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Brief Report

Effects of flusulfamide on spore germination of *Plasmodiophora brassicae*

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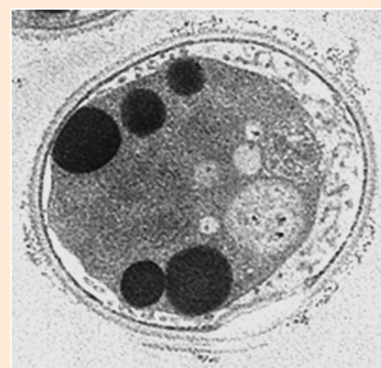
Supplementary material

Flusulfamide inhibits germination of *Plasmodiophora brassicae* resting spores to suppress clubroot disease, but its mechanism of action on the germination of *P. brassicae* resting spores remains unclear. In this study, *P. brassicae* resting spores were treated with flusulfamide and visualized using transmission electron microscopy (TEM). The gene expression of *P. brassicae* resting spores was analyzed using RT-PCR, followed by immunoblotting analysis. TEM results revealed that flusulfamide suppressed the primary zoosporogenesis of *P. brassicae* resting spores during the early phase, and RT-PCR results revealed that flusulfamide affected the gene expression during the germination of the resting spores. Immunoblot and RT-qPCR analyses revealed that *PbCyp3*, an immunophilin (peptidyl-prolyl-isomerase) gene, was highly expressed, resulting in the unusual accumulation of PbCYP3 protein in *P. brassicae* resting spores immediately after treatment with flusulfamide. This suggests that flusulfamide may cause aberrant folding of proteins involved in primary zoosporogenesis, thereby inhibiting germination.



Keywords: flusulfamide, clubroot, resting spores, immunophilin, primary zoosporogenesis.

Introduction

Clubroot is an important disease of Brassica crops worldwide, and it is caused by the obligate biotrophic protist *Plasmodiophora brassicae*. The pathogen is responsible for the formation of large galls on the roots of hosts and many resting spores in the galls. These spores released into the soil can remain viable for at least seven years, resulting in significant yield and economic losses.[1,2]

Although there are fungicides that can be used to control clubroot disease, their efficacy against high density of resting spores and highly virulent populations of *P. brassicae* is limited.[3] In Japan, fluazinam, cyazofamid, amisulbrom, ethaboxam, chlorothalonil, and flusulfamide are registered fungicides used to control clubroot disease. Of these, flusulfamide has been reported to control clubroot in fields that are highly infested with *P. brassicae*.[4] Tanaka *et al.*[5] investigated the mode of action of flusulfamide against *P. brassicae* and reported that flusulfamide acts fungistatically on resting spores and suppresses clubroot disease by inhibiting the germination of *P. brassicae* resting spores. However, the detailed mechanism of action of flusulfamide on the germination of *P. brassicae* resting spores is unclear. Therefore, flusulfamide is categorized among the fungicides with unknown mode of action in the FRAC (Fungicide Resistance Action Committee) mode-of-action classification list (FRAC Code

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List 2022: Fungal control agents sorted by cross-resistance pattern and mode of action, 2022).[6] In this study, we examined the morphological changes and gene expression of *P. brassicae* resting spores treated with flusulfamide to elucidate its mechanism of inhibiting germination.

Materials and methods

1. Preparation of P. brassicae *mature resting spores*

P. brassicae (Williams' pathotype 4) resting spore suspension was prepared from Chinese cabbage (*Brassica rapa*) clubroot galls, which had been frozen and stored at −40°C, as described by Tanaka *et al.*[7] The Chinese cabbage (cultivar: Nozaki Nigo) was sown on commercial artificial soil (Tanemaki Baido, Takii, Shiga, Japan) and then grown in a greenhouse. Three days after germination, each Chinese cabbage seedling was inoculated with 2 mL of *P. brassicae* resting spore suspension (1.0×10^7 spores/mL). After inoculation, the plants were grown in a greenhouse for 60 days under the long-day conditions, 15.0 to 35.4°C, and clubroot galls were collected and washed with tap water. The washed galls were transferred to polyethylene bags filled with autoclaved river sand, left at 25°C for one month to mature *P. brassicae* resting spores in the galls, and then stored at 4°C. At the time of use, the mature resting spores were prepared according to the description of Tanaka *et al.*[7]

2. Transmission electron microscopy

Resting spores were suspended (1×10^9 spores/mL) in distilled water or a cabbage hydroponic solution[8] in the presence or absence of flusulfamide (1 ppm) and incubated at 25°C in the dark. The concentration (1 ppm) of flusulfamide was determined based on the practical dosage of flusulfamide dust formulation for clubroot control in Japan (0.6–0.9 µg active ingredient/g soil).[9] The resting spores were recovered from the suspension three days after incubation and fixed with 1% glutaraldehyde followed by 1% osmium tetraoxide as described by Tanaka *et al.*[10] The resting spores were collected *via* centrifugation and embedded in 2% agar. The agar block was then cut into approximately 1-mm^3 pieces and dehydrated in a 50–100% ethanol series. The resultant specimens were embedded in Spurr's resin.[11] Ultrathin sections were cut using a Reichert-Jung Ultracut-E Ultramicrotome (Reichert, Inc., Depew, NY, USA) and observed using a transmission electron microscopy (JEM-1400; JEOL, Tokyo, Japan).

3. RNA sequencing and expression analysis of P. brassicae *genes related to resting spore germination*

Seeds of cabbage (YCR Rinen, Nippon Norin), which is susceptible to the *P. brassicae* used in this study, were sown on commercial artificial soil (Tanemaki Baido) containing *P. brassicae* resting spores (1.0×10^7 spores/g dry soil) and incubated in a chamber at 25°C, with light and dark periods of 16 and 8 hr, respectively. Five days after inoculation, the seedlings were removed from the cell trays, and the roots with attached rhizosphere soil were flash frozen in liquid nitrogen. Frozen roots were stored at −80°C until further use. Total RNA was extracted from the frozen roots using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. RNA quality was determined using Qubit (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity (RIN) was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total of 500 ng of RNA per sample was used for cDNA library preparation using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA). cDNA libraries were sequenced on an Illumina MiniSeq platform (Illumina, San Diego, CA, USA) and 75 bp single-end raw reads were generated. Before the assembly, various quality control measures were applied to the raw data. High-quality sequences were obtained from original offline data sequences by removing the reads containing adapters and low-quality reads. All subsequent analyses were based on filtered and clean data. The clean data were aligned to the reference genome (*Brassica oleracea* var. *capitata* NCBI: txid3716) using CLC Genomics Workbench software. Reads that did not map to the cabbage reference sequence were subjected to *de novo* assembly to obtain contigs using the RNA-Seq analysis tool in the CLC Genomics Workbench, and they were mapped to the reference sequence of *P. brassicae* at EnsemblProtists database (GCA_001049375.1, https://protists.ensembl.org/Plasmodiophora_brassicae_gca_001049375/Info/Index). The sequences mapped to the *P. brassicae* genome were further confirmed to be *P. brassicae* genes *via* an NCBI BLAST homology search. RT-PCR primers were designed from *P. brassicae* genes using Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

4. Effect of flusulfamide on the expression of genes related to resting spore germination

P. brassicae resting spores (1.0×10^8 spores/mL) were suspended in cabbage hydroponic solution[8] in the presence or absence of flusulfamide (1 ppm) to induce zoosporogenesis (differentiation of primary zoospores), as described by Tanaka *et al.*[10] Resting spores were collected over time, and total RNA was extracted using the NucleoBond RNA Soil Mini kit (Takara Bio, Shiga, Japan). RT-PCR was performed using 20 RT-PCR primers (PBRA primers, Supplemental Table S1), and the amplification products were visualized using 1.5% agarose gel electrophoresis and stained with ethidium bromide.

5. Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA of *P. brassicae* resting spores was extracted following the protocol outlined in section 4. Reverse transcription was performed with 500 ng total RNA in a 20 µL reaction volume using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Shiga, Japan) according to the manufacturer's instructions. The primers used are listed in Supplemental Table S1. cDNA was diluted (1 : 1), and 1 µL was used as a template in a 20 µL reaction containing THUNDERBIRD SYBR

qPCR Mix (Toyobo). We assessed three technical replicates and three biological replicates for each gene. *P. brassicae actin1* gene (*PbACT1*, GenBank accession number AAR88383) was used as a reference to normalize the gene expression in each sample. RT-qPCR analysis was performed using the Applied Biosystems QuantStudio Real-Time PCR *system* (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA). The amplification program consisted of an initial denaturation at 95°C for 1 min, followed by 40 cycles of 95°C for 15 sec, and 60 to 95°C for 60 sec. Relative quantification was performed using the $\Delta\Delta C_T$ method.[12)]

6. Immunoblotting

P. brassicae resting spores (1×10^9 spores/mL) were suspended in a cabbage hydroponic solution adjusted to pH 6.0, treated with 10 mM MES containing flusulfamide (1 ppm), and incubated at 25°C for 24 hr. Proteins were extracted from *P. brassicae* resting spores using ULTRA RIPA Kit (Biodynamics Laboratory, Tokyo, Japan) and the RIPA-soluble fraction (cytoplasmic and membrane proteins) was subjected to protein analysis. The protein concentration was determined using the Bradford method with bovine serum albumin (BSA) as the standard. Subsequently, 50 μg of proteins from *P. brassicae* resting spores was subjected to immunoblot analysis using the IgG fraction of the anti-PbCYP3 antibody, following sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A peptide (SIYGAKFEDENFKLKHC) was synthesized based on the amino acid sequence of the PbCYP3 protein (PBRA_003184) and used as an antigen to raise the anti-PbCYP3 antibody. ECL-prime (GE Healthcare, Princeton, NJ, USA) was used to detect protein signals according to the manufacturer's instructions.

Results and discussion

1. Transmission electron microscopy (TEM)

TEM revealed the primary zoosporogenesis of *P. brassicae* resting spores suspended in cabbage hydroponic solution (Fig. 1A). The primary zoosporogenesis was characterized as stage 0 (resting state), stage 1 (a space between the cell wall and cell membrane indicated as "s" in Fig. 1A and B), stage 2 (autophagosome-like vacuoles indicated as "v" in Fig. 1A), and stage 3 (vesicles containing an electron-dense granule indicated as "ve" and microvillus-like vesicles indicated as "ms" in Fig. 1A).[10)] TEM images revealed that a specific count of spores suspended in cabbage hydroponic solution (CHS) were observed to be in stages 2 and 3 (Fig. 1B), indicating their active involvement in the primary zoosporogenesis process. However, the TEM of *P. brassicae* resting spores suspended in CHS containing flusulfamide (1 ppm) showed evidence of stage-1 zoosporogenesis, and it did not display images of stages 2 and 3 zoosporogenesis. This suggests that flusulfamide does not inhibit the progression from stage 0 to stage 1 but instead inhibits the progression from

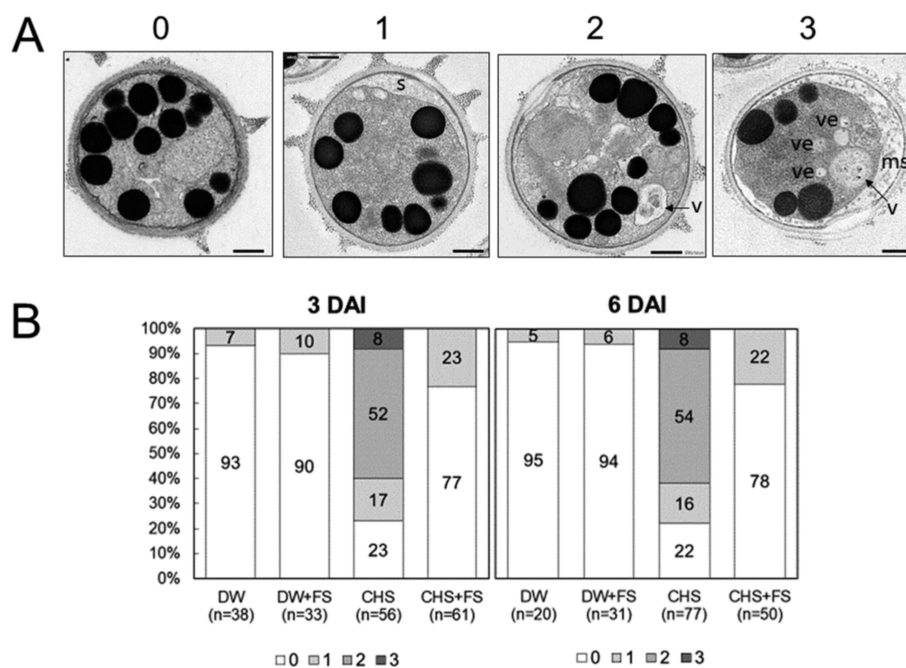

Fig. 1. Transmission electron microscopy (TEM). (A) TEM images showing different stages of primary zoosporogenesis of *P. brassicae* resting spores. Numerals 0, 1, 2, and 3 correspond to the stages of primary zoosporogenesis. The following annotations are used: s, space between the cell wall and the cell membrane; v, vacuole; ve, vesicle containing an electron-dense granule; ms, microvillus-like vesicle. Black circles in the cytoplasm of resting spores indicate lipid bodies. (B) Percentage of the four stages (0, 1, 2, and 3) of primary zoosporogenesis of *P. brassicae* resting spores. The four stages of primary zoosporogenesis at 3- and 6-days post-incubation (DPI) were determined by quantifying TEM images. *P. brassicae* resting spores were incubated in distilled water (DW), distilled water containing flusulfamide (1 ppm) (DW+FS), cabbage hydroponic solution (CHS), or cabbage hydroponic solution containing flusulfamide (1 ppm) (CHS+FS). Numerals 0, 1, 2, and 3 represent the stages of primary zoosporogenesis of *P. brassicae* resting spores in each treatment group.

stage 1 to stage 2 in the primary zoosporogenesis of *P. brassicae* resting spores (Fig. 1B). The TEM images of *P. brassicae* resting spores also showed that very little difference existed between 3 dpi and 6 dpi, suggesting that flusulfamide inhibited, rather than delayed, the progression from stage 1 to stage 2 in the primary zoosporogenesis of the resting spores. However, most of the resting spores suspended in distilled water or flusulfamide (1 ppm) solution were observed at stage 0, indicating that there was no commencement of primary zoosporogenesis of *P. brassicae* resting spores. TEM results in this study revealed for the first time that flusulfamide inhibited the germination of *P. brassicae* resting spores by preventing the progress of primary zoosporogenesis at the early stage (stage 1) rather than keeping the resting spores in a dormant state (stage 0).

2. *Effect of flusulfamide on the expression of* P. brassicae *germination-related genes*

Primary infection (root hair infection) was observed 3 days after cabbage plants were inoculated with *P. brassicae*, and the root hair infection rate increased till 5 days after inoculation (Supplemental Figure S1). We observed that the host plants inoculated with *P. brassicae* were susceptible to *P. brassicae* and showed clubroot symptoms (Supplemental Fig. 2). To identify *P. brassicae* genes during zoosporogenesis, germination of primary zoospore, and primary infection, we performed an RNA-seq analysis using RNA extracted from inoculated cabbage roots with attached rhizosphere soil at 5 days after inoculation and screened the sequences that map to the *P. brassicae* genome. A total of 1562 *P. brassicae* genes (corresponding to 16% of the total number of *P. brassicae* genes[13)]) were mapped to the reference genome of *P. brassica* (Supplemental Table S2).

P. brassicae is an obligate parasite that grows only in host root cells and cannot be cultured in artificial media; however, it is known that some of the resting spores suspended in the host hydroponic solution develop into primary zoospores that sprout (swim out) from the germination pore of the resting spores.[8,10)] We initially hypothesized that the genes expressed during zoosporogenesis and germination would be downregulated by flusulfamide, consequently inhibiting the gemination of the resting spores. To confirm this hypothesis, we selected 39 genes, including those associated with flagellar components, morphogenesis, signal transduction, protein degradation and biosynthesis, and spore cell wall degradation, from the 1562 *P. brassicae* genes obtained *via* RNA-seq. We then used RT-PCR to examine whether their expression was suppressed by flusulfamide. We used RNA from the resting spores suspended in CHS and conducted the experiment in the presence (0.5, 1, and 2 ppm) or absence of flusulfamide for 5 days. RT-PCR analysis of the *P. brassicae* resting spores yielded non-reproducible results for some genes, possibly due to physiological conditions such as maturity, viability, and vigor of the resting spores. Among the 39 *P. brassicae* genes, RT-PCR analysis revealed that 20 genes showed reproducible expression patterns in the presence or absence of flusulfamide. The expression patterns were similar among the three concentrations (0.5, 1, and 2 ppm) of flusulfamide. The result revealed that flusulfamide downregulated the expression of 80% (16 out of 20) of the *P. brassicae* genes expressed in the resting spores during germination (Groups 2 and 3), indicating that the normal differentiation process was inhibited by flusulfamide. However, there were genes that consistently showed expression without being affected by flusulfamide (Group 1) and genes whose expression was increased by flusulfamide treatment (Group 4). This suggests that flusulfamide not only downregulates *P. brassicae* genes expressed during germination but also upregulates some of them (Supplemental Figure S3).

RT-PCR results were consistent with TEM results because autophagosome-like vacuoles and vesicles containing an electron-dense granule were not observed in *P. brassicae* resting spores treated with flusulfamide. The expressions of genes thought to be involved in autophagosome-like vacuole ("v" in Fig. 1A) formation (SCF ubiquitin ligase and ubiquitin hydrolase 1)[14)] and vesicle trafficking (RAB GTPase),[15)] which are probably involved in primary zoosporogenesis, were down-regulated in the flusulfamide-treated *P. brassicae* resting spores at 3 dpi and afterward (Supplementary Figure S3).

Murakami *et al.* (2003)[16)] reported that the incidence of clubroot disease was significantly reduced for a long period in a field treated with flusulfamide, even during the second cultivation of Chinese cabbage, and the density of *P. brassicae* resting spores in the soil was not reduced, suggesting that the germination inhibitory effect of flusulfamide may persist for a long period. Our study showed that the expression of genes in Groups 1 and 4 continued or increased in the flusulfamide-treated resting spores, suggesting that the resting spores continued to consume energy even without germination. Continuous energy consumption may cause depletion of energy sources, such as lipid bodies in the resting spores, leading to insufficient energy for germination and primary infection process, which may result in suppressed clubroot disease. The relationship between flusulfamide treatment period and primary infection ability of resting spores needs to be further investigated.

3. *Effect of flusulfamide on the expression of immunophilin genes*

Cyclophilin 3 (peptidyl-prolyl *cis-trans* isomerase) gene was highly induced in *P. brassicae* resting spores treated with flusulfamide at 1 dpi and afterward, suggesting the important roles of the gene in the response of *P. brassicae* resting spores to flusulfamide (Supplemental Figure S3). Immunophilins are ubiquitous proteins that possess peptidyl-prolyl *cis–trans* isomerase (PPIase) activity and are involved in protein folding and transportation, RNA splicing, and regulation of multi-protein complexes in cells.[17)] *P. brassicae* possesses 20 immunophilin (11 cyclophilins (*PbCYP*), seven FK506 binding proteins (*FKBP*), and two parvulin-like proteins (*PAR*) genes.[18)] RT-qPCR analysis of the 20 immunophilin genes showed that the expression of most of the immunophilin genes was increased by flusulfamide (1 ppm) (Fig. 2A), suggesting that aberrant expression of the immunophilin genes was induced in *P. brassicae* resting spores treated

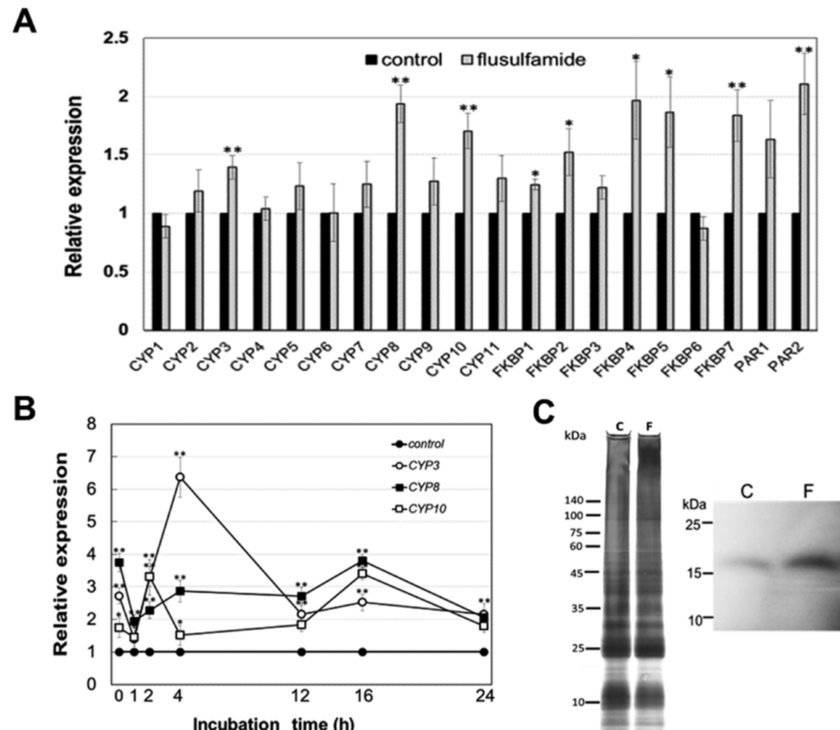

Fig. 2. Effect of flusulfamide on the expression of immunophilin genes. (A) RT-qPCR analyses of 20 immunophilins (11 cyclophilins (*PbCYP*), seven FK506 binding proteins (*FKBP*), and two parvulin-like proteins (*PAR*) genes in *P. brassicae* resting spores treated with flusulfamide for 24 hr. Values represent means±S. E. Statistical significance was determined using Student's *t*-test. Asterisks indicate significant differences between *P. brassicae* resting spores treated with water (control) and flusulfamide (1 ppm); *$p<0.05$ and **$p<0.01$. (B) Time–course gene expression analyses of three cyclophilin genes: *PbCYP3* (open circles), *PbCYP8* (filled squares), and *PbCYP10* (open squares). Control (filled circles) corresponds to the reference gene (*PbACT1*) used for RT-qPCR normalization. Values represent means±S. E. Statistical significance was determined using Student's *t*-test. Asterisks indicate significant differences between *P. brassicae* resting spores treated with water (control) and flusulfamide (1 ppm); *$p<0.05$ and **$p<0.01$. (C) Silver staining of SDS-PAGE (left) and immunoblot analysis (right) of proteins extracted from *P. brassicae* resting spores treated with flusulfamide for 24 hr: anti-PbCYP3 IgG was used as the primary antibody. C, flusulfamide-untreated; F, flusulfamide-treated.

with flusulfamide 24 hr after treatment.

We further analyzed the expression of immunophilin genes, such as cyclophilin 3 (*PbCYP3*), in *P. brassicae* resting spores treated with flusulfamide using quantitative RT-PCR (RT-qPCR). Time-course gene expression analysis was conducted for the three cyclophilin genes (*PbCYP3*, *PbCYP8*, and *PbCYP10*) in which their expression was already up-regulated in the resting spores of *P. brassicae* treated with flusulfamide at 24 hr after treatment. The results showed that all of them were upregulated in the flusulfamide-treated resting spores immediately after treatment; however, the expression peaks were different from each other (Fig. 2B), thereby confirming the results indicated in Fig. 2A.

To determine whether the gene of PbCYP3, significantly up-regulated in RT-PCR analysis, was accumulated in *P. brassicae* resting spores treated with flusulfamide (1 ppm), proteins from *P. brassicae* resting spores treated with flusulfamide for 24 hr were analyzed *via* immunoblotting using anti-PbCYP3 IgG as the primary antibody. The results showed increased accumulation of a protein with the expected size of PbCYP3 (17.5 kDa) in the flusulfamide-treated resting spores as early as at 24 hr after the treatment (Fig. 2C). Although the function of PbCYP3 in *P. brassicae* resting spores is unclear, PbCYP3 has been revealed to play a role during an infection process such as peg formation or generation of turgor pressure in *P. brassicae* zoospore *via* genetic complementation experiments on a cyclophilin (*CYP1*) gene-deficient mutant of *Magnaporthe oryzae*.[18] PbCYP3 may function in the process of root hair infection of germinated zoospores after reaching the host, rather than in the process of zoosporogenesis. In the present study, the accumulation of PbCYP3 increased in the flusulfamide-treated resting spores but not in the untreated resting spores, indicating that PbCYP3 accumulation was induced by flusulfamide in *P. brassicae* resting spores at inappropriate times. The amount of PbCYP3 in the cell is strictly regulated because cyclophilins are proteins that maintain normal post-translational protein structure through peptidyl-prolyl-isomerase activity. Resting spore cells with abnormally increased amounts of PbCYP3 are expected to have abnormal post-translational protein structure maintenance mechanisms. In such resting spores, it is probably possible that the primary zoosporogenesis may not proceed normally. Further study is needed to elucidate the mechanisms by which flusulfamide triggers abnormal up-regulation of *PbCYP3* gene in *P. brassicae* resting spores.

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Electronic supplementary materials

The online version of this article contains supplementary materials (Supplementary Figure S1, Supplementary Tables S1, S2).

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