Regular Article

The target site of the novel fungicide quinofumelin, *Pyricularia oryzae* class II dihydroorotate dehydrogenase

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Ⓢ *Supplementary material*

The target site of the novel fungicide quinofumelin was investigated in the rice blast fungus *Pyricularia oryzae*. Quinofumelin-induced mycelial growth inhibition was reversed by orotate but not by dihydroorotate. Recovery tests suggested that the target site of quinofumelin was dihydroorotate dehydrogenase (DHODH), which catalyzes the oxidation of dihydroorotate to orotate. Quinofumelin strongly inhibited *P. oryzae* class 2 DHODH (DHODH II) (IC$_{50}$: 2.8 nM). The inhibitory activities of mycelial growth and DHODH II were strongly positively correlated, indicating that DHODH II inhibition by quinofumelin lead to antifungal activity. A *P. oryzae* DHODH II gene (*PoPYR4*) disruption mutant (*ΔPopyr4*), showing the same tendency as the quinofumelin-treated wild strain in recovery tests, was constructed, and disease symptoms were not observed in rice plants infected by *ΔPopyr4*. Thus, DHODH II, which plays an important role in pathogenicity and mycelial growth, is found to be the target site of quinofumelin.
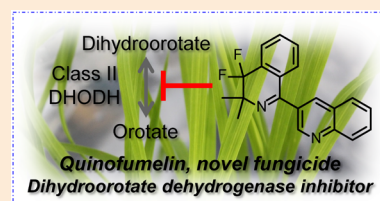
Keywords: pyrimidine biosynthesis, dihydroorotate dehydrogenase, quinofumelin, *Pyricularia*, rice blast.

Dihydroorotate
Class II DHODH
Orotate
Quinofumelin, novel fungicide
Dihydroorotate dehydrogenase inhibitor

Introduction

Plant pathogens reduce agricultural production, causing substantial economic losses and threatening global food security. Preferably, food production should be increased by reducing losses and retaining natural ecosystems rather than by increasing crop areas. Fungicides help farmers increase food production on less land by protecting crops from disease; therefore, they play an important role in improving global food security. However, the effectiveness of fungicides is threatened by the evolution of resistant fungal strains. Indeed, various resistance mechanisms exist, such as target site mutations and metabolic breakdown. Fungicide resistance mostly appears to evolve by mutations in the target-site encoding genes.[1] Thus, the development of new fungicides with novel modes of action is needed to manage the evolution of crop pathogens resistant to existing fungicides.

Pyrimidines, such as uracil and cytosine, are important substrates in constructing DNA and RNA. Pyrimidines also contribute to synthesizing phospholipids, proteins, and uridine diphosphate sugars.[2] Two pyrimidine synthesis routes exist: (1) the *de novo* pathway, in which pyrimidines are synthesized by several enzyme reactions with L-glutamine as the starting material, and (2) the salvage pathway, in which pyrimidines are recycled from DNA and RNA (Fig. 1).[3] Dihydroorotate dehydrogenase (DHODH), which catalyzes the reaction from L-dihydroorotate to orotate, is the fourth enzyme in the *de novo* pathway of pyrimidine biosynthesis.[3,4] DHODH is divided into two classes—class 1 DHODH (DHODH I) and class 2 DHODH (DHODH II)—by the characteristics of amino acid sequence homology, cellular location, and co-substrate specificity. DHODH II is a monomeric protein bound to the mitochondrial inner membrane of eukayotes.[5–7] In addition, DHODH II exists in the cytoplasmic membrane of some prokaryotes, such as *Escherichia coli*, categorized as gram-negative bacteria.[8] In med-
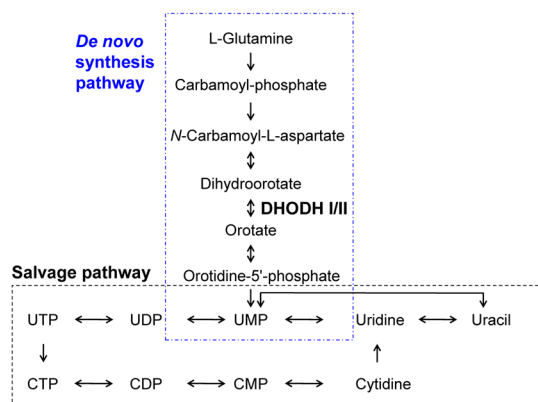
Fig. 1. Pyrimidine biosynthesis pathways in *P. oryzae*. Pathways are drawn based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Abbreviations: UTP, uridine triphosphate; UDP, uridine diphosphate; UMP, uridine monophosphate; CTP, cytidine triphosphate; CDP, cytidine diphosphate; CMP, cytidine monophosphate.

icine, DHODH II has been actively studied as a drug discovery target for cancer, immunological disorders, bacterial and parasitic diseases, and bacterial, viral, and fungal infections.[4,9,10] For example, the drugs leflunomide (Arava) for rheumatoid arthritis and teriflunomide A77-1726 (Aubagio) for multiple sclerosis are now available.[11,12] The antifungal drug olorofim (formerly F901318) for the DHODH of *Aspergillus fumigatus*[10,13] and the antimalarial drug for the DHODH of *Plasmodium falciparum*[14] are under development. DHODH is reportedly the target site of LY214352, an analog of quinoxyfen, which has a mode of action different from that of LY214352 discovered by DowElanco[15,16] in the agrochemical field; however, this product has yet to be launched. In addition, the novel DHODH-inhibiting herbicide tetflupyrolimet is under development by FMC.[17]

Pyricularia oryzae (synonym *Magnaporthe oryzae*) is an ascomycete fungus that causes rice blast, which is one of the most serious fungal diseases affecting global rice production.[18] *P. oryzae* has been studied extensively as a model pathogenic fungus.[19,20] Quinofumelin, 3-(4,4-difluoro-3,3-dimethyl-3,4-dihydroisoquinolin-1-yl)quinoline (Fig. 2), a novel fungicide developed by Mitsui Chemicals Agro, Inc., shows potent fungicidal activity against a broad range of ascomycete fungi, including *P. oryzae*.[21,22] In the present study, using recovery tests and *P. oryzae* DHODH II (PoDHODH II) assays, the target site of quinofumelin was investigated. The indicated target site was DHODH II, and the inhibition of DHODH II caused quinofumelin-mediated inhibition of mycelial growth. Additionally, when using a DHODH II gene (*PoPYR4*)-disruption mutant (*ΔPopyr4*), DHODH II was essential for *P. oryzae* infection of rice plants.

Materials and methods

1. Chemicals, culture media, and P. oryzae *strain*
Quinofumelin (Fig. 2), its analogs, and penthiopyrad were synthesized at Agrochemicals Research Center, Mitsui Chemicals Agro, Inc. (Chiba, Japan). Other chemicals were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan)

and Sigma-Aldrich (St. Louis, MO, USA). Potato dextrose agar (PDA; 4 g of potato starch from the infusion, 20 g of dextrose, and 15 g of agar per liter of formula as a natural agar medium), Czapek Solution Agar (CZA; 30 g of saccharose, 2 g of sodium nitrate, 1 g of dipotassium phosphate, 0.5 g of magnesium sulfate, 0.5 g of potassium chloride, 0.01 g of ferrous sulfate, and 15 g of agar per liter formula as a minimal agar medium), yeast extract, and Luria–Bertani (Miller) broth (LB) were purchased from Becton, Dickinson, and Company (Sparks, MD, USA). Restriction enzymes and plasmid vectors were purchased from Takara (Shiga, Japan) and TOYOBO (Osaka, Japan). *P. oryzae* Ina-86-137 (MAFF accession number: 151011) and the fungal disruption plasmids pETHG and pCAMBIA-Bar-RfA were obtained from Dr. Yoko Nishizawa of the Genetically Modified Organism Research Center, National Institute of Agrobiological Sciences. *P. oryzae* 40901 and M-0-001 were the in-house strains maintained at Mitsui Chemicals Agro, Inc.

2. Recovery test
P. oryzae 40901 was preincubated on a natural medium agar plate (PDA plate) to form a mycelial colony. The resultant mycelial colony disc (4 mm in diameter) was removed and inoculated onto a fresh minimal medium agar plate (CZA plate) containing quinofumelin at 0.01–10.00 ppm and dihydroorotate or orotate at 50 ppm. Inoculated plates were subsequently incubated at 25°C for 12 days and observed visually for the growth of mycelial colonies.

P. oryzae Ina-86-137 and *ΔPopyr4* were preincubated on a natural medium agar plate to form a mycelial colony. The resultant mycelial colony disc (4 mm in diameter) was removed and inoculated onto a fresh minimal medium agar plate containing dihydroorotate at 250 ppm, orotate at 250 ppm, uridine at 50 ppm, or uracil at 50 ppm. Inoculated plates were subsequently incubated at 25°C for 7 days and observed visually for the growth of mycelial colonies.

3. Construction of a bacterial expression vector of the PoDHODH II gene PoPYR4
All kits and the DNA sequencing analyzer were used per the manufacturer's protocols. *P. oryzae* 40901 was grown aerobically in 100 mL of potato dextrose broth (4 g of potato starch from the infusion and 20 g of dextrose per liter of formula) in a 300 mL Erlenmeyer flask at 25°C for 7 days. Total RNA was extracted from 0.2 g of *P. oryzae* 40901 mycelium using a RNeasy Plant Mini Kit (QIAGEN). *PoPYR4* was amplified from the total RNA using a PrimeScript High-Fidelity RT-PCR Kit (TaKaRa) with
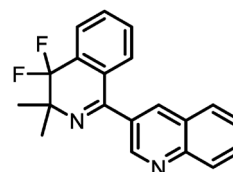

Fig. 2. Structure of quinofumelin.

primer pairs#1 and#2 (see Supplemental Table S1 for primer sequences).

The amplified fragment was ligated into the *Hinc*II site of cloning vector pUC118 using a Mighty Cloning Kit (TaKaRa). The ligated plasmid solution was used to transform *E. coli* DH5α cells. Plasmid DNA was extracted using a QuickLyse Miniprep Kit (QIAGEN), and its DNA sequence was confirmed using an ABI310 genetic analyzer (Applied Biosystems).

The positive clone, named pUC-Po-Mt-F, was digested using *Xba*I and *Hind*III, and the resulting fragment was purified using a MinElute Gel Extraction Kit (QIAGEN). The purified fragment was ligated into the *Xba*I/*Hind*III site of expression vector pET-21b(+) (Novagen) using a Ligation Mighty Mix (TaKaRa), and the ligated vector solution was used to transform *E. coli* DH5α cells. Plasmid DNA was extracted using a QuickLyse Miniprep Kit, and positive clones were identified using restriction enzyme digestion analysis. The resultant plasmid, named pET-Po-Mt-F, which encodes *PoPYR4* fused in a frame to eight histidines and two stop codons, was used to transform *E. coli* BL21(DE3)pLysS cells (Novagen).

The gene sequence of *PoPYR4* retrieved from *P. oryzae* 70-15 was previously submitted to GenBank (accession number: XM_003719109). In the present study, *PoPYR4* was cDNA cloned from in-house *P. oryzae* 40901. The in-house *PoPYR4* differed from 70-15 at nucleotide position 1204, which was guanine in the in-house gene and adenine in 70-15. Amino acid position 402 was valine in the in-house gene rather than methionine in 70-15.

4. Expression and purification of the recombinant PoDHODH II protein PoPYR4

Cells were grown to a density of A600=0.6–0.7 in LB medium with 50 μg/mL of ampicillin. Protein expression was induced by adding 1 mM isopropyl-*β*-D-thiogalactopyranoside. The cells were kept at 20°C for 5 hr in the presence of 100 μM flavin mononucleotide (FMN), after which they were harvested using centrifugation at 5,000 *g* and 4°C for 10 min and stored at −80°C until further use.

For purification of recombinant PoPYR4, the frozen cells were suspended in 10 mL of extraction buffer [50 mM Tris-HCl, 150 mM KCl, 10% glycerol, 0.1% Triton X-100, 1 mM DTT, and 0.5 mM FMN (pH 8.0)] per gram of wet weight. The cells were disrupted using an ultrasonic disrupter UD-200 (TOMY) and centrifuged at 12,000 *g* and 4°C for 40 min. The resultant supernatant was diluted using 2 M imidazole solution to produce a 40 mM solution, which was applied to a His GraviTrap (GE Healthcare). After washing the His GraviTrap with 10 mL of 60 mM imidazole in extraction buffer, recombinant PoPYR4 was eluted from the His GraviTrap using 2.5 mL of 200 mM imidazole in extraction buffer. The elute fraction was applied to a PD-10 column (GE Healthcare) and eluted again with 3.5 mL of elution buffer [50 mM Tris-HCl, 150 mM KCl, 10% glycerol, and 0.1% Triton X-100 (pH 8.0)]. The total protein content was estimated using a protein assay kit (Bio-Rad Laboratories) with bo-
vine serum albumin used as a standard.

5. PoDHODH II assay

PoDHODH II activity was measured using histidine-tagged recombinant PoPYR4 according to a protocol described previously.[23,24] Oxidation of the substrate dihydroorotate with the quinone co-substrate was coupled to reduce the chromogen 2,6-dichloroindophenol (DCIP). Specifically, 100 μL of reaction mixture containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% TritonX-100, 200 μM DCIP, 2 mM dihydroorotate, 100 μM decylubiquinone, approximately 10 μg/mL of recombinant PoPYR4 suspension, and various concentrations of test compounds dissolved in 1% DMSO (or no compound in the control) was incubated at 30°C for 20–30 min. After incubation, 10 μL of 10% sodium dodecyl sulfate was added to each sample and mixed well to stop the reaction. The absorbance at 595 nm was then measured. The inhibitory rate was calculated as (1−T/C), where C and T represent the decreasing quantity of the absorbance at 595 nm with the control and test sample, respectively. The IC$_{50}$ (half-inhibition concentration) values for PoDHODH II inhibitory activity were determined using a four-parameter logistic curve-fitting program (GraphPad Prism 6.00), in which two parameters were constrained (the top and bottom were fixed as 1 and 0, respectively). The Michaelis constant values of dihydroorotate and decylubiquinone were 129.1±18.9 μM and 494.6±52.2 μM, respectively.

6. Mycelial growth assay

To form conidia, *P. oryzae* M-0-001 was preincubated on an oatmeal agar medium containing 40 g of oatmeal, 20 g of saccharose, 50 mg of chloramphenicol, and 15 g of agar per liter of formula. Conidia were harvested, and a conidial suspension was prepared with a spore concentration of 3.0×10⁴ spores/mL in a suspension medium containing 250 μL/L of Tween 80, 100 mg/L of streptomycin sulfate, and 100 mg/L of chloramphenicol. Subsequently, 100 μL of the conidial suspension was mixed with 100 μL of assay medium containing 35 g of Czapek–Dox broth, 1 mg of thiamine HCl, 0.005 mg of biotin, and 20 mM sodium phosphate buffer (pH 7.0) per liter of formula as well as various concentrations of test compounds dissolved in<1% DMSO (or no compound in the control). The mixed medium was then incubated at 28°C for 3 days, after which 8 μL of Alamar Blue (Invitrogen) was added to each sample and mixed well. After another 4–11 hr of incubation, fluorescence was measured at 535 nm excitation and 590 nm emission using an ARVO SX 1420 multilabel counter (Wallac). The inhibitory rate was calculated as (1−T/C), where C and T represent the quantity of fluorescence at 590 nm with the control and test samples, respectively. Experiments were performed at least in duplicate. IC$_{50}$ values for the inhibitory activity of mycelial growth containing conidial germination were determined using probit analysis.

7. Construction of PoPYR4 gene-disrupted P. oryzae

PoPYR4 gene-disrupted *P. oryzae* was constructed according to

Quinofumelin
0ppm 10ppm 1ppm 0.1ppm 0.01ppm

Control

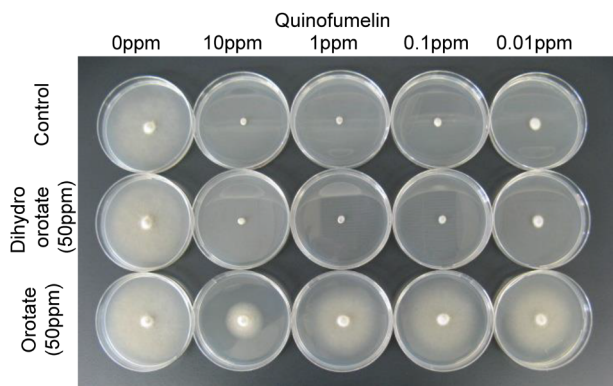Dihydro orotate (50ppm)

Orotate (50ppm)

Fig. 3. Recovery test of *P. oryzae* on minimal medium agar plates. Recovery of mycelial growth was tested by adding dihydroorotate or orotate at 50 ppm to plates on which quinofumelin had been added at 0–10 ppm.

the method of Saitoh,[25)] and the detailed procedure is described in the Supplemental Material. Briefly, genomic DNA was extracted from *P. oryzae* Ina-86-137 mycelium, and the 5′ and 3′ flanking regions of *PoPYR4* were subcloned. A disruption entry plasmid was constructed by introducing ~700 bp DNA fragments of the 5′ and 3′ flanking regions of *PoPYR4* at both sides of the synthetic GFP (S65T) gene (*sGFP*) and PtrpC-HPT [hygromycin B phosphotransferase (HPT) under the control of the *Aspergillus nidulans trpC* promoter] region of pETHG. The disruption fragment of *PoPYR4* containing the DNA fragment of the 5′ and 3′ flanking regions of *PoPYR4* and the sGFP and HPT region was introduced into pCAMBIA-Bar-RfA using an LR reaction *via* the GATEWAY cloning system (Invitrogen, Carlsbad, CA). Ina-86-137 was transformed to generate a *PoPYR4*-disruption mutant (*ΔPopyr4*) by introducing the disruption cassette *via Agrobacterium tumefaciens*.

8. Pathogenicity test of P. oryzae

Conidia were suspended at 3×10^5 spores/mL in a spray solution containing 0.01% Gramin S to conduct pathogenicity tests on rice plants. At the three-leaf stage, the conidial suspension (25 mL) was sprayed onto rice plant seedlings (*Oryza sativa* cv. Sachikaze). Inoculated rice plants were kept in a growth chamber at 20°C under humid conditions and a 12/12 hr light/dark cycle. Disease severity was assessed 6 days after inoculation.

Results

1. Recovery test of P. oryzae

The mycelial growth of *P. oryzae* 40901 could not be recognized on minimal medium agar plates containing quinofumelin at concentrations from 0.01 to 10.00 ppm (Fig. 3). In order to investigate whether exogenous dihydroorotate or orotate could recover the mycelial growth of *P. oryzae*, minimal medium agar plates containing quinofumelin were supplemented with dihydroorotate or orotate. Although exogenous dihydroorotate at 50 ppm did not recover mycelial growth, exogenous orotate at 50 ppm recovered quinofumelin-induced mycelial growth inhibition. Thus, quinofumelin apparently inhibited the orotate for-

mation step in the pyrimidine biosynthesis pathways (Fig. 1). Orotate formation from dihydroorotate is catalyzed by DHODH; thus, the quinofumelin target site seemed to be DHODH.

2. Effect of quinofumelin on the inhibitory activity of recombinant PoDHODH II

The gene *PoPYR4* (MGG_08814) was found from the *P. oryzae* gene database at NCBI (https://www.ncbi.nlm.nih.gov/gene/2678980). It potentially encoded a 514-amino acid polypeptide possessing a DHOD II-like region (https://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=240089), and the amino acid sequence of *PoPYR4* has a high degree of similarity to that of *Neurospora crassa* PyrE with 61% identity. To determine the enzymatic function of *PoPYR4*, the gene *PoPYR4* (MGG_08814) was subcloned, introduced into the expression vector, and then expressed in *E. coli*. The enzymatic activity of class II dihydroorotate dehydrogenase (quinone type) of the purified PoPYR4 protein was confirmed by the method described in the material and methods section in accordance with those of the references.[23,24)] Because it possessed the enzymatic activity of class II dihydroorotate dehydrogenase (quinone type), the gene *PoPYR4* (MGG_08814) was confirmed to encode DHODH II. Based on these results, we named the gene *P. oryzae* PYR4 *PoDHODH II*. The potential inhibition of recombinant PoDHODH II by quinofumelin was then tested. Penthiopyrad, the target site of which is succinate dehydrogenase, did not inhibit PoDHODH II up to 10 μM (Fig. 4). In contrast, quinofumelin strongly inhibited the enzymatic activity of PoDHODH II (IC$_{50}$ value: 2.8 nM; Fig. 4).

3. Relationship between PoDHODH II inhibitory activities and the mycelial growth inhibitory activities of P. oryzae

In order to determine the biologically relevant target of quinofumelin, PoDHODH II inhibitory activity was compared with the inhibitory activity of *P. oryzae* mycelial growth containing conidial germination. When plotted in logarithmic form, there was
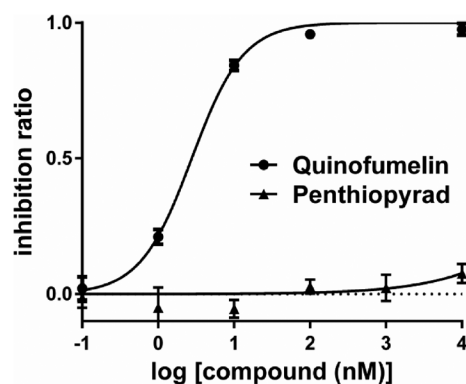

Fig. 4. Effects of quinofumelin and penthiopyrad on recombinant PoDHODH II. Inhibition ratios of PoDHODH II are shown as circles (quinofumelin) and triangles (penthiopyrad). Mean values and standard deviations were calculated from three independent experiments conducted in quadruplicate.
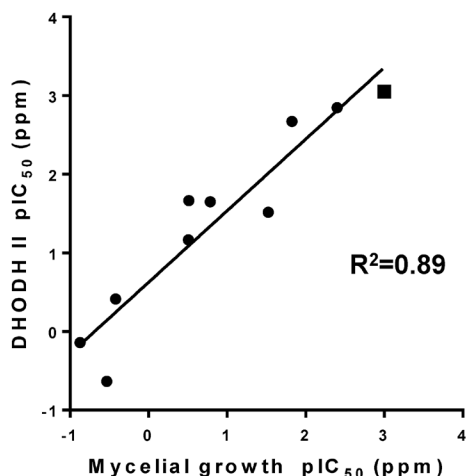
Fig. 5. Correlation of pIC$_{50}$ values between *P. oryzae* mycelial growth inhibitory activity (mycelial growth pIC$_{50}$) and DHODH II inhibitory activity (DHODH II pIC$_{50}$) with the use of quinofumelin and its analogs. pIC$_{50}$=−log$_{10}$IC$_{50}$; square, quinofumelin; circles, analogs.

a strong linear relationship (R^2=0.89) between the IC$_{50}$ values of PoDHODH II inhibitory activity and mycelial growth inhibitory activity for all compounds listed in Supplemental Table S2 (Fig. 5). Based on this strong correlation, we confirmed that DHODH II was the target site of quinofumelin.

4. Mycelial growth of the P. oryzae *DHODH II gene-disruption mutant recovered by supplementation of orotate*

Disruption of *PoPYR4* was confirmed using Southern blot and RT-PCR analyses by comparing *ΔPopyr4* mutant#3102 and wild-type Ina-86-137 (Supplemental Fig. S1). The *ΔPopyr4* mutant could not grow on a minimal medium agar plate, whereas mycelial growth was recovered with 250 ppm of exogenous oro-
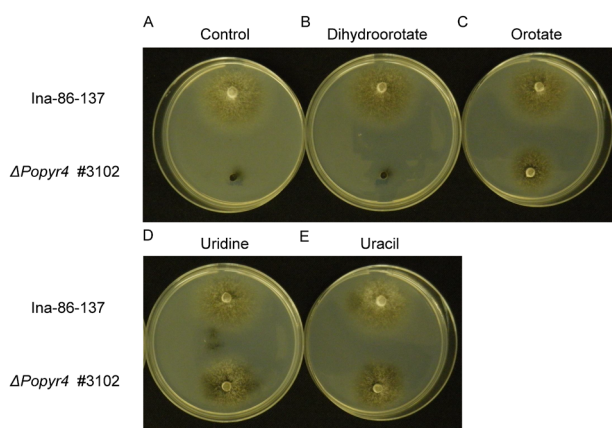

Fig. 6. Recovery test of *P. oryzae* on a minimal medium agar plate using wild-type (Ina-86-137) and DHODH II gene-disruption mutant (*ΔPopyr4* #3102) strains. Plates show the additive-free control (A) and added dihydroorotate (B), orotate (C), uridine (D), and uracil (E). Dihydroorotate or orotate was added at 250 ppm, and uridine or uracil was added at 50 ppm.
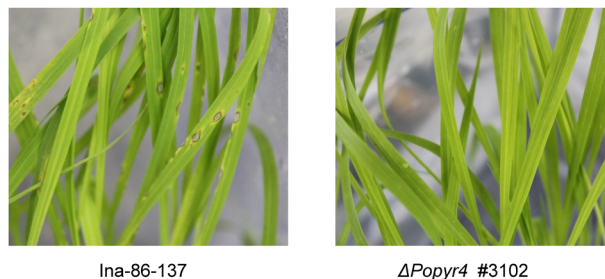

Ina-86-137   *ΔPopyr4* #3102

Fig. 7. Pathogenicity test of the *P. oryzae PYR*4 disruption mutant. Rice leaves (*Oryza sativa* cv. Sachikaze) were sprayed with conidial solution from the wild-type (Ina-86-137) and disruption mutant (*ΔPopyr4* #3102).

tate, 50 ppm of exogenous uridine, or 50 ppm of exogenous uracil, but not with 250 ppm of exogenous dihydroorotate (Fig. 6). Thus, DHODH II was essential for the mycelial growth of *P. oryzae* on a minimal medium agar plate.

5. Reduced pathogenicity in rice plants with P. oryzae *DHODH II gene-disruption mutant*

The pathogenicity of the *ΔPopyr4* mutant against rice plants was evaluated 6 days after inoculation of a conidial suspension of *ΔPopyr4* mutant#3102 and wild-type Ina-86-137. Typical necrotic lesions appeared on rice plant leaves inoculated with wild-type Ina-86-137, whereas no such lesions appeared on rice plant leaves inoculated with *ΔPopyr4* mutant#3102 (Fig. 7). Therefore, DHODH II is apparently essential for developing *P. oryzae* pathogenicity in rice plants.

Discussion

Using *P. oryzae*, one of the most important plant pathogens due to its serious effects on rice farming, we performed various experiments to determine the mode of action of quinofumelin. It was recently reported that quinofumelin showed strong antifungal activity on PDA medium against *Fusarium graminearum* or *Sclerotinia sclerotiorum*.[21,22] Although quinofumelin also showed moderate antifungal activity on PDA medium against *P. oryzae* (Supplemental Fig. S2), it showed much weaker activity when using PDA medium than when using a minimal medium (Fig. 1). We focused attention on the difference in the antifungal activity when using PDA medium as opposed to a minimal medium, and we selected a possible target site of quinofumelin using a minimal medium for the recovery test. Fungal growth tests indicated that quinofumelin might inhibit the biosynthesis of substances essential for cell growth, and these substances could be exogenously absorbed from the medium. We also found that exogenous uracil restored mycelial growth following inhibition by quinofumelin on minimal medium agar plates (Supplemental Fig. S3). Furthermore, exogenous orotate but not exogenous dihydroorotate recovered mycelial growth inhibited by quinofumelin at 0.01–10.00 ppm. These recovery tests suggested that quinofumelin inhibited the orotate formation step in the pyrimidine biosynthesis pathway (Fig. 1). Moreover, as DHODH catalyzes orotate formation, it was indicated as the

likely target site of quinofumelin.

To confirm DHODH as the target site of quinofumelin, we tested whether quinofumelin inhibited recombinant PoDHODH II, finding that quinofumelin strongly inhibited the enzymatic activity of PoDHODH II (Fig. 4). The IC_{50} value of F901318 for the DHODH of *A. fumigatus* was reported previously as 44 ± 10 nM,[10] and the IC_{50} values of A77-1726 for the DHODH of mice, rats, and humans are reportedly 82 ± 21, 18 ± 3, and 773 ± 78 nM, respectively.[26] Although IC_{50} values are dependent on assay conditions and inhibitory mechanisms, the inhibitory activity of quinofumelin against PoDHODH II ($IC_{50}=2.8$ nM) was strong enough to exert a biological effect.

PoDHODH II inhibitory activity was compared with the inhibitory activity of *P. oryzae* mycelial growth containing conidial germination to determine the biologically relevant target of quinofumelin. The strong linear relationship ($R^2=0.89$) between the IC_{50} values of PoDHODH II inhibitory activity and those of *P. oryzae* mycelial growth inhibitory activity (Fig. 5) verified the direct relationship between these inhibitory activities, *i.e.*, that *P. oryzae* mycelial growth inhibition was caused directly by PoDHODH II inhibition.

Although the recovery tests indicated that DHODH II was important for *P. oryzae* mycelial growth, pyrimidines could potentially be supplied using two other routes: the salvage pathway and the *de novo* pathway *via* DHODH I (Fig. 1). DHODH II has been reported in several eukaryotes.[9] *Saccharomyces cerevisiae*, *S. bayanus*, and *S. castelli* have cytoplasmic-type DHODH I, whereas *Schizosaccharomyces pombe* and *Pichia stipites* have DHODH II, and *Saccharomyces kluyveri* has both types.[27] Based on the *P. oryzae* genomic DNA annotation, *P. oryzae* also has DHODH I (GenBank accession number: XM_003715437) and II. Thus, *P. oryzae* seems to survive using rescue routes (DHODH I and the salvage pathway) when DHDOH II is inhibited. In order to determine the biological function of DHODH II, the PoDHODH II gene-disruption mutant *ΔPopyr4* #3102 was constructed and used in tests. The colony morphology of the *ΔPopyr4* #3102 mutant growing on a PDA plate was the same as that of the wild-type strain Ina-86-137 on a PDA plate. The mycelium growth of the *ΔPopyr4* #3102 mutant was recovered with exogenous orotate, uracil, or uridine, but not with exogenous dihydroorotate (Fig. 6). Our recovery tests confirmed that DHODH II was essential for mycelial growth on minimal medium agar plates. For the *ΔPopyr4* #3102 mutant, the recovery test results and medium-dependent growth were consistent with those in a quinofumelin-treated wild-type strain, confirming that the target site of quinofumelin is DHODH II. Moreover, the supplementation of pyrimidines from DHODH I and the salvage pathway was seemingly insufficient to recover mycelial growth in the minimal medium condition. The recovery of mycelial growth by exogenous uracil and uridine indicated that the pyrimidine salvage pathways are sufficient for mycelial growth. In contrast, the *de novo* biosynthesis pathway *via* DHOHD I is insufficient for such growth in the minimal medium condition.

The mycelial growth speed of the *ΔPopyr4* #3102 mutant on a natural medium agar plate was a little slower than that of the wild-type strain Ina-86-137 (data not shown). In recovery tests, the wild-type strain treated with 10 ppm quinofumelin and exogenous orotate also showed slow mycelial growth (Fig. 3), possibly due to insufficient absorption of exogenous orotate or the imbalance in the ubiquinone pool of the mitochondrial respiratory chain complex, given that DHODH II is involved in the ubiquinone–ubiquinol cycle.[28] However, a secondary target site of quinofumelin might also be involved in this slow growth.

In order to investigate the effect of DHODH II on pathogenicity, a pathogenicity test of the *ΔPopyr4* mutant in rice plants was conducted. Typical lesions rarely occurred on rice plant leaves inoculated with *ΔPopyr4* #3102 (Fig. 7). *De novo* pyrimidine biosynthesis reportedly plays an important role in potato leaves during the fast replicative early infection stages of *Phytophthora infestans*.[29] The DHODH II disruption mutant of *Ustilago maydis* causes a loss of pathogenicity in corn plants.[30] When orotate phosphoribosyl transferase, the fifth-step enzyme in the *de novo* pyrimidine biosynthesis pathway, was disrupted in the *P. oryzae* genome in a previous study, the gene-disruption mutant lost pathogenicity.[31] In these studies, the loss of pathogenicity in the disruption mutants was consistent with our result, *i.e.*, that the *PoPYR4* gene-disruption mutant lost pathogenicity in rice plants. According to our rice pathogenicity test results, the pyrimidines supplied through *de novo* biosynthesis *via* DHODH II are essential for developing *P. oryzae* pathogenicity in rice plants. In contrast, the salvage pathway and *de novo* biosynthesis *via* DHODH I are not sufficient for rice necrotic lesions as an alternative to DHODH II. Based on our recovery tests and pathogenicity tests, we conclude that DHODH II is a promising target site for fungicides.

In conclusion, the mode of action of quinofumelin is the inhibition of DHODH II, which is the fourth-step enzyme of the *de novo* pyrimidine biosynthesis pathway. We found that DHODH II is essential for mycelial growth and is a promising target for fungicide discovery. Thus, quinofumelin, with its novel mode of action, is expected to contribute to the development of new strategies for managing fungicide resistance as well as food safety and security.

Electronic supplementary materials

The online version of this article contains supplementary material (Supplemental Tables S1 and S2 and Supplemental Figs. S1–S3), which is available at https://www.jstage.jst.go.jp/browse/jpestics/.

References

1) N. J. Hawkins, C. Bass, A. Dixon and P. Neve: The evolutionary origins of pesticide resistance. *Biol. Rev. Camb. Philos. Soc.* **94**, 135–155

(2019).

2) M. Löffler, L. D. Fairbanks, E. Zameitat, A. M. Marinaki and H. A. Simmonds: Pyrimidine pathways in health and disease. *Trends Mol. Med.* **0**, 430–437 (2005).

3) M. E. Jones: Pyrimidine nucleotide biosynthesis in animals: Genes, enzymes, and regulation of UMP biosynthesis. *Annu. Rev. Biochem.* **49**, 253–279 (1980).

4) R. A. G. Reis, F. A. Calil, P. R. Feliciano, M. P. Pinheiro and M. C. Nonato: The dihydroorotate dehydrogenase: Past and present. *Arch. Biochem. Biophys.* **632**, 175–191 (2017).

5) R. W. Miller: A high molecular weight dihydro-orotate dehydrogenase of *Neurospora crassa*. Purification and properties of the enzyme. *Can. J. Biochem.* **53**, 1288–1300 (1975).

6) V. Hines, L. D. Keys III and M. Johnston: Purification and properties of the bovine liver mitochondrial dihydroorotate dehydrogenase. *J. Biol. Chem.* **261**, 11386–11392 (1986).

7) M. Löffler, W. Knecht, J. Rawls, A. Ullrich and C. Dietz: *Drosophila melanogaster* dihydroorotate dehydrogenase: The N-terminus is important for biological function *in vivo* but not for catalytic properties *in vitro. Insect Biochem. Mol. Biol.* **32**, 1159–1169 (2002).

8) J. N. Larsen and K. F. Jensen: Nucleotide sequence of the pyrD gene of *Escherichia coli* and characterization of the flavoprotein dihydroorotate dehydrogenase. *Eur. J. Biochem.* **151**, 59–65 (1985).

9) H. Munier-Lehmann, P. O. Vidalain, F. Tangy and Y. L. Janin: On dihydroorotate dehydrogenases and their inhibitor and uses. *J. Med. Chem.* **56**, 3148–3167 (2013).

10) J. D. Oliver, G. E. M. Sibley, N. Beckmann, K. S. Dobb, M. J. Slater, L. McEntee, S. du Pré, J. Livermore, M. J. Bromley, N. P. Wiederhold, W. W. Hope, A. J. Kennedy, D. Law and M. Birch: F901318 represents a novel class of antifungal drug that inhibits dihydroorotate dehydrogenase. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 12809–12814 (2016).

11) S. Greene, K. Watanabe, J. Braatz-Trulson and L. Lou: Inhibition of dihydroorotate dehydrogenase by the immunosuppressive agent leflunomide. *Biochem. Pharmacol.* **50**, 861–867 (1995).

12) P. W. O'Connor, D. Li, M. S. Freedman, A. Bar-Or, G. P. A. Rice, C. Confavreux, D. W. Paty, J. A. Stewart and R. Scheyer; Teriflunomide Multiple Sclerosis Trial Group; University of British Columbia MS/MRI Research Group: A Phase II study of the safety and efficacy of teriflunomide in multiple scrlerosis with relapses. *Neurology* **66**, 894–900 (2006).

13) M. Hoenigl, R. Sprute, M. Egger, A. Arastehfar, O. A. Cornely, R. Krause, C. Lass-Flörl, J. Prattes, A. Spec, G. R. Thompson III, N. Wiederhold and J. D. Jenks: The antifungal pipeline: Fosmanogepix, ibrexafungerp, olorofim, opelconazole, and rezafungin. *Drugs* **81**, 1703–1729 (2021).

14) J. M. Coteron, M. Marco, J. Esquivas, X. Deng, K. L. White, J. White, M. Koltun, F. E. Mazouni, S. Kokkonda, K. Katneni, R. Bhamidipati, D. M. Shackleford, I. Angulo-Barturen, S. B. Ferrer, M. B. Jimenez-Diaz, F.-J. Gamo, E. J. Goldsmith, W. N. Charman, I. Bathurst, D. Floyd, D. Matthews, J. N. Burrows, P. K. Rathod, S. A. Charman and M. A. Phillips: Structure-guided lead optimization of triazolopirimidine-ring substituents identifies potent plasmodium falciparum dihydroorotate dehydrogenase inhibitors with clinical candidate potential. *J. Med. Chem.* **54**, 5540–5561 (2011).

15) G. D. Gustafson, G. E. Davis, C. Waldron, A. Smith and M. Henry: Identification of a new antifungal target site through a dual biochemical and molecular-genetics approach. *Curr. Genet.* **30**, 159–165 (1996).

16) G. Gustafson, M. J. Henry, A. J. Smith, S. Aigle, A. Hannum, W. Arnold, M. Barkham, H. Hill, A. Orth and C. Longhurst: Evidence that the phenoxyquinoline fungicides LY214352 and quinoxyfen have different modes of action. "Modern Fungicides and Antifungal Compounds II. 12th International Reinhardsbrunn Symposium, Friendrichroda, Thuringa, Germany, 24th–29th May 1998," eds. by H. Lyr, P. E. Russell, H. -W. Dehne, and H. D. Sisler, Intercept Limited, Andover, pp.93-100, 1999.

17) N. Umetsu and Y. Shirai: Development of novel pesticides in the 21st century. *J. Pestic. Sci.* **45**, 54–74 (2020).

18) R. A. Wilson and N. J. Talbot: Under pressure: Investigating the biology of plant infection by *Magnaporthe oryzae. Nat. Rev. Microbiol.* **7**, 185–195 (2009).

19) B. Valent: Rice blast as a model system for plant pathology. *Phytopathology* **80**, 33–36 (1990).

20) D. J. Ebbole: Magnaporthe as a model for understanding host-pathogen interactions. *Annu. Rev. Phytopathol.* **45**, 437–456 (2007).

21) X. Tao, H. Zhao, H. Xu, Z. Li, J. Wang, X. Song, M. Zhou and Y. Duan: Antifungal activity and biological characteristics of the novel fungicide quinofumelin against *Sclerotinia sclerotiorum. Plant Dis.* **105**, 2567–2574 (2021).

22) Q. Xiu, L. Bi, H. Xu, T. Li, Z. Zhou, Z. Li, J. Wang, Y. Duan and M. Zhou: Antifungal activity of quinofumelin against *Fusarium graminearum* and its inhibitory effect on DON biosynthesis. *Toxins (Basel)* **13**, 348 (2021).

23) J. Baldwint, C. H. Michonoff, N. A. Malmquist, J. White, M. G. Roth, P. K. Rathod and M. A. Phillips: High-throughput screening for potent and selective inhibitors of *Plasmodium falciparum* dihidroorotate dehydrogenase. *J. Biol. Chem.* **280**, 21847–21853 (2005).

24) E. Zameitat, Z. Gojković, W. Knecht, J. Piskur and M. Löffler: Biochemical characterization of recombinant dihydroorotate dehydrogenase from the opportunistic pathogenic yeast *Candida albicans. FEBS J.* **273**, 3183–3191 (2006).

25) K. Saitoh, M. Nishimura, Y. Kubo, N. Hayashi, E. Minami and Y. Nishizawa: Constructuion of a binary vector for knockout and expression analysis of rice blast fungus genes. *Biosci. Biotechnol. Biochem.* **72**, 1380–1383 (2008).

26) A. Ullrich, W. Knecht, M. Fries and M. Löffler: Recombinant eapression of N-terminal truncated mutants of the membrane bound mouse, rat and human flavoenzyme dihydroorotate dehydrogenase. *Eur. J. Biochem.* **268**, 1861–1868 (2001).

27) Z. Gojković, W. Knecht, E. Zameitat, J. Warnebolt, J.-B. Coutelis, Y. Pynyaha, C. Neuveglise, K. Møller, M. Löffler and J. Piskur: Horizontal gene transfer promoted evolution of the ability to propagate under anaerobic conditions in yeasts. *Mol. Genet. Genomics* **271**, 387–393 (2004).

28) B. L. Trumpower: New concepts on the role of ubiquinone in the mitochondrial respiratory chain. *J. Bioenerg. Biomembr.* **13**, 1–24 (1981).

29) L. García-Bayona, M. F. Garavito, G. L. Lozano, J. J. Vasquez, K. Myers, W. E. Fry, A. Bernal, B. H. Zimmermann and S. Restrepo: *De novo* pyrimidine biosynthesis in the oomycete plant pathogen *Phytophthora infestans. Gene* **537**, 312–321 (2014).

30) E. Zameitat, G. Freymark, C. D. Dietz, M. Löffler and M. Bölker: Functional expression of human dihydroorotate dehydrogenase (DHODH) in pyr4 mutants of *Ustilago maydis* allows target validation of dhodh inhibitors *in vivo. Appl. Environ. Microbiol.* **73**, 3371–3379 (2007).

31) Z. Qi, M. Liu, Y. Dong, J. Yang, H. Zhang, X. Zheng and Z. Zhang: Orotate phosphoribosyl transferase MoPyr5 is involved in uridine 5′-phosphate synthesis and pathogenesis of *Magnaporthe oryzae. Appl. Microbiol. Biotechnol.* **100**, 3655–3666 (2016).