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Regular Article

Effect of flutianil on the morphology and gene expression of powdery mildew

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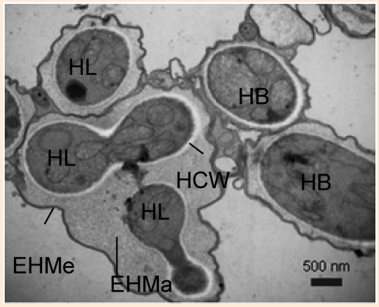
Supplementary material

Flutianil, a fungicide effective only on powdery mildew, was previously reported to affect the host cell's haustorial formation and nutrient absorption. Studies were conducted to investigate flutianil's primary site of action on *Blumeria graminis* morphology using transmission electron microscope (TEM) observation and RNA sequencing (RAN-seq) techniques. TEM observation revealed that flutianil caused the extra-haustorial matrix and fungal cell wall to be obscured, without remarkable changes of other fungal organelles. RNA-seq analysis indicated that, unlike other powdery-mildew fungicides, flutianil did not significantly affect the constantly expressed genes for the survival of *B. graminis*. Genes whose expression is up- or downregulated by flutianil were found; these are the three sugar transporter genes and various effector genes, mainly expressed in haustoria. These findings indicate that the primary site of action of flutianil might be in the haustoria.



Keywords: flutianil, powdery mildew, haustorium, *Blumeria graminis*, microscopy, ultrastructure, RNA-seq.

Introduction

Powdery-mildew fungi are widespread plant pathogens that cause devastating damage to a large number of monocotyledonous and dicotyledonous plants, including many agriculturally important crops.[1,2] These obligate biotrophic ascomycetes are entirely dependent on living host cells for their nutrient supply for growth and reproduction. The powdery-mildew infection process starts when a conidium lands on a leaf surface and germinates to form an appressorium, with which it attempts to penetrate the plant cell. During penetration, the conidium differentiates into a specialized feeding organ, called a haustorium,[3] in the host epidermal cell. The haustorium is involved not only in nutrient acquisition but also in biosynthetic pathways, suppressing host defense mechanisms, and redirecting the metabolic flow of the host.[4–6]

Modern fungicides provide excellent control of powdery mildew but tend to act at single target sites,[7] meaning that a single gene mutation can alter the target site, reducing its sensitivity and leading to resistance. Therefore, to maintain effective control of powdery mildew, fungicides with a novel mode of action are required. Studies on the mode of action are very difficult when the fungicides are only active against obligate biotrophs, such as powdery mildew, downy mildew, and rust, as these pathogens cannot be grown in culture and are difficult to separate from their host.[8] Under these circumstances, microscopic morphological analysis has proven to be a valuable tool for determining the site of action for fungicides during the infection process of fungal pathogens. In addition, transcriptional profiling has recently become a powerful tool that provides unique information on regulatory pathways and gene function.[9–11] RNA-seq allows us to monitor the expression of most genes simultaneously and can be used to anticipate the mode of action by comparing the gene expression profiles of fungal cells treated with the novel compounds whose modes of action are known.

In previous studies, we have reported the biological properties of the fungicide flutianil[12] (Gatten®, (2*Z*)-{[2-fluoro-

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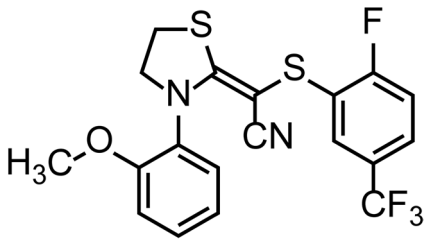

Fig. 1. Chemical structure of flutianil.

5-(trifluoromethyl)phenyl]sulfanyl}[3-(2-methoxyphenyl)-1,3-thiazolidin-2-ylidene]acetonitrile) (chemical structure shown in Fig. 1), which is effective to control only powdery mildew. In those reports, we revealed that flutianil inhibits the haustorial formation and secondary hyphal elongation of *Blumeria graminis* f. sp. *hordei* (*Bgh*) by inhibiting its nutrient absorption from the host cell.[13] However, the biochemical target site of flutianil has not been completely clarified. In order to find the primary site of action of flutianil, a detailed analysis of the effect of flutianil on *Bgh* morphology was conducted using microscopic observation techniques with a transmission electron microscope.

We also performed a comprehensive gene expression analysis using RNA-seq to determine the mode of action and to identify the target genes of flutianil. We compared the gene expression profiles of *Bgh* in response to flutianil with those of other existing powdery-mildew fungicides metrafenone and cyflufenamid, which are used to control powdery mildew in various crops, such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), mode of action of cyflufenamid being unclear, but known with metrafenone as disruption of the actin cytoskeleton at the hyphal tip.[14]

Materials and methods

1. Chemicals and reagents

The flutianil used in this study was a technical-grade material with purity greater than 99%, synthesized at OAT Agrio Co., Ltd. (Tokyo, Japan). Metrafenone was purchased from Sigma-Aldrich (St. Louis, MO, USA), while cyflufenamid was purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan). Stock fungicide solutions were prepared by dissolving each compound in acetone and diluting it with distilled water to make a test solution with $<$1% acetone (v/v).

2. Plant materials, pathogens, and inoculation

Barley seeds were sown in 9 cm polyethylene pots filled with culture soil, a 50 : 50 mixture of Nippi Horticultural Growing Media No. 1 (Nihonhiryo, Tokyo, Japan) and a soil mix (Sakata Seed Corp., Yokohama, Japan). The pots were placed in a growth chamber at 23°C with 60% relative humidity under a 24 hr photoperiod for 7 days. In the case of microscopy and RNA-seq studies, a dust of *Blumeria graminis* f. sp. *hordei* Race1,[15] which had been maintained on the barley cv. Kobinkatagi in a growth chamber at 23°C under a 24 hr photoperiod, was inoculated onto barley plants. Flutianil and other fungicides were sprayed onto the barley plants, which were inoculated with a conidia dust 2 days after inoculation. The treated plants were maintained in a growth chamber at 15°C under a 24 hr photoperiod for the predetermined number of days for each study.

3. Transmission electron microscopy

Barley plants were inoculated with *Bgh* and incubated in a growth chamber at 23°C under a 24 hr photoperiod for 7 days. Flutianil (10 mg/L) was applied to the inoculated *Bgh* plants and then maintained for 3 days in the growth chamber. The treated barley leaves were then cut and put in microtubes and were used as a test sample in the next tests. To observe the structure of *Bgh via* transmission electron microscope (TEM) H-7500 (Hitachi, Japan), barley leaves inoculated with *Bgh* were collected 2 days after spraying with flutianil solution (50 mg/L), which took place 7 days after the pathogen inoculation. Small segments (approximately 1 mm$\times$2 mm) excised from the middle portion of the leaf blades were fixed in 3% (w/v) glutaraldehyde in a 50 mM sodium phosphate buffer (pH 7.2) for 10–12 hr at 4°C. After being rinsed in the buffer for 2 hr, the segments were postfixed in 2% osmium tetroxide in the same buffer for 3 hr at 4°C. The segments were rinsed with the buffer and distilled water, followed by dehydration in a graded acetone series (30, 50, 70, 90, 99, and 100%) and treatment with propylene oxide, and then embedded in resin (Agar Low Viscosity Resin, Agar Scientific, UK). Ultrathin (90 nm thick) transverse sections of the embedded leaf were cut with a diamond knife on an ultramicrotome (EM UC6, Leica, Germany). The sections were placed on 150-mesh copper grids and were double stained with 2% (w/v) uranyl acetate for 20 min, followed by staining with the lead stain solution (Sigma-Aldrich, Japan) for 5 min. The specimens were then observed with a TEM at an accelerating voltage of 100 kV and were photographed with a CCD (Charge-Coupled Dvices) camera (Advanced Microscopy Technique, MA, USA) connected to the microscope.

4. Comparative analysis of flutianil and existing fungicides on the morphology of Bgh

The methodology of inoculation with *Bgh* and application of fungicide were undertaken as described in the section 2. Flutianil and other fungicides were sprayed onto the barley plants, which were inoculated with a conidia dust 2 days after inoculation. The treated plants were then washed, and fluorescence was observed *via* CLSM. Morphological effects of flutianil (10 mg/L), cyflufenamid (17 mg/L), and metrafenone (90 mg/L) on the fungal cell walls and septa of *Bgh* were observed *via* CLSM. Samples treated with each fungicide were incubated for 5 min at room temperature in calcofluor white (0.1 mg/mL; Sigma-Aldrich) 3 days after fungicide spraying. The concentration of fungicides was set as stated on the product labels.

5. RNA sequencing

Flutianil (10 mg/L), cyflufenamid (17 mg/L), and metrafenone

(90 mg/L) were sprayed onto pathogen-infected barley leaves 7 days after inoculation. Fungal mycelia on the leaf surface (epiphytic tissue) were then collected using a microspatula 24 hr after application. Total RNA was isolated from the sample using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. RNA-seq was carried out by BGI Japan (Kobe, Japan). The quality and integrity of the total RNA were assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Tokyo, Japan), and only RNA samples with RIN integrity numbers of >6.5 were used for sequencing. The cDNA libraries were prepared from the RNA samples of four treatment groups (control, flutianil, cyflufenamid, and metrafenone) using the Illumina mRNA-seq Sample Prep Kit (Illumina, San Diego, CA, USA) and were sequenced using the Illumina HiSeq 2500 System (101 bp, paired end) according to the manufacturer's protocol. Transcriptome analyses were performed using the reference genome of *Blumeria graminis* f. sp. *hordei* strain DH14 produced by BluGen, the Blumeria Sequencing Project (http://www.blugen.org/). Reads were mapped onto reference genomes using annotated coding sequences (CDS). The sequence reads were filtered for quality in FASTAQ format, after which the total reads per sample were standardized. The reads were aligned to the annotated coding sequences (bgh_dh14_v3) by TopHat.[16)]

Results

1. *Effects of flutianil on the morphologies of the hyphae and haustoria of* Bgh

The structure of *Bgh* was then observed using a transmission electron microscope (TEM). Figure 2 (panels A–E) shows normal (untreated) haustoria, which contained complements of cytoplasm, nuclei, mitochondria, and vacuoles. The cytoplasm was surrounded by a plasma membrane and haustorial cell wall (HCW), and the haustorial body (HB) and lobes (HL) were embedded within an extra-haustorial matrix (EHMa). The haustoria were separated from the extra-haustorial membrane (EHMe) by the EHMa. In order to make it easier to understand the composition of organs such as the HB, HCW, EHMa, and EHMe, schematic diagrams of the organs, untreated and treated with flutianil are shown in Fig. 2, panels G and H, respectively.

Following flutianil treatment, different ultrastructural modifications occurred in the haustoria, as shown in Fig. 2. The electron density of the matrix region of the EHMa of flutianil-treated *Bgh* was lower than that of the untreated control (as indicated by white arrows in Fig. 2, panels B and F). The HCW became obscured after treatment with flutianil (as indicated by black arrows in Fig. 2, panels B, D, and F). In some cases, an image of very electron-dense materials, which were constituents of the EHMa shrinking close to the cell wall, was observed in the EHMa region (as indicated by white arrows in Fig. 2, panel D).

The transverse section of the hyphae of *Bgh* observed using TEM is shown in Fig. 3. The flutianil-treated cell walls of the hyphae became obscured (as indicated by arrows in Fig. 3, panels E–L), and the vacuoles in the hyphal cytoplasm became larger (indicated by a "V" in Fig. 3, panels F and K).

Overall, these results indicated that flutianil affects the HCW, extra-haustorial matrix, and hyphal cell wall. By contrast, in the haustoria and hyphae of *Bgh* on barley leaves treated with flutianil, remarkable degeneration of cell organelles could not be found.

2. *Comparison of the effect of flutianil with that of other existing fungicides on the morphologies of the hyphae and conidiophores of* Bgh

The effect of flutianil treatment with cyflufenamid and metrafenone treatments on the morphologies of the hyphae and conidiophores of *Bgh* was investigated. *Bgh* was treated with flutianil, cyflufenamid, and metrafenone and stained with calcofluor white, and the formation of hyphae and conidiophores was observed using CLSM. The CLSM micrographs obtained are shown in Fig. 4. Conidia and septa were normally observed along with the conidiophores and hyphae in the untreated control, as shown in Fig. 4, panels C and D, respectively. After the treatment with flutianil, aberrant hyphae and conidiophores were observed, and the conidiophores formed elongated tubes of a uniform diameter or a chain of conidiophores with irregular or no septation (Fig. 4, panel C). The hyphae also developed irregular septation (Fig. 4, panel D). In contrast, the collapse of hyphae and conidiophores was observed 3 days after *Bgh* exposure to cyflufenamid and metrafenone (Fig. 4, panels E–H). Our previous study indicated that on flutianil-treated leaves, conidiophores had formed abnormally as elongated tubes, but the collapse of conidiophores or mycelia was not observed.[13)] These findings indicate that the morphological change in *Bgh* by treatment with flutianil is completely different from that by treatment with the existing fungicides cyflufenamid and metrafenone.

3. *RNA sequencing*

3.1. *Gene expression differences between flutianil and other existing fungicides*

RNA-seq was conducted to examine gene expression differences between flutianil and existing powdery-mildew fungicides, cyflufenamid and metrafenone after spraying the fungicides onto *Bgh*-infected barley leaves 7 days after inoculation. In total, 85,678,844, 91,548,658, 85,419,090, and 83,604,864 successful sequences (clean reads) were produced using water (untreated control), flutianil, cyflufenamid, and metrafenone, respectively. The RNA-seq expression values in this study were divided into four categories: (1) downregulated (<0.7-fold change), (2) low upregulated (>1.5-fold change), (3) medium upregulated (>2-fold change), and (4) high upregulated (>3-fold change), as shown in Table 1.

Compared with the gene expression of the untreated control, there were 248 differentially expressed genes in response to flutianil treatment. Of these, 205 were upregulated and 43 were downregulated. In a case of cyflufenamid treatment, 638 differentially expressed genes were identified. Of these, 379 were upregulated and 259 were downregulated. In a case of metrafenone

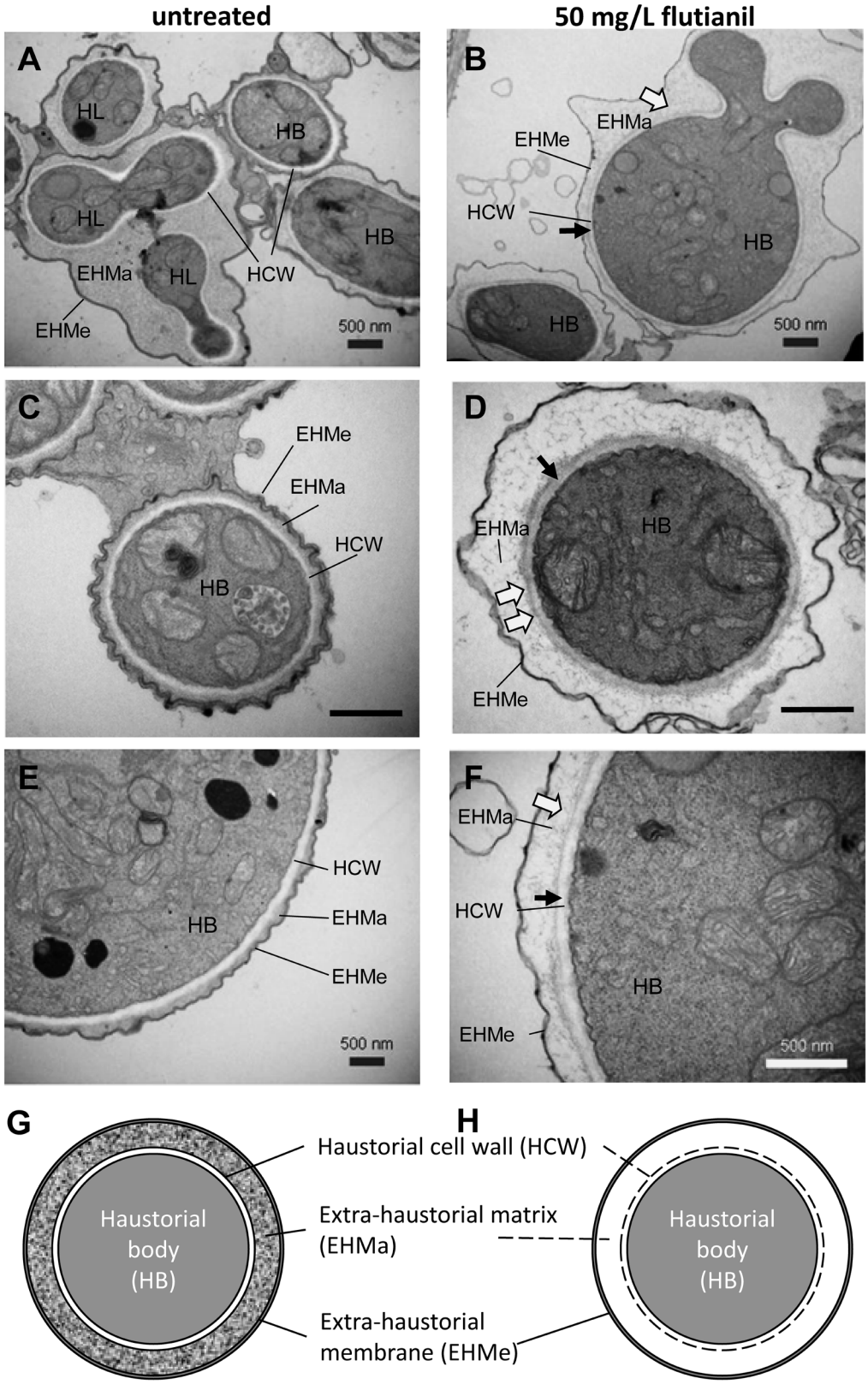

Fig. 2. Transmission electron micrographs of the haustoria of *Bgh* in barley epidermal cells and a schematic of a section of haustoria (A, C, and E) Haustoria of *Bgh* without flutianil treatment. (B, D, and F) Flutianil-treated haustoria showing the shrinkage or no development of the extra-haustorial matrix. Schematic diagrams of untreated haustoria (G) and flutianil-treated (H) sections. Bars, 500 nm. Organ abbreviations: HB, haustorial body; HL, haustorial lobe; EHMe, extra-haustorial membrane; EHMa, extra-haustorial matrix; HCW, haustorial cell wall.

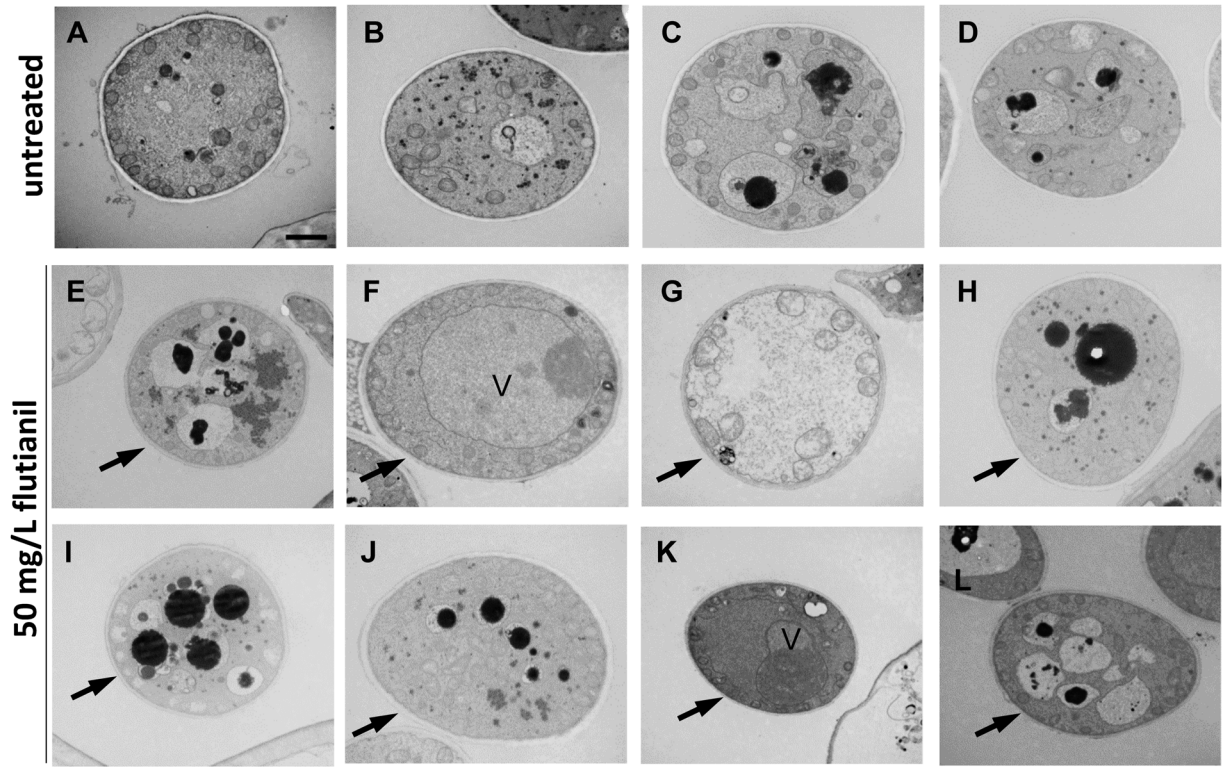

Fig. 3. Transmission electron micrographs of the hyphae of *Bgh* (A–D). Hyphae of *Bgh* without flutianil treatment. (E–L) Hyphae of *Bgh* in barley epidermal cells 2 days after treatment with flutianil (50 mg/L). Bars, 500 nm.

treatment, 1504 differentially expressed genes were identified. Of these, 745 were upregulated and 549 were downregulated. Thus, >1.5-fold or <0.7-fold transcriptional changes were remarkably infrequent with flutianil as compared with cyflufenamid and metrafenone.

3.2. *Effects of flutianil on gene expression in the basic cell function of* Bgh

The effect of flutianil on the genes of *Bgh* that are constantly expressed under normal growth conditions was investigated. Among the transcriptionally active genes, the top 100 most highly expressed genes were identified in the untreated control 9 days after inoculation with *Bgh* (Supplemental Table S1). The most upregulated genes involved basic cell functions such as housekeeping, transcription, protein biosynthesis, lipid metabolism, cellular respiration enzymes, cell wall structure, and effector proteins. These genes play an important role in maintaining the life cycle of powdery mildew. For example, bgh00776 and bgh00774 (β-1,3-glucanosyltransferase)[17] play key roles in cell wall maintenance. Eight candidate secreted effector protein genes (bgh02536,[18] bghG000714000001001, bgh02072, bgh02386, bgh04794, bgh01776, bgh04522, and bghG004378000001001) have been studied and shown to have a function in virulence.[17,19] The bgh04203 (CAP20-like protein) gene was reported as a strong candidate for pathogenicity factors in *Bgh*.[20]

The flutianil treatment did not affect those constantly expressed genes of *Bgh*. In contrast, the expression of 31 of these genes was significantly downregulated by the metrafenone treatment, and the expression of seven of these genes was significantly downregulated by the cyflufenamid treatment. Thus, unlike other powdery-mildew fungicides, flutianil did not significantly affect the genes constantly expressed for the survival of *Bgh*. There was also no correlation in the expression profiles between flutianil and cyflufenamid or metrafenone. Therefore, it appears that flutianil has a different mode of action than cyflufenamid and metrafenone.

3.3. *Up- and downregulated genes in response to flutianil treatment*

The top 100 upregulated and downregulated genes in response to flutianil treatment are shown in Supplemental Tables S2 and S3, respectively. In addition, the genes that specifically exhibited a change in expression in response to flutianil treatment are listed in Table 2. Interestingly, three genes, bgh00499, bgh00500, and bgh00501,[21,22] known to be involved in pathogenicity and responsible for transmembrane transporters such as haustorium-specific sugar transporters, exhibited increased expression in response to flutianil treatment (Tables 2 and Supplemental Table S2). Other than these, no genes exhibiting a specific function in response to flutianil were found. There is no apparent correlation between the expression profiles of flutianil and the other two existing fungicides.

Discussion

Our previous report indicated that in the infection process of

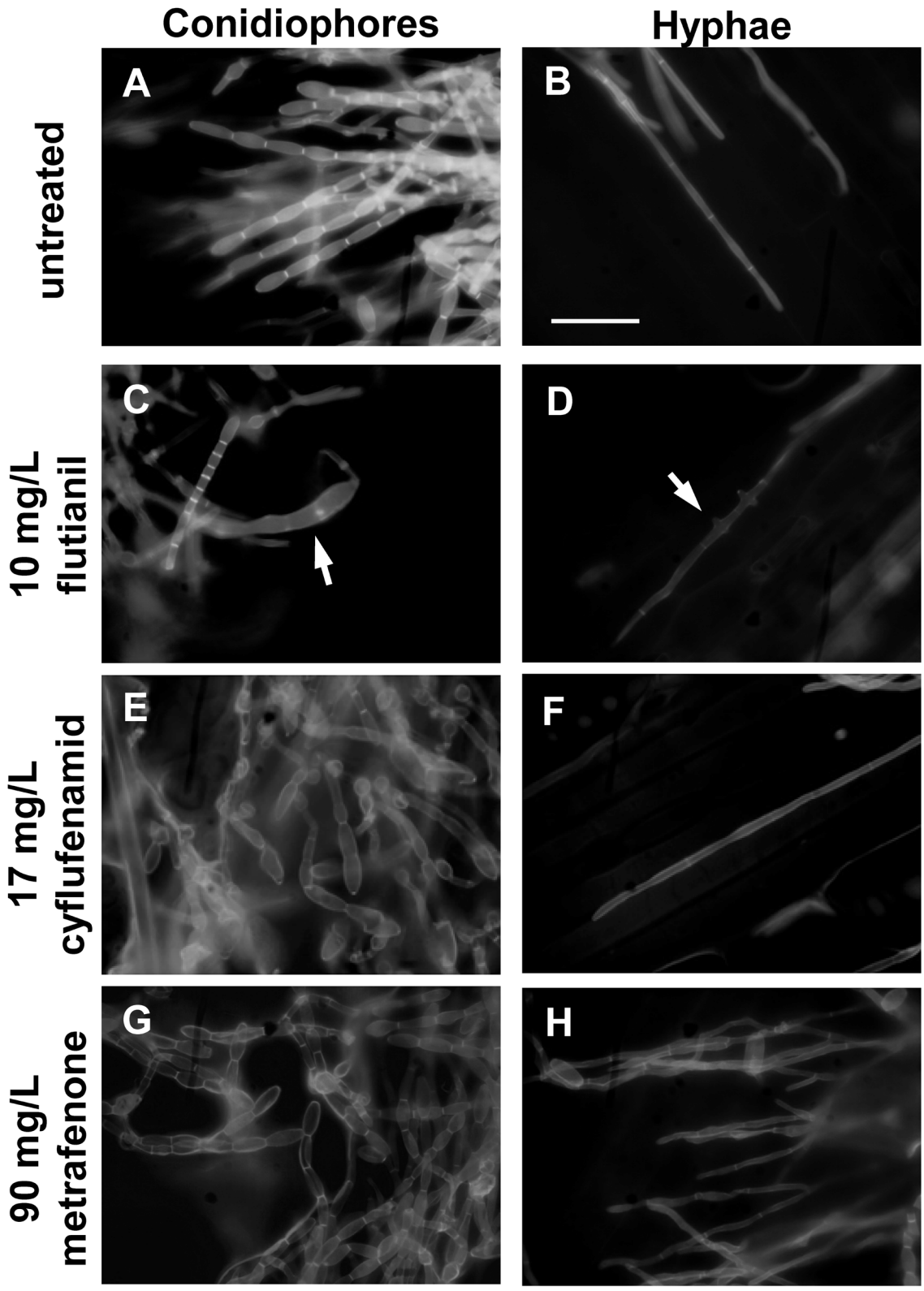

Fig. 4. Comparison of the effect of flutianil with that of cyflufenamid or metrafenone on the morphologies of the hyphae and conidiophores of *Bgh* observed using a confocal laser scanning microscope. Barley leaves were treated with fungicides 7 days after *Bgh* inoculation and subsequently stained with calcofluor white to visualize chitin deposition in the cell wall and septa. (A and B) Untreated conidiophores and secondary hyphae, respectively. (C and D) Flutianil-treated (10 mg/L) conidiophores and secondary hyphae, respectively. (E and F) Cyflufenamid-treated (17 mg/L) conidiophores and secondary hyphae, respectively. (G and H) Metrafenone-treated (90 mg/L) conidiophores and secondary hyphae, respectively. Bars, 50 μm.

Table 1. Differentially-expressed genes from fungal mycelia in the flutianil, cyflufenamid, and metrafenone treatmented *Bgh*-infected leaves

Directionality	Fold change	10 mg/L flutianil	17 mg/L cyflufenamid	90 mg/L metrafenone
Up-regulated	>3	8	9	21
	>2	38	71	189
	>1.5	159	299	535
Down-regulated	<0.7	43	259	759

Bgh, flutianil inhibited the haustorial formation and the prevention of pathogen nutrient acquisition from the host cells.[13] In current studies, the most noteworthy morphological and ultrastructural changes as a result of treating *Bgh* with flutianil were in the haustoria. Flutianil causes the EHMa to be obscured, resulting in the HCW being obscured. The EHMa is believed to represent a major battleground[23,24] and is also a formidable trading site for nutrient exchange and effector delivery, plant–fungal recognition, and plant-defense molecules exchange.[25,26] Interestingly, an important site of action of flutianil is the haustorium an indispensable organ for the infection strategy of the pathogen.

This change in haustorium morphology without any noticeable change in the cytoplasm of fungus cells in response to flutianil is similar to those occurring in powdery mildew or rust in response to plant-defense activators such as benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl[27] (BTH) ester or amino acid L-methionine.[28] Plant-defense activators are not effective when applied directly to the parasites. No difference was found in the ultrastructure of fungi that contained normal cell organelles in untreated or BTH- or L-methionine-treated plants, but remarkable degeneration was found in the host cell.

Simons[29] showed the effects of two fungicides (oxycarboxin and benoyl) on cell organelles in the haustoria of *Puccinia coronata* var. *avenae* generated primarily, but no degeneration occurred in the cytoplasm of the host cells. Richmond and Pring[30] reported the effects of benomyl on *Botrytis fabae* observing the fungal structure's disorganization without any change in the host cells' cytoplasm. Together with our results of morphological change on haustoria in the flutianil-treated *Bgh*, these results indicate that flutianil is not a plant-defense activator but affects fungi directly as an antifungal agent.

Regarding gene expression studies, the most upregulated genes identified in response to flutianil treatment are the sugar transporter genes, such as bgh00499, bgh00500, and bgh00501, and the effector genes, which are specifically localized in haustoria. Given the results of previous studies, in which that flutianil inhibited the formation of haustoria in *Bgh* and nutrient absorption from the host cell, it is interesting to note that flutianil affected the expression of these genes. Other genes with a clear function in response to flutianil could not be identified in this study. Moreover, we analyzed RNA-seq data obtained from the epiphytic structures of *Bgh* tissues isolated following the removal of the haustorial tissue containing plant epidermis. Our study of the RAN-seq analysis demonstrated that flutianil treatment had little effect on gene expression in the epiphytic tissue of *Bgh* as compared with the powdery-mildew fungicides metrafenone and cyflufenamid, confirming that the main target of flutianil is not the epiphytic tissue but the haustorial tissue. Further RNA-seq analysis using haustoria-containing or haustoria-solo tissues is required.

It should be mentioned that there were few similarities in the gene expression profiles of *Bgh* treated with flutianil and that treated with the other powdery-mildew fungicides metrafenone and cyflufenamid, suggesting that the mode of action of flutianil

Table 2. UP- and downregulated genes specifically in response to flutianil treatment (<0.7, and 2-fold or greater difference)

Gene ID[a]	Description[b]	WT (FPKM)	FL/WT	CY/WT	MT/WT
bgh00499	CELP0012 (sugar transporter)	59.5	4.112	1.788	1.582
bgh00500	sugar transporter (major facilitator superfamily)	110.1	2.31	1.793	1.474
bgh00501	sugar transporter (major facilitator superfamily)/MFS glucose transporter	135.8	2.282	1.89	1.518
bgh00059	Chi1/extracellular endochitinase	10.8	2.987	1.361	1.138
bgh06353	conserved hypothetical protein/Sialidase superfamily/BNR/Asp-box repeat protein	161.3	2.473	1.448	1.072
bgh04412	endonuclease/reverse transcriptase	6.2	2.443	1.197	1.497
bghG001399000001001	hypothetical protein	0.2	2.155	1.211	1.424
bgh01106	putative leucine Rich Repeat domain-containing protein	53.5	2.013	1.168	0.995
bgh05200	blue light-inducible protein Bli-3	2.3	0.66	1.051	0.955
bgh00522	protein BCP1	37.4	0.67	1.004	1.217

Red: high upregulated (>3-fold change), Orange: medium upregulated (>2-fold change), Yellow: low upregulated (>1.5-fold change), Blue: downregulated (<0.7-fold change).

[a] Gene identification numbers retrieved *via* www.blugen.org/.

[b] Predicted function for genes.

Abbreviations: WT, water; FL, flutianil; CY, cyflufeanmid; MT, metrafenone.

is entirely different from that of other existing fungicides. In a previous report, the change in morphology of *Bgh* in the early infection stage differed between flutianil- and metrafenone- or cyflufenamid-treated *Bgh*. In this study, the change in morphology of *Bgh* in the late infection stage also differed among flutianil, metrafenone, and cyflufenamid treatments. Metrafenone and cyflufenamid caused the collapse of the fungal body.

In summary, morphological and ultrastructural analyses and comprehensive gene expression analysis using RNA-seq suggest that the primary site of action of flutianil might be in the haustoria.

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Electronic supplementary materials

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

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