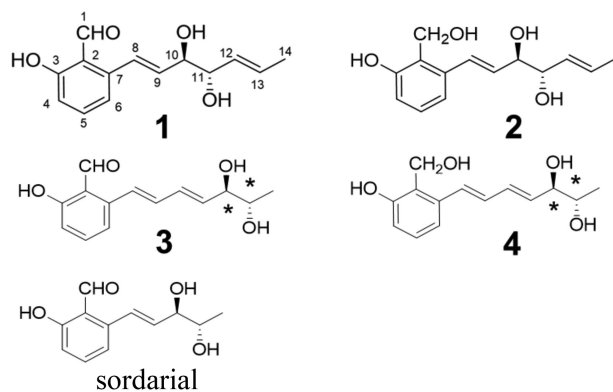


Figure 1. Structures of pyriculols and a related compound (sordarial). The stereochemical configurations shown at the asterisks indicate relative stereochemistry.

Filamentous fungi typically produce a variety of secondary metabolites (SMs). It is known that some of these SMs play pivotal roles in fungal life cycles and affect surrounding ecosystems (Macheleidt et al., 2016, Keller, 2019). For example, certain SMs produced by plant pathogens are thought to be phytotoxins (Horbach et al., 2011). To develop a method for controlling plant pathogenic fungi, elucidating the biological function of such SMs in their life cycle is of vital importance. *Pyricularia oryzae* is one of the most devastating plant pathogens in the world (Dean et al., 2012). Pyriculol (1) (Iwasaki et al., 1973) is a well-known SM produced by *P. oryzae*. Several analogs of pyriculol have been noted; the main analogs are dihydropyriculol (2) (Kono et al., 1991), pyriculariol (3) (Nukina et al., 1981), and dihydropyriculariol (4) (Tanaka et al., 2011; Figure 1). The pyriculols are classified into two groups: aldehyde-type (pyriculol and pyriculariol) and alcohol-type (dihydropyriculol and dihydropyriculariol). Some studies have demonstrated that aldehyde derivatives induce lesion-like necrosis on rice leaves, while alcohol derivatives are inactive in this aspect (Kono et al., 1991, Tanaka et al., 2011, Jacob et al., 2017).

In axenic cultures, four analogs are produced at a time, indicating interconversion between oxidized aldehyde analogs and reduced alcohol derivatives (Jacob et al., 2017). This leads to the question: why and how *P. oryzae* produces both alcohol and aldehyde analogs? Identification of the genes responsible for this oxidoreductive conversion will provide an answer to this question. The polyketide synthase (PKS) gene and other genes responsible for the biosynthesis of pyriculols have been reported (Jacob et al., 2017). It has been suggested that aldehyde-type analogs are produced first, followed by the reduction reaction to yield alcohol analogs (Tanaka et al., 2011). Although the gene (MGG_10961/PYC10) responsible for the oxidation of alcohol derivatives to aldehyde derivatives has been reported (Jacob et al., 2017), the gene catalyzing the adverse reductive reaction has not yet been identified. In *Neurospora crassa*, the biosynthetic mechanism of a structurally related salicylaldehyde sordarial (Figure 1) has been reported (Zhao et al., 2019), but the gene responsible for the reduction of the aldehyde moiety has not been identified.

In this study, we identified the gene for the reduction of the pyriculol congeners in *P. oryzae*. We demonstrated that the alcohol-type compound dihydropyriculol is a precursor of the aldehyde-type compound pyriculol, contrary to the previous prediction (Tanaka et al., 2011, Jacob et al., 2017).

Materials and methods

Strains, media, culture, and transformation

P. oryzae Kita1 (Hoku1) was used as the parental strain. *P. oryzae* was grown either on OMA plates (5% oatmeal agar; Difco Co., Franklin Lakes, NJ), in PDB medium with 0.1% agar [2.4% potato dextrose broth (Difco Co.), 0.1% agar] or in FDY medium (1.5% glucose, 1.5% soluble starch, 1% corn steep liquor, 1% dried yeast, 0.3% malt extract, 0.03% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% KH_2PO_4 , 0.1% agar, pH 6.0) at 25 or 28 °C. *P. oryzae* was transformed by *Agrobacterium tumefaciens* mediated transformation (ATMT), as described previously (Motoyama et al., 1998). *A. tumefaciens* strain C58 was cultured with a conidial suspension of *P. oryzae* that was prepared as described previously (Sambrook et al., 1989). Transformants were selected by 500 $\mu\text{g}/\text{mL}$ hygromycin B (Wako Pure Chemical Co.). In subsequent cultures of *P. oryzae* transformants, the appropriate selection reagent (200 $\mu\text{g}/\text{mL}$ hygromycin B) was added as required. Plasmids were amplified in *Escherichia coli* DH5 α . *E. coli* was cultured in lysogeny broth (LB), and the transformation was performed by following the standard method (Sambrook et al., 1989).

DNA manipulations

Total DNA was extracted from *P. oryzae* using the DNeasy plant total DNA isolation kit (Qiagen KK, Tokyo, Japan). PCR amplifications were performed using a thermal cycler (Model PTC-200, Bio-Rad Laboratories, Inc., Hercules, CA) and either KOD Plus Neo (Toyobo, Tokyo, Japan) or KOD FX Neo (Toyobo) DNA polymerases. KOD Plus Neo is a proofreading DNA polymerase that was used for plasmid construction, and KOD FX Neo was used for routine PCR. The PCR products were cloned using the InFusion system (Takara Shuzo, Kyoto, Japan) and sequenced, where necessary. Sequencing reactions were performed using BigDye Terminator version 3.1 cycle sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ). Gene prediction was performed by using 2ndFind (<http://biosyn.nih.gov.jp/2ndfind/>).

Gene disruption

The target genes were disrupted by exchanging a whole-gene ORF with the hygromycin B-resistance gene expression unit via homologous recombination. Gene disruptants were constructed as follows. The primers used are listed in Table S1. Two kilobases located upstream of each gene were amplified from the genomic DNA of *P. oryzae* via PCR with the primers InFu_RB-5' gene_F and InFu_3HPH-5' gene_R (fragment 1). The hygromycin B resistance gene expression unit from pCSN45 (Motoyama et al., 2005) was amplified via PCR with primers 5HPH and 3HPH (fragment 2). Two kilobases located downstream of each gene were amplified from the genomic DNA of *P. oryzae* via PCR with the primers InFu_LB-3' gene_F and InFu_RB-5' gene_R (fragment 3). The vector sequence of pBI121 (Clontech) between the right and left borders was amplified via PCR with primers pBI121-RB and pBI121-LB (fragment 4). All fragments were gel purified and cloned using the In-Fusion cloning technology to yield gene knockout vectors. The In-Fusion reaction mixtures were used to transform *E. coli* DH5 α , and the transformants were selected with kanamycin (50 $\mu\text{g}/\text{mL}$). After verification of the DNA sequence, plasmid DNA containing the correct insert was transformed into *A. tumefaciens*, and the transformants were used for ATMT. Target gene disruptants were selected via PCR with primers gene number-check-F and gene number-check-R that hybridize just

upstream and downstream of the deleted ORF, respectively. This primer set can amplify the following sequences: the ORF from the wild-type strain, the 1.6-kb hygromycin B-resistance gene expression unit from gene disruptants, and both fragments from ectopic transformants (Figures S1–S3). In the case of PYC10 gene knockout, the PCR products were digested with *Bgl*II, yielded 0.5- and 1.2-kb fragments from the ORF and an intact 1.6-kb fragment from the hygromycin B-resistance gene expression unit.

DNA microarray analysis

The wild-type strain was cultured in FDY medium and potato dextrose broth medium with 0.1% agar for 2 days at 28°C. RNA extraction and DNA microarray analysis were performed as described in previous reports (Yun et al., 2015, Motoyama et al., 2019).

Analysis of the metabolites

The Waters Acquity UPLC H-Class system equipped with a mass spectrometer (API 3200, Applied Biosystems) was used to analyze *P. oryzae* metabolites. A reversed-phase column (BEH C18, 2.1 × 50 mm, 1.7 μm, Waters, Milford, MA) was used with a flow rate of 0.5 mL/min. The gradient system was MeCN (solvent B) in 0.05% aqueous formic acid (solvent A): 5–100% B from 0 to 4 min and 100% B from 4 to 6 min. Negative ion electrospray ionization method was used for the detection of the pyriculols.

Isolation and characterization of compounds

Pyriculol (1) was isolated from the wild-type strain. This strain was inoculated in 500 mL PDB + 0.1% agar liquid medium in a 2 L Erlenmeyer flask. All cultures were incubated at 25°C for 7 days. The culture broth was extracted with ethyl acetate to obtain the organic extract. It was subjected to silica gel column chromatography and eluted with CHCl₃/MeOH stepwise gradient to yield eight fractions. The fraction containing compound 1 was further purified by C18-HPLC, eluting with a linear gradient of MeOH/H₂O (PEGASIL ODS SP100 column, Φ10 × 250 mm, Senshu Scientific Co. Ltd., Tokyo, Japan) to obtain compound 1 (0.9 mg) as an amorphous solid. The NMR data, obtained using a JEOL JNM-ECA-500 spectrometer (JEOL, Tokyo, Japan) at 500 MHz for ¹H NMR and 125 MHz for ¹³C NMR, were in accordance with the previously reported data (Kim et al., 1998, Jacob et al., 2017). Optical rotation was recorded on a HORIBA SEPA-300 High Sensitivity Polarimeter (HORIBA, Kyoto, Japan). High resolution mass spectra were recorded on a Waters IMS Vion QToF coupled to a Waters UPLC H-class system.

Pyriculol (1); [α]^{23.0}_D + 147.0° (c 0.09 CHCl₃). HR-ESI-TOF-MS found *m/z*: 247.0971 [M-H]⁻, calculated for C₁₄H₁₅O₄: 247.0975. ¹H NMR (CDCl₃) (Figure S1) δ_H: 11.84 (s, 1H, 3-OH), 10.29 (s, 1H, H-1), 6.89 (1H, d, 7.0, H-4), 7.43 (1H, t, 7.8, H-5), 6.87 (1H, d, 8.8, H-6), 7.14 (1H, d, 15.5, H-8), 6.09 (1H, dd, 16.0, 6.0, H-9), 4.37 (1H, br s, H-10), 4.21 (1H, br s, H-11), 5.55 (1H, ddq, 15.5, 7, 2, H-12), 5.82 (1H, m, H-13), 1.73 (3H, dd, 6.5, 1.5, H-14), 2.28 (1H, br s, OH). ¹³C NMR (CDCl₃) (Figure S2) δ_C: 195.6 (C-1), 142.6 (C-2), 163 (C-3), 119.1 (C-4), 137.3 (C-5), 117.3 (C-7), 126.8 (C-8), 135.4 (C-9), 75.4 (C-10), 77.2 (C-11), 130.9 (C-12), 128.9 (C-13), 18.1 (C-14).

Dihydropyriculol (2) was isolated from the Δ*osm1* Δ*Poypd1* strain (Motoyama et al., 2019). This strain was cultured at 28°C for 3 days in 10 cylindrical flasks containing 70 mL of FDY medium. The culture (700 mL) was treated with 2 volumes of acetone and subsequently filtered to remove mycelia. The supernatant was

concentrated in *vacuo* to remove acetone. The resulting aqueous solution was extracted twice with 1 L ethyl acetate and once with 1 L CHCl₃. The organic layer was combined and dried in *vacuo*. Half of the dried material was separated by C18-HPLC (PEGASIL ODS, 20 × 250 mm, Senshu Scientific Co. Ltd., Tokyo, Japan) eluted with MeCN/H₂O gradient system at a flow rate of 8 mL/min. A fraction containing compound 2 was purified by C18-HPLC using the same column by isocratic elution with 20% MeCN at a flow rate of 8 mL/min to afford 10.0 mg of the compound 2 as an amorphous solid. The NMR and optical rotation data obtained were in accordance with the previously published data (Kim et al., 1998, Jacob et al., 2017, Masi et al., 2020).

Dihydropyriculol (2); [α]^{22.8}_D + 11.5 (c 0.1 EtOH). HR-ESI-TOF-MS found *m/z*: 249.1125 [M-H]⁻, calculated for C₁₄H₁₇O₄: 249.1132. ¹H NMR (CD₃OD) (Figure S3) δ_H: 4.73 (s, 2H, H-1), 6.67 (1H, d, 8.0, H-4), 7.02 (1H, t, 8.0, H-5), 6.94 (1H, d, 8.0, H-6), 6.92 (1H, d, 15.5, H-8), 6.10 (1H, dd, 15.5, 6.5, H-9), 4.10 (1H, dd, 6.5, 5.5, H-10), 3.99 (1H, dd, 6.5, 5.5, H-11), 5.55 (1H, dd, 14.5, 6.5, H-12), 5.70 (1H, dq, 14.5, 6.2, H-13), 1.68 (3H, d, 6.2, H-14). ¹³C-NMR (CD₃OD) (Figure S4) δ_C: 56.7 (C-1), 125.2 (C-2), 157.3 (C-3), 115.4 (C-4), 129.7 (C-5), 118.7 (C-6), 139.7 (C-7), 130.5 (C-8), 132.6 (C-9), 77.0 and 77.1 (C-10 and C-11), 131.7 (C-12), 129.2 (C-13), 18.1 (C-14).

Protein expression using a wheat germ cell-free system

PYC7 (MGG_16813) was amplified from the cDNA of *P. oryzae* via PCR with primers Infu-pEU_Nhis_PYC7F and Infu-pEU_Nhis_PYC7R. The amplified fragment was inserted into the pEU01 vector (Cell-Free Science, Matsuyama, Japan) amplified with primers Liner_pEU_Nhis_R and Liner_pEU_Nhis_F using the In-Fusion HD cloning system (Clontech). The resulting expression plasmid for 6 × His-tagged PYC7 was designated as pEU01_PYC7. Cell-free protein synthesis with wheat embryo extracts was performed with a WEPRO7240 Expression kit (CellFree Sciences) using pEU01_PYC7 in a 226-mL bilayer format on 96-well flat-bottom plates (Iwaki) according to the manufacturer's instructions. The solubility of the translation product was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The reaction mixture was centrifuged at 13,000 × *g* for 30 min at 4°C, and the supernatant was used for the subsequent *in vitro* enzyme assay.

In vitro enzyme assay

PYC7 activity assay was conducted in a 100 μL reaction mixture containing 100 mM potassium phosphate (pH 7.5), 4 mM NADPH (Sigma-Aldrich), 5 μL soluble crude proteins containing PYC7, and 1 mM 5 or 1. The reaction was performed at 25°C, followed by quenching and subsequent extraction with ethyl acetate. Ethyl acetate was evaporated with a N₂ stream, and finally dissolved in 200 μL methanol for UPLC-MS analysis.

Results

Identification of the PYC7 gene in *P. oryzae*

We performed DNA microarray analysis to identify biosynthetic genes for the pyriculols. We compared the expression of genes around the PKS gene (MGG_10912/MoPKS19) for the biosynthesis of the pyriculols under a condition inducing the production of pyriculols (PDB + 0.1% agar) as well as a non-inducing condition (FDY) (Figure S5). We predicted that ten genes are involved in the biosynthesis of the pyriculols (Figure 2). We named them PYC genes in this study (Table 1 and Figure 3). *N. crassa* has

Table 1. Gene names and putative functions of the pyriculol biosynthesis genes

MGG-no.	Gene name in previous study (Jacob et al. 2017)	Gene name in this study	Protein size (aa)	Protein domains (pfam)	Putative function	High homology protein (SWISS-PROT)
MGG_10912	MoPKS19	PYC1	2571	ADH_N Acyl_transf_1 KR Ketoacyl-synt_C PP-binding PS-DH adh_short ketoacyl-synt Cupin_2	Polyketide synthase (PKS)	Highly reducing polyketide synthase (G4N296.1)
MGG_10911	Hypothetical protein	PYC2	198			
MGG_10910	MoC10RED3	PYC3	277	adh_short	Cupin domain containing protein short chain dehydrogenase (SDR)	Cupin-domain-containing oxidoreductase (G9N4B0.1) Short-chain dehydrogenase RED3 (G4N292.1)
MGG_12981	Hypothetical protein	PYC4	205	Cupin_2	Cupin domain containing protein	Cupin-domain-containing oxidoreductase (G9N4B0.1)
MGG_12982	MoC19RED2	PYC5	316	adh_short	short chain dehydrogenase (SDR)	Short-chain dehydrogenase RED2 (G4N290.1)
MGG_15114	Hypothetical protein	PYC6	458	FAD_binding_3 Lycopene_cycl	FAD binding domain containing protein	FAD-dependent monoxygenase (A0A3G9GX61.1)
MGG_16813	Hypothetical protein	PYC7	324	Aldo_ket_red	Aldo/keto Reductase	Pyridoxal reductase (O14295.1)
MGG_16812	MoC19OXR2	PYC8	520	BBE FAD_binding_4	FAD binding domain containing protein	FAD-linked oxidoreductase OXR2 (G4N287.1 FA)
MGG_12983	MoC19RED1	PYC9	299	adh_short	short chain dehydrogenase (SDR)	Short-chain dehydrogenase RED1 (G4N286.1)
MGG_10961	MoC19OXR1	PYC10	507	BBE FAD_binding_4	FAD binding domain containing protein	FAD-linked oxidoreductase OXR (G4N285.1)

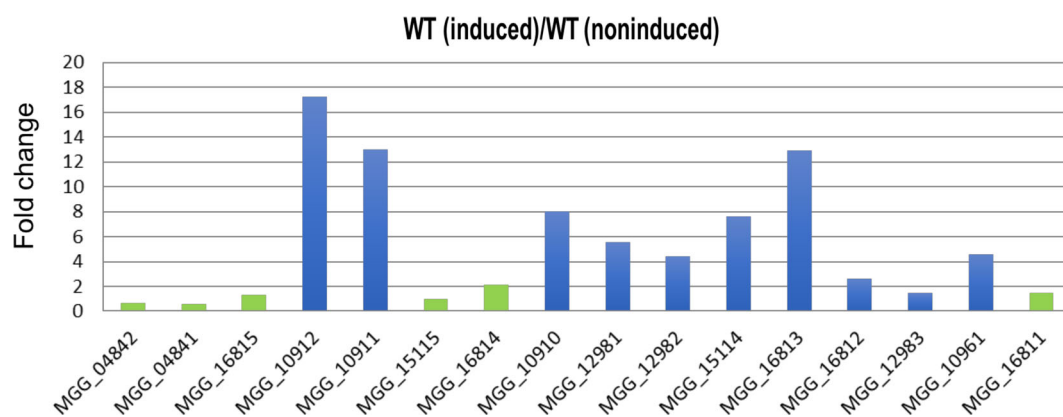


Figure 2. DNA microarray analysis of MGG_10912 (*MoPKS19*) and neighboring genes. The figure shows relative gene expression of the wild-type strain cultured in the condition inducing pyricularin production (PDB + 0.1% agar) and non-inducing condition (FDY). Vertical axis represents fold change of gene expression.

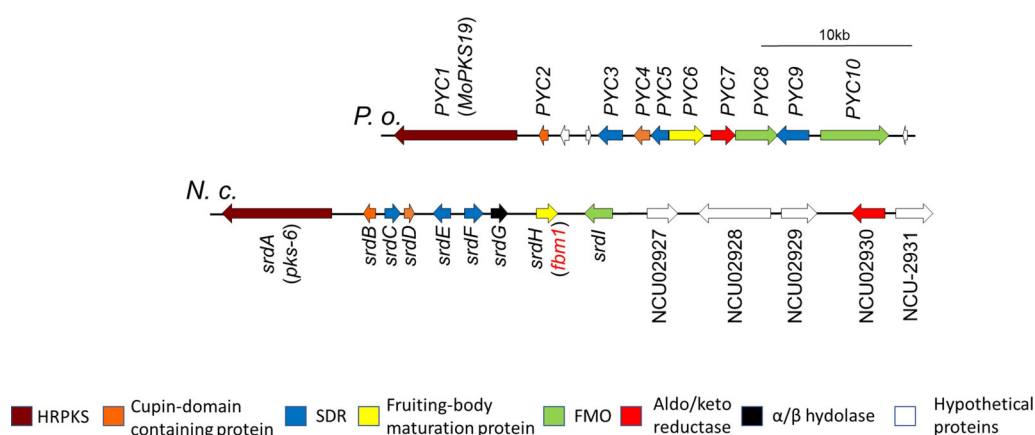


Figure 3. Comparison between the *P. oryzae* *MoPKS19* gene cluster (*PYC* gene cluster) and *N. crassa* *pks-6* gene cluster (*srd* gene cluster).

homologous genes (*srd* genes) and is involved in the biosynthesis of sordarial (Figure 3; Zhao et al., 2019), which is an analog of pyricularin. The sordarials are also classified into 2 groups: aldehyde-type and alcohol-type. However, the gene responsible for the reduction reaction has not yet been identified. In another study, *PYC3* (MGG_10910/*MoC19RED3*) was expected to be responsible for the conversion of aldehyde-type analogs to alcohol-type analogs in *P. oryzae* (Jacob et al., 2017). We disrupted *PYC3* in *P. oryzae* (Figure S6). Regardless, the production of alcohol 2 was still observed in $\Delta pyc3$ strain (Figure S7), indicating that this gene is not required to produce alcohol-type derivatives. One of the hypothetical protein gene *PYC7* (MGG_16813) possesses an aldo/keto reductase domain (Table 1). We hypothesized that this gene would be involved in the reduction of the aldehyde residue of the pyricularins.

Functional analysis of *PYC7*

To analyze the function of *PYC7*, we disrupted *PYC7* in *P. oryzae* (Figure S8). The $\Delta pyc7$ strain was cultured for 7 days in PDB medium with 0.1% agar, which is a compound 2 producing medium. Under this condition, the wild-type strain mainly produced compound 2 and an unidentified compound 2'. Analysis of the organic extract of the $\Delta pyc7$ strain demonstrated the emergence of other compounds, 1 and 1' (Figure 4). We purified and identified one of these compounds as 1 by comparing the NMR spectra. UV and mass spectra suggested that compounds

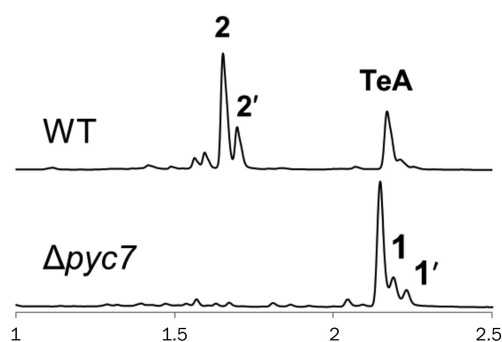


Figure 4. Metabolite analysis of *PYC7* knockout strain. The wild-type strain and the $\Delta pyc7$ strain were cultured in PDB medium with 0.1% agar for 7 days, and the cultures were analyzed by UPLC. Pyricularin (1) and dihydropyricularin (2) were detected at OD 293. 1' and 2' are putative diastereomers of 1 and 2, respectively. TeA represents tenuazonic acid.

1' and 2' are isomers of 1 and 2, respectively (Figures S9 and S10). We speculate that 1' and 2' are epipyricularin (Kono et al., 1991) and epidihydropyricularin (Kono et al., 1991; Masi et al., 2020), respectively. In addition, the BLAST search of the homologous gene of *PYC7* in *N. crassa* demonstrated that a homologous gene *NCU02930* is located close to the *srd* genes (Figure 3). Next, we attempted to express the *PYC7* gene in *E. coli* to obtain *PYC7* recombinant protein. However, we could not express *PYC7* as a soluble protein in this system. We successfully expressed *PYC7*

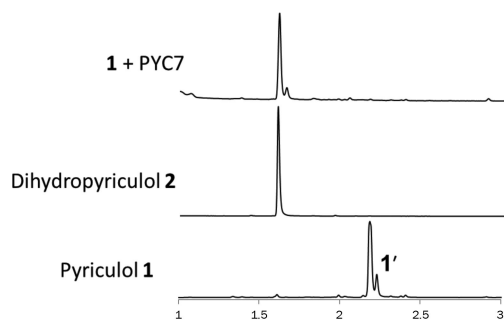


Figure 5. *In vitro* assay of PYC7 with 1. UPLC analysis of *in vitro* conversion assay leading to the conversion of pyriculol (1) to dihydropyriculol (2) using crude expression mix containing PYC7. Products were detected at OD 293 and identified by comparison with purified compounds.

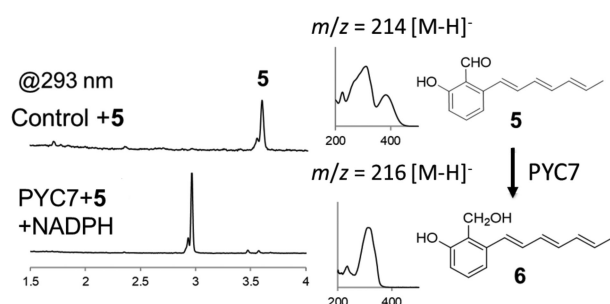


Figure 6. *In vitro* assay of PYC7 with the putative intermediate compound 5. UPLC analysis of *in vitro* 5 to 6 conversion assays using crude expression mix containing PYC7. The structure of 6 was predicted by MS (Figure S4).

protein using the wheat germ cell-free protein synthesis system (Figure S11). PYC7 recombinant protein expressed in the cell-free system converted compound 1 to 2 *in vitro* (Figure 5). The aldo/keto reductase family possess a NADPH-binding motif. Therefore, NADPH was used as a cofactor in this assay. Kiyota's group reported the structure of the putative intermediate compound (5) of the pyriculols (Figure 6), and the involvement of a similar intermediate was predicted in sordarial biosynthesis (Tanaka et al., 2011, Zhao et al., 2019). Thus, we conducted an *in vitro* compound 5 conversion assay using PYC7. PYC7 converted α -deuterated 5 (5-*d*, m/z 214 [M-H]⁻, Figure S12; Tanaka et al., 2011) to another compound (Figure 6). Mass chromatogram (m/z 216 [M-H]⁻, Figure S13) suggested that the product of the conversion reaction was 6-*d*. These results indicate that PYC7 reduces the aldehyde residue of the pyriculols. Next, the conversion efficiencies of 1 and 5 were compared. Time course analysis indicated that 1 was a better substrate than 5 (Figure S14). To analyze cofactor specificity, NADH was added to the reaction mixture. The result showed that NADH can also be used as a cofactor (Figure S15). In addition, 5-*d* was subjected to a bio-transformation experiment. A solution of 5-*d* in MeOH was introduced into the culture broth of WT or $\Delta pyc7$ strains. Each broth was extracted with EtOH and analyzed. The extract of the WT strain contained labeled 2 (2-*d*), while the extract of the $\Delta pyc7$ strain did not contain 2-*d* (Figure S16). This indicated that the WT strain can convert 5-*d* but the $\Delta pyc7$ strain cannot.

Analysis of the biosynthetic mechanism of pyriculols using gene disruptants

To determine the early intermediate compound, we analyzed the extract obtained from the culture broth of $\Delta pyc7$ strain at

different time points. Interestingly, the $\Delta pyc7$ strain produced alcohol compound 2 on the third day. Following that, on day 10 aldehyde compound 1 was observed instead of compound 2 (Figure 7). These results indicated that alcohol compound 2 can be produced without PYC7 and then converted to aldehyde compound 1. A recent study demonstrated that the biosynthetic intermediate of flavoglucan, a pyriculol-related compound, is released as an alcohol by SDR (FogD) from PKS (FogA) in *Aspergillus ruber* (Nies et al., 2020). PYC5 is a homolog of FogD in *P. oryzae*. The $\Delta pyc5$ strain did not produce pyriculols (Figure S17), suggesting that the polyketide chain is released from PYC1 by PYC5. To analyze the release product from PKS (PYC1), two types of mutant strains were constructed (Figures S18 and S19). The only PYC1-expressing strain ($\Delta pyc2-10$) did not produce pyriculols (Figure S18). The PYC1 and PYC5-expressing strain ($\Delta pyc2-10::PYC5$) also did not produce pyriculols (Figure S19). However, this strain produced new peaks (Figure S19). These new peaks are predicted to be the release product from PYC1 or its derivatives. Although further studies about structural determination of these metabolites are needed, our data suggest that the polyketide chain is released from PYC1 by PYC5.

Discussion

It was predicted earlier that in *P. oryzae*, compound 1 is first produced from an aldehyde intermediate compound 5, and subsequently reduced to yield compound 2 (Tanaka et al., 2011). However, in *N. crassa*, an aldehyde sordarial was predicted to be the final product (Zhao et al., 2019). In addition, the interconversion of aldehyde and alcohol analogs in *P. oryzae* has been predicted (Jacob et al., 2017). These studies raise a question: which analog is the final product? This question is related to the biological function of the pyriculols. Based on previous results, we predicted that analyzing the interconversion mechanism would be helpful in answering this question. To analyze the interconversion mechanism, the identification of the gene responsible for the enzyme catalyzing the reduction of aldehyde to alcohol is necessary.

In this study, we demonstrated that the PYC7 gene in the pyriculol biosynthetic gene cluster is involved in this reduction reaction. By performing an *in vitro* assay, we confirmed that PYC7 is responsible for the reduction of the aldehyde residue of pyriculols (1 and 5, Figures 5 and 6), indicating that the reduction of the aldehyde moiety is controlled by an enzymatic reaction. Interestingly, the $\Delta pyc7$ strain could produce alcohol compound 2 in the early phase of culture (Figure 7), indicating that the reduction reaction by PYC7 is not required for the biosynthesis of alcohol compound 2. Based on these data, we propose a new biosynthetic pathway for the pyriculols (Figure 8). To form an alcohol compound 2 without reduction by PYC7, the presence of alcohol-type intermediates is required. Based on this fact, we predicted that the polyketide chain is released as an alcohol from PKS. The remaining enzymes modify this intermediate to yield compound 2. PYC10 then oxidizes compound 2 to 1. In this model, compound 2 is a biosynthetic intermediate of compound 1 and is produced first from an alcohol-type intermediate compound 6. PYC10 oxidizes compound 2 to 1 and PYC7 reduces compound 1 to 2. We propose that the wild-type strain mainly produces compound 2 under our culture condition as the reduction reaction by PYC7 is dominant. We also propose that the $\Delta pyc7$ strain accumulates compound 1 as PYC7 is absent and PYC10 is active. Kiyota and colleagues observed the conversion of a putative biosynthetic intermediate compound 5 to the pyriculols and concluded that compound 5 is a

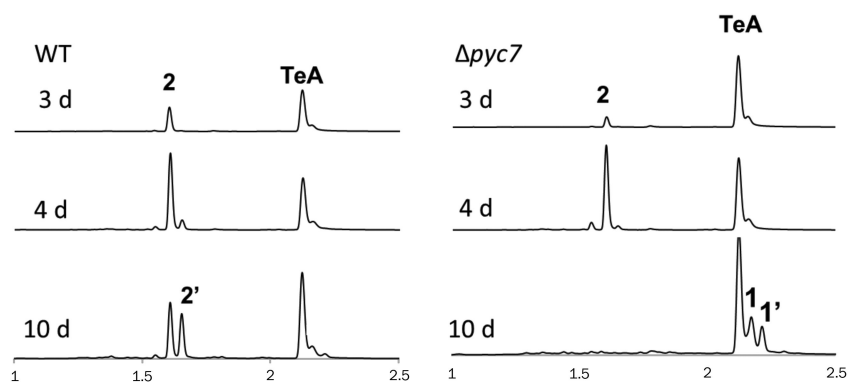


Figure 7. Time-course metabolite analysis of the wild-type strain and the $\Delta pyc7$ strain. The wild type strain and the $\Delta pyc7$ strain were cultured in PDB medium with 0.1% agar and sampled on days 3, 4, and 10. The samples were analyzed by UPLC. Pyriculol (1) and dihydropyriculol (2) were detected at OD 293. 1' and 2' are putative diastereomers of 1 and 2, respectively. TeA represents tenuazonic acid.

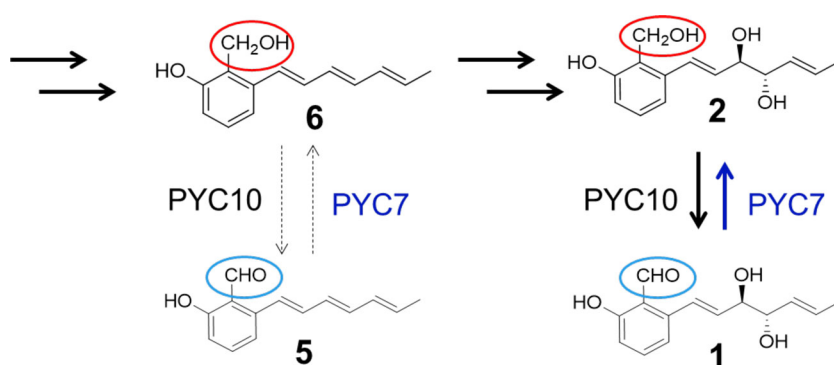


Figure 8. Proposed new biosynthetic pathway for the pyriculols in *P. oryzae*.

biosynthetic intermediate (Tanaka et al., 2011). This conversion can be explained by the new biosynthetic model in this study (Figure 8) In our model, compound 5 can be converted to compound 6 by PYC7 and then converted to compound 2 and 1. We analyzed the conversion of 5-d in $\Delta pyc7$ and found that the $\Delta pyc7$ strain cannot convert 5-d (Figure S16), supporting our biosynthesis model in Figure 8.

In pyriculol biosynthesis, we proposed that the polyketide chain is released as an alcohol from PKS. As shown before, a recent study showed that the pyriculol-related compound flavoglaucin is biosynthesized via alcohol intermediates in *Aspergillus ruber* (Nies et al., 2020). In sordarial biosynthesis in *N. crassa*, it was predicted that a similar alcohol-type intermediate is produced from an aldehyde congener by an endogenous reductase enzyme (Zhao et al., 2019). In addition, the BLAST search revealed that *N. crassa* has a PYC7 homolog (NCU02930) and a PYC10 homolog (*srdI*). Therefore, we speculate that *N. crassa* also employs a similar mechanism. We also speculate that an alcohol-type compound might be the release product from PKS (SrdA). In the current model of sordarial biosynthesis, it was predicted that an aldehyde-type intermediate is released from PKS (SrdA) and cyclized by SrdC/D/E. These proteins are homologs of PYC9, 4, and 5, respectively. SrbB, a homolog of PYC2, is predicted to be involved in following epoxidation reaction. In this pathway, an alcohol-type intermediate is thought to be produced from an aldehyde congener by an endogenous reductase. However, this endogenous reductase gene has not yet been identified. *N. crassa* has a PYC7 homolog (NCU02930) and we speculate that this gene has the same function as PYC7 in *N. crassa*. A PYC10 homolog (*srdI*) is predicted to be in-

involved in the oxidation of this alcohol-type intermediate (Nies et al., 2020).

Our data suggest that alcohol 2 is a precursor of aldehyde 1. If so, why does this fungus reduce 1 to 2 again by an enzymatic reaction? In a previous study, it was reported that compound 1 inhibited the germination of *P. oryzae*, whereas compound 2 did not demonstrate this activity (Namai et al., 1996), suggesting that aldehyde derivatives are toxic to *P. oryzae* itself. We predict that PYC7 and PYC10 are involved in the control of equilibrium between compounds 2 and 1 to avoid the accumulation of toxic compounds. *P. oryzae* may stock a non-active form and quickly convert to the active form when they need to produce phytotoxins.

In summary, we observed that PYC7 is involved in the reduction of pyriculols. In addition, we revealed that alcohol 2 is the precursor of an aldehyde 1. Our results suggest that this fungus controls the amount of pyriculol analogs by 2 oxidoreductases PYC7 and PYC10, as the aldehyde form is toxic to *P. oryzae* itself.

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Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Author contribution

Y.F. and T.N. purified and determined the chemical structures of the compounds. Y.F. and T.M. isolated and analyzed the biosynthetic genes. H.K. synthesized compound 5. T.M., T.K., and H.O. designed the research. Y.F. and T.M. wrote the paper.

Disclosure statement

No potential conflict of interest was reported by the authors.

References

- Dean R, Van Kan JAL, Pretorius ZA et al. The top 10 fungal pathogens in molecular plant pathology. *Mol Plant Pathol* 2012;**13**:414-30.
- Horbach R, Navarro-Quesada AR, Knogge W et al. When and how to kill a plant cell: Infection strategies of plant pathogenic fungi. *J Plant Physiol* 2011;**168**:51-62.
- Iwasaki S, Muro H, Sasaki K et al. Isolations of phytotoxic substances produced by *Pyricularia oryzae* cavara. *Tetrahedron Lett* 1973;**14**:3537-42.
- Jacob S, Grötsch T, Foster AJ et al. Unravelling the biosynthesis of pyriculol in the rice blast fungus *Magnaporthe oryzae*. *Microbiology* 2017;**163**:541-53.
- Keller NP. Fungal secondary metabolism: regulation, function and drug discovery. *Nat Rev Microbiol* 2019;**17**:167-80.
- Kim JC, Mir JY, Kim HT et al. Pyriculol, a new phytotoxin from *Magnaporthe grisea*. *Biosci Biotechnol Biochem* 1998;**62**:173-4.
- Kono Y, Sekido S, Yamaguchi I et al. Structures of two novel pyriculol-related compounds and identification of naturally produced epipyriculol from *Pyricularia oryzae*. *Agric Biol Chem* 1991;**55**:2785-91.
- Macheleidt J, Mattern DJ, Fischer J et al. Regulation and role of fungal secondary metabolites. *Annu Rev Genet*. 2016;**50**:371-92.
- Masi M, Santoro E, Clement S et al. Further secondary metabolites produced by the fungus *Pyricularia grisea* isolated from buffelgrass (*Cenchrus ciliaris*). *Chirality* 2020;**1**-9.
- Motoyama T, Imanishi K, Kinbara T et al. Inhibition of scytalone dehydratase in melanin biosynthesis by carpropamid, a novel rice blast controlling agent. *J Pest Sci* 1998;**23**:58-61.
- Motoyama T, Kadokura K, Ohira T et al. A two-component histidine kinase of the rice blast fungus is involved in osmotic stress response and fungicide action. *Fungal Genet Biol* 2005;**42**:200-12.
- Motoyama T, Nogawa T, Hayashi T et al. Induction of nectriapyrone biosynthesis in the rice blast fungus *Pyricularia oryzae* by disturbance of the two-component signal transduction system. *ChemBioChem*. 2019;**20**:693-700.
- Namai T, Nukina M, Togashi J. Effects of two toxins and a derivative of one toxin produced by rice blast fungus on its infection to inner epidermal tissue of rice leaf sheath. *Jpn J Phytopathol* 1996;**62**:114-8.
- Nies J, Ran H, Wohlgenuth V et al. Biosynthesis of the prenylated salicylaldehyde flavoglucan requires temporary reduction to salicyl alcohol for decoration before reoxidation to the final product. *Org Lett* 2020;**22**:2256-60.
- Nukina M, Sassa T, Ikeda M et al. Pyriculariol, a new phytotoxic metabolite of *Pyricularia oryzae* cavara. *Agric Biol Chem*. 1981;**45**:2161-2.
- Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. 1989.
- Tanaka K, Sasaki A, Cao HQ et al. Synthesis and biotransformation of plausible biosynthetic intermediates of salicylaldehyde-type phytotoxins of rice blast fungus, *Magnaporthe grisea*. *Eur J Org Chem* 2011;**2011**:6276-80.
- Yun CS, Motoyama T, Osada H. Biosynthesis of the mycotoxin tenuazonic acid by a fungal NRPS-PKS hybrid enzyme. *Nat Commun* 2015;**6**:1-9.
- Zhao Z, Ying Y, Hung Y-S et al. Genome mining reveals *Neurospora crassa* can produce the salicylaldehyde sordarial. *J Nat Prod* 2019;**82**:1029-33.