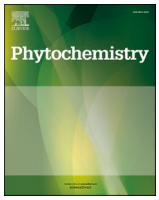
Phytochemistry 184 (2021) 112650

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem



Identification of methoxylchalcones produced in response to $CuCl_2$ treatment and pathogen infection in barley

Naoki Ube [a], Yuhka Katsuyama [b], Keisuke Kariya [c], Shin-ichi Tebayashi [d], Masayuki Sue [e], Takuji Tohnooka [f], Kotomi Ueno [b], Shin Taketa [g], Atsushi Ishihara [b,*]

[a] Arid Land Research Center, Tottori University, Tottori, 680-8553, Japan
[b] Faculty of Agriculture, Tottori University, Tottori, 680-8553, Japan
[c] Graduate School of Sustainability Science, Tottori University, Tottori, 680–8553, Japan
[d] Faculty of Agriculture and Marine Science, Kochi University, Monobe, Nankoku, Kochi, 783–8502, Japan
[e] Department of Agricultural Chemistry, Tokyo University of Agriculture, Tokyo, 243-0034, Japan
[f] National Agriculture and Food Research Organization, Tsukuba, 305-8518, Japan
[g] Institute of Plant Science and Resources, Okayama University, Kurashiki, 710-0046, Japan

ARTICLE INFO

Keywords:
Hordeum vulgare
Poaceae
Phytoalexin
Methoxylchalcone
Flavonoid
Pathogen infection

ABSTRACT

Changes in specialized metabolites were analyzed in barley (*Hordeum vulgare*) leaves treated with $CuCl_2$ solution as an elicitor. LC-MS analysis of the $CuCl_2$-treated leaves showed the induced accumulation of three compounds. Among them, two were purified by silica gel and ODS column chromatography and preparative HPLC and were identified as 2′,3,4,4′,6′-pentamethoxychalcone and 2′-hydroxy-3,4,4′,6′-tetramethoxychalcone by spectroscopic analyses. The remaining compound was determined as 12-oxo-phytodienoic acid (OPDA), a major oxylipin in plants, by comparing its spectrum and retention time from LC-MS/MS analysis with those of the authentic compound. The accumulation of these compounds was reproduced in leaves inoculated with *Bipolaris sorokiniana*, the causal agent of spot blotch of the Poaceae species. This inoculation increased the amounts of other oxylipins, including jasmonic acid (JA), JA-Ile, 9-oxooctadeca-10,12-dienoic acid (9-KODE), and 13-oxooctadeca-9,11-dienoic acid (13-KODE). The treatments of the barley leaves with JA and OPDA induced the accumulation of methoxylchalcones, but treatment with 9-KODE did not. These methoxylchalcones inhibited conidial germination of *B. sorokiniana* and *Fusarium graminearum*, thereby indicating that these compounds possessed antifungal activity. Consequently, they are considered to be involved in the chemical defense processes as phytoalexins in barley. Accumulation of methoxylchalcones in response to JA treatment was observed in all seven barley cultivars tested, but was not detected in other wild Hordeum species, wheat, and rice, thus indicating that their production was specific to cultivated barley.

1. Introduction

Plants employ various defense mechanisms to prevent pathogen infection, including phytoalexin accumulation. Phytoalexins are low-molecular-weight, anti-microbial compounds produced by plants in response to pathogen infection, and their accumulation leads to the formation of an inducible chemical barrier against pathogen attacks. The accumulation of phytoalexins is induced by oxylipins such as jasmonic acid, heavy-metal ions, and UV irradiation, as well as pathogen infection (Nojiri et al., 1996; Rakwal et al., 1996; Kato et al., 1994; Schmelz et al., 2011). The significance of phytoalexin accumulation in the interaction between plants and pathogenic microorganisms has been demonstrated by various experiments. For example, a phytoalexin-deficient mutant (*pad3*) was shown to be more susceptible to pathogenic infection than phytoalexin-producing wild type plants in *Arabidopsis thaliana* (L.) Heynh. (Thomma et al., 1999). Genetically modified tobacco (*Nicotiana tabacum* L.) plants that accumulate a foreign phytoalexin, resveratrol, showed enhanced resistance to the gray mold fungus *Botrytis cinerea* Persoon: Fries (Hain et al., 1993).

Flavonoids are widely distributed specialized metabolites in the plant kingdom. They are biosynthesized from L-phenylalanine via the phenylpropanoid pathway in common with other phenolic metabolites. The first committed step in the biosynthetic pathway is a reaction catalyzed by chalcone synthase (EC 2.3.1.74), where one molecule of

* Corresponding author.
E-mail address: aishihara@tottori-u.ac.jp (A. Ishihara).

https://doi.org/10.1016/j.phytochem.2020.112650
Received 14 September 2020; Received in revised form 8 December 2020; Accepted 23 December 2020
Available online 30 January 2021

hydroxycinnamoyl-CoA is condensed with three molecules of malonyl-CoA to form a chalcone, which is subsequently converted to flavanone by chalcone isomerase (EC 5.5.1.6.). Flavonoids can be extensively modified through hydroxylation, *O*-methylation, and *C*- or *O*-glycosylation, depending on the plant species, organs, and development stages. Flavonoids provide UV protection and pigmentation, protect against pathogens and pests, promote pollen fertility, communicate with microorganisms, and regulate auxin transport (Winkel-Shirley, 2001).

Some flavonoids contribute to the inducible, chemical defense responses as phytoalexins. Sakuranetin (7-*O*-methylnaringenin) is a flavonoid phytoalexin in rice (*Oryza sativa* L.) and is crucial for disease resistance because of its strong antifungal activity against pathogens (Kodama et al., 1992; Hasegawa et al., 2014; Murata et al., 2020). 3-Deoxyanthocyanidins are flavonoid phytoalexins in sorghum (*Sorghum bicolor* (L.) Moench) (Nicholson et al., 1987), and their accumulation is stimulated by inoculation with either pathogenic or non-pathogenic fungi in sorghum (Nicholson et al., 1987; Snyder and Nicholson, 1990). When the *yellow seed1* (*y1*) allele that encodes the transcription factor MYB loses its functionality, 3-deoxyanthocyanidine accumulation decreases and the susceptibility to sorghum pathogens is enhanced (Ibraheem et al., 2010).

Oxylipins such as jasmonic acid (JA) and 12-oxo-phytodienoic acid (OPDA) have been shown to induce defensive responses in plants. "Oxylipins" are oxidized fatty acids with unique physiological roles. Specific oxylipins function as plant signaling molecules that regulate diverse processes such as development, reproduction, stress acclimation, and innate immune responses against pests and pathogens (Farmer et al., 2003; Wasternack and Hause, 2013; Alméras et al., 2003). Recently, the accumulation of 9-oxooctadeca-10,12-dienoic acid (9-KODE) and 13-oxooctadeca-9,11-dienoic acid (13-KODE) was shown to be induced in response to JA treatment and pathogen infection in rice leaves (Nishiguchi et al., 2018). Furthermore, JA and these KODEs induced defensive metabolites, such as sakuranetin and serotonin, in rice leaves. In wheat (*Triticum aestivum* L.), JA treatment led to accumulation of wheat phytoalexins, triticamides A and B (Ube et al., 2019a). The production of maize (*Zea mays* L.) phytoalexins, such as kauralexins and zealexins, is stimulated by co-treatment with JA and ethylene (Schmelz et al., 2011; Huffaker et al., 2011).

Changes in flavonoid metabolism in response to exogenous application of JA have been found in barley (*Hordeum vulgare* L., Poaceae). The barley plant constitutively accumulates the *C*-glycosylated flavones isovitexin-7-*O*-glucoside (saponarin) and isoorientin-7-*O*-glucoside (lutonarin) during primary leaf development (Reuber et al., 1996). Saponarin was shown to be modified by acylation to generate 6‴-sinapoyl-saponarin, 6‴-feruloyl-saponarin, and 4′-glucosyl-6‴-sinapoyl-saponarin in JA-treated young leaves (Ishihara et al., 2002).

Recently, we observed an increased accumulation of the dimer of serotonin and 3-(2-aminoethyl)-3-hydroxyindolin-2-one after infection with *Bipolaris sorokiniana* (Sacc. ex Sorok.) Shoem. and $CuCl_2$ treatment in barley leaves and proposed that they served as phytoalexins in barley (Ishihara et al., 2017). Furthermore, the production of phenylamide phytoalexins, triticamides A–C, was also induced in pathogen-infected tissues (Ube et al., 2019a). In the present study, metabolic changes in barley leaves that received exogenous stimuli were analyzed, and two inducible chalcones with antifungal activity were identified. Additionally, JA and OPDA were shown to induce chalcone accumulation in barley leaves. These findings indicate that chalcone phytoalexin accumulation plays a role in the inducible defensive responses of barley, and that the response is regulated by oxylipins such as JA and OPDA. The discovery of this new phytoalexin class in barley emphasizes the multiplicity of its chemical defense responses.

2. Results

2.1. Detection of inducible metabolites in barley leaves treated with $CuCl_2$

Barley leaves were treated with a $CuCl_2$ solution by the floating method, and the effects of the treatment on specialized metabolite accumulation were analyzed. The $CuCl_2$ solution was used as an elicitor because it has been shown to be effective in inducing the accumulation of specialized metabolites in various plants (Rakwal et al., 1996; Oikawa et al., 2001; Ube et al., 2019a). The third leaves of 3-week-old barley seedlings were treated with a 0.5 mM solution of $CuCl_2$ because this treatment effectively induced the accumulation of phytoalexin triticamides A and B in wheat (Ube et al., 2019a,b), and the metabolites in the leaves were extracted with 80% methanol 72 h after commencement of the treatment. The extracts were subjected to liquid chromatography mass spectrometry (LC-MS) analysis. We manually checked ions corresponding to the peaks detected in the total ion chromatograms (TICs, Fig. 1), and generated selected ion chromatograms (SICs). In the SICs, other than the peaks of known phytoalexins, triticamides A (*m/z* 303 $[M\text{-}OH]^+$, retention time: 6.75 min) and B (*m/z* 305 $[M+H]^+$, retention time: 7.22 min), only three SIC peaks of the ions at *m/z* 359, 293, and 345 showed prominent increases in the peak area in the $CuCl_2$-treated leaves as compared to the peaks in control leaves. The compounds corresponding to these peaks were referred to as **1**–**3**. Triticamide C was not detected under these analytical conditions due to its small accumulated amount. Although other peaks showed slightly increased in peak area in the SICs, their fold changes were less than 3-fold.

2.2. Identification of 1 and 3

We purified **1** and **3** from barley leaves treated with 0.1 mM $CuCl_2$ for 72 h. The molecular formula of **1** was found to be $C_{20}H_{22}O_6$, as determined by HR-MS (*m/z* 359.1487 $[M+H]^+$). The ^1H NMR spectrum of **1** (Table 1, Fig. S1) showed two groups of aromatic proton signals: one at 6.17 ppm represented a tetrasubstituted benzene ring, and the other had peaks at 6.85, 7.06, and 7.09 ppm, corresponding to a trisubstituted benzene ring. Additionally, the spectrum revealed the coupling of two double-bond protons (6.84 and 7.28 ppm, $J = 16.2$ Hz) and the presence of five methoxy groups (3.77–3.91 ppm). Considering the molecular formula and partial structures, a carbonyl group was assumed to be present. The ^1H–^1H COSY spectrum showed a correlation between the double-bond proton signals and those of the trisubstituted benzene ring (Fig. 2A and Fig. S2). These partial structures suggested that **1** possesses a chalcone skeleton. Five methoxy groups were considered to be bound to the two benzene rings of this skeleton because one ring had three positions and the other two positions were available for substitution by methoxy groups. In the ^1H–^1H COSY spectrum, three methoxy groups at 3.77 and 3.86 ppm showed correlations to aromatic protons at 6.17 ppm in the tetrasubstituted ring, while two methoxy groups at 3.91 and 3.90 ppm showed correlations to aromatic protons at 6.85 and 7.06 ppm in the trisubstituted ring, respectively. Two protons of the tetrasubstituted benzene ring were observed as one signal at 6.17 ppm, indicating that this benzene ring possessed a symmetric structure, giving either a 2,4,6- or 3,4,5-trimethoxybenzene ring. The relatively high magnetic field shift of this signal suggested that it was likely a 2,4,6-trimethoxybenzene ring. The three proton signals from the trisubstituted benzene ring appeared at 6.85, 7.06, and 7.09 ppm and indicated occurrence of *ortho* and *meta* coupling. Considering their chemical shifts and coupling patterns, the trisubstituted benzene ring could possess three protons at positions 2, 5, and 6 or positions 2, 4, and 5. Based on the ^1H NMR spectra of pentamethoxychalcones from previous reports, we found the spectrum of **1** to be identical to that of 2′,3,4,4′,6′-pentamethoxychalcone (PMC, Chu et al., 2004). Thus, the identity of **1** was determined and the structure has been shown in Fig. 2B.

The molecular formula of **3** was found to be $C_{19}H_{20}O_6$, as determined by HRMS (*m/z* 345.1331 $[M+H]^+$), which showed that the methyl

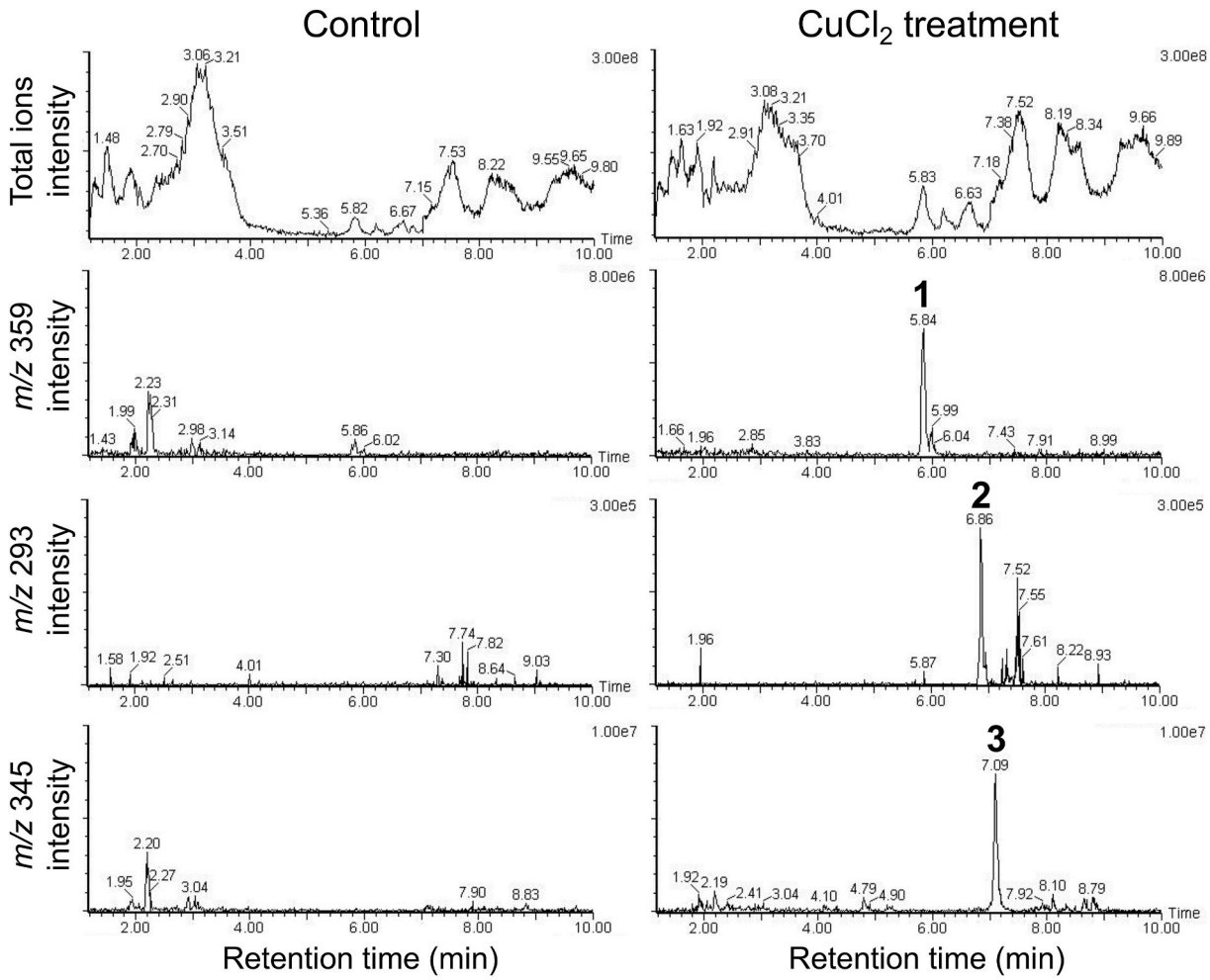


Fig. 1. Comparison of total ion and selected ion chromatograms of extracts from $CuCl_2$-treated and control barley leaves.
The barley leaves were treated with 0.5
mM $CuCl_2$ solution by the floating method. Metabolites in the leaves were extracted 72 h after treatment and analyzed by LC-MS. Three independent samples (3 plant/sample) from either $CuCl_2$ treated or control leaves were analyzed, and increased accumulation of **1**–**3** in the $CuCl_2$ treated leaves was detected in all analyses.

Table 1
^{1}H NMR spectral data (600 MHz) for **1** and **3** in $CDCl_3$.

Compound 1		Compound 3	
Position	^{1}H multi, *J* (Hz)	Position	^{1}H multi, *J* (Hz)
H-3, 5	6.17 (2H, s)	H-3	6.12 (1H, d, 2.4)
H-7	6.84 (1H, d, 16.2)	H-5	5.97 (1H, d, 2.4)
H-8	7.28 (1H, d, 16.2)	H-7	7.80 (1H, d, 15.6)
H-2′	7.06 (1H, d, 1.8)	H-8	7.77 (1H, d, 15.6)
H-5′	6.85 (1H, d, 8.4)	H-2′	7.13 (1H, d, 1.8)
H-6′	7.09 (1H, dd, 8.4, 1.8)	H-5′	6.90 (1H, d, 8.4)
MeO x 5	3.91 (3H, s)	H-6′	7.22 (1H, dd, 8.4, 1.8)
	3.90 (3H, s)	MeO x 4	3.94 (3H, s)
	3.86 (3H, s)		3.93 (3H, s)
	3.77 (6H, s)		3.92 (3H, s)
			3.84 (3H, s)
		OH	14.4 (1H, brs)

group present in **1** was lost in **3**. As such, the ^{1}H NMR spectrum of **3** was similar to that of **1**, except for a methoxy signal that was replaced with a hydroxy proton signal (Table 1, Fig. S3). The four remaining methoxy groups of **3** gave four signals with different chemical shifts, indicating asymmetrical substitution of both benzene rings. Furthermore, the tetrasubstituted benzene ring protons were observed as two signals at 6.12 and 5.97 ppm. These differences in the ^{1}H NMR spectra of **1** and **3** indicated that the tetrasubstituted benzene ring of **3** was asymmetric and the methoxy group that was replaced by a hydroxy group was located at the C-2 position. Indeed, the ^{1}H NMR spectrum of **3** was identical to that of 2′-hydroxy-3,4,4′,6′-tetramethoxychalcone (TMC, Detsi et al., 2009). Thus, the identity of **3** was determined and the structure has been shown in Fig. 2B.

To confirm the structures of **1** and **3**, these compounds were synthesized by the condensation reaction of 3,4-dimethoxybenzaldehyde with 2,4,6-trimethoxy- and 2-hydroxy-4,6-dimethoxy-acetophenones to give **1** and **3**, respectively (Chu et al., 2004), with corresponding yields of 98% and 31.4%, respectively. The ^{1}H NMR spectra of the synthesized compounds were identical to those of **1** and **3**, thereby confirming their identities.

2.3. Identification of 2

Compound **2** was purified by preparative HPLC and subjected to LC-MS analysis. The analysis indicated that the molecular weight of **2** was 292 based on the presence of the $[M\text{-}H]^-$ ion at *m/z* 291. LC-MS/MS analysis of **2** showed fragment ions at *m/z* 273, 247, and 165 in the product ion scan mode (Fig. S4). We speculated that **2** might be OPDA based on the molecular weight of 292 and the detection of $[M\text{-}H\text{-}CO_2]^-$ ion at *m/z* 247. The accumulation of OPDA was previously demonstrated in barley leaves in response to infection by *Fusarium graminearum* Schw., the causal agent of *Fusarium* head blight, and to osmotic stress caused by

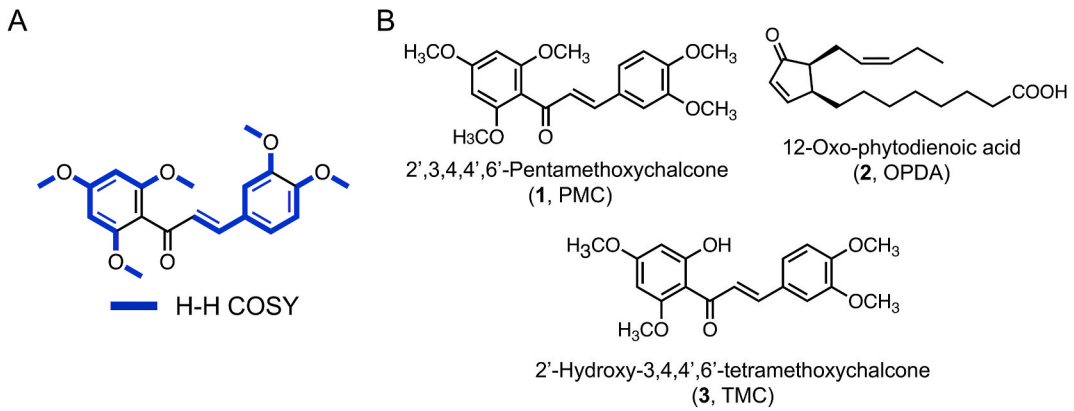


Fig. 2. ^{1}H–^{1}H COSY correlations (indicated by blue bold line) of **1** (A) and chemical structures of compounds **1**–**3** (B). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

sorbitol (Chamarthi et al., 2014; Kramell et al., 2000). The spectra and retention time of **2**, which were determined by LC-MS and LC-MS/MS analysis, were compared with those of authentic OPDA, which confirmed that this compound was the identity of **2**.

2.4. Accumulation of methoxychalcones and oxylipins after pathogen infection in barley leaves

The kinetics of the accumulation of PMC (**1**, Fig. 3A), TMC (**3**, Fig. 3B), and OPDA (**2**, Fig. 3C) were investigated in *B. sorokiniana*-infected leaves. The amounts of **1** and **3** gradually increased 24 h after inoculation, showing a monotonically increasing trend up to 72 h. On the other hand, **2** showed different kinetics, wherein after the inoculation, the amount of **2** began to increase at an earlier point than those of PMC and TMC, and reached a maximum 12 h later. The amounts of **1**–**3** did not change in the intact and control leaves although a marginal increase in the amount of **3** was detected in the control leaves at 72 h. Compound **2** showed a slight increase at 6–12 h in the control leaves.

Additionally, we investigated the accumulation of other oxylipins, JA, JA-Ile, 9-KODE, and 13-KODE because the accumulation of these oxylipins was observed in response to pathogen infection in other plants (Wakuta et al., 2011; Vollenweider et al., 2000; Horie et al., 2015; Nishiguchi et al., 2018). The amounts of these oxylipins also increased after inoculation with *B. sorokiniana*, where JA and JA-Ile started increasing 6 h after inoculation and reached their maxima at 12 h, whereas 9- and 13-KODEs gradually increased throughout the experimental period up to 72 h after inoculation (Fig. 3D–G).

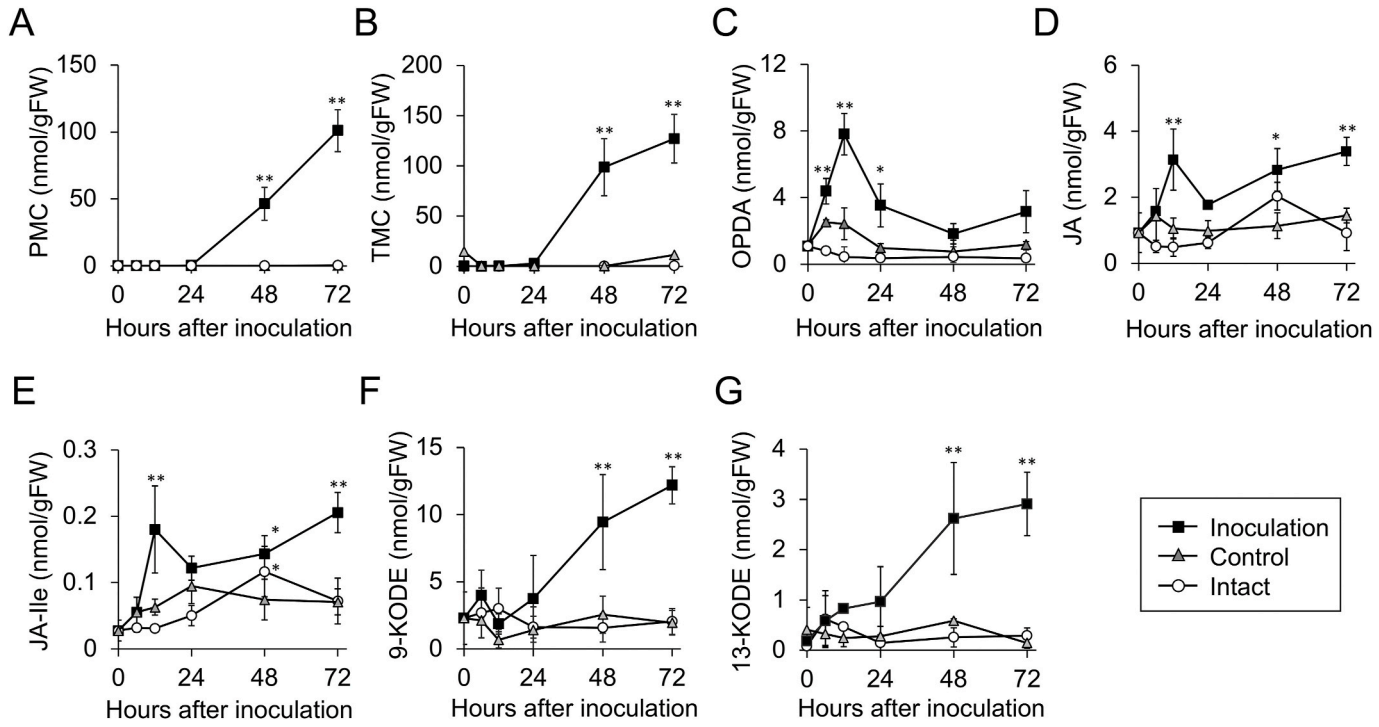


Fig. 3. The kinetics of the accumulation of 2′,3,4,4′,6′-pentamethoxychalcone (PMC, A), 2′-hydroxy-3,4,4′,6′-tetramethoxychalcone (TMC, B), 12-oxo-phytodienoic acid (OPDA, C), jasmonic acid (JA, D), JA-Ile (E), 9-oxooctadeca-10,12-dienoic acid (9-KODE, F), and 13-oxooctadeca-9,11-dienoic acid (13-KODE, G) in *B. sorokiniana*-infected leaves. The metabolites in the leaves were extracted at 0, 6, 12, 24, 48, and 72 h after inoculation.
Data represent mean and standard deviation values obtained from three independent samples (3 plant/sample). Asterisks indicate a statistically significant difference from the values of the intact leaves (*$p < 0.05$; **$p < 0.01$; Dunnett's test).

2.5. Accumulation of 1–3 in leaves treated with $CuCl_2$ and oxylipins

We treated the leaf segments with 0.5 mM $CuCl_2$ solution for 72 h and the amounts of **1**–**3** in the treated leaves were found to be 4.8-, 58.8-, 14.8, -fold higher, respectively, than those in control leaves (Fig. 4A).

To examine the effects of oxylipin treatment on the accumulation of **1**–**3**, droplets of 1 mM JA, OPDA, and 9-KODE solutions were placed on the third leaves. Their concentrations were considered on the basis of the effective concentrations for the induction of metabolic changes in barley and rice (Ishihara et al., 2002; Nishiguchi et al., 2018). Treatment with JA and OPDA elicited the accumulation of **1** and **3** (Fig. 4B). The respective concentrations of **1** and **3** in JA-treated leaves were 25.5 and 15.3 nmol/g FW, and their concentrations in OPDA-treated leaves were 23.4 and 10.6 nmol/g FW, respectively. In contrast, 9-KODE treatment did not significantly affect the accumulation of PMC or TMC. We also included triticamides A–C in the analysis because they have been denoted as phytoalexins of barley (Ube et al., 2019b). However, triticamides were not detected in JA-treated leaves after treatment with any oxylipins.

2.6. Antifungal activities of 1 and 3

The antifungal activities of **1** and **3** against *B. sorokiniana* and *F. graminearum* were evaluated in an assay for the inhibition of conidial germination. Compounds **1** and **3** showed inhibitory effects on the conidial germination of both fungi, and almost completely inhibited them at a concentration of 1000 μM (Fig. 5).

2.7. Accumulation of 1 and 3 in barley cultivars, wild Hordeum species, wheat, and rice

The general accumulation of **1** and **3** in barley cultivars was investigated using seven cultivars: 'Shunrei', 'Morex', 'Bowman', 'Steptoe', 'Yumesakiboshi', 'CDC-Fibar', and 'Sachiho-Golden'. As JA is a defensive hormone and induces metabolic changes in various plant species, we used 0.5 M solution of JA as an elicitor. The increased accumulation of both **1** and **3** in the JA-treated leaves was detected in all cultivars, and their amounts were 21.2–71.3 nmol/g FW for PMC and 6.7–36.0 nmol/g FW for TMC, indicating that these compounds were ubiquitously present in barley cultivars (Fig. 6).

We then expanded the analysis to 10 wild *Hordeum* species: *H. vulgare* L. ssp. *spontaneum* (C. Koch.) in the H clade; *H. murinum* subsp. *glaucum* (Steudel.) Tzvelev. and *H. murinum* subsp. *leporinum* (Link.) in the Xu clade; *H. marinum* subsp. *marinum* Hudson. in the Xa clade, and *H. bogdanii* Wil., *H. brachyantherum* Nevski. ssp. *californicum* (Cov. & Steb.) Both. & et al., *H. chilense* Roem. & Schult., *H. flexuosum* Steud., *H. pusillum* Nutt., and *H. jubatum* L. in the I clade. We also included wheat and rice in the analysis. However, no accumulation of **1** or **3** was detected in any of the analyzed species, suggesting that the accumulation of methoxylchalcones was specific to cultivated barley (data not shown).

3. Discussion

To date, 2′,3,4,4′,6′-pentamethoxychalcone (PMC) has not been isolated from plants although the other PMC, 2′-hydroxy-3,4,3′,4′,6′-pentamethoxychalcone, has been identified in *Citrus* species (Iwase et al., 2001; Li et al., 2006). Presence of 2′-Hydroxy-3,4,4′,6′-tetramethoxychalcone (TMC) has been reported in the root bark of *Pongamia pinnata* L. (Tanaka et al., 1992) and *Bidens parviflora* Willd. (Li et al., 2008). We detected PMC and TMC at concentrations higher than 100 nmol/g FW in *B. sorokiniana*-infected leaves (Fig. 3), and these concentrations were in the range where inhibition of fungal germination was observed (Fig. 6). Thus, PMC and TMC likely exert antifungal activity as chalcone-type phytoalexins and function as a part of the chemical barrier against pathogens.

The finding of induced accumulation of PMC and TMC expands the list of phytoalexins in barley. In this study, their accumulation was observed in the pathogen-infected and $CuCl_2$-treated leaves (Fig. 1). In addition to methoxylchalcones, triticamides and indole amines were induced in pathogen infected and $CuCl_2$-treated barley leaves (Ishihara et al., 2017; Ube et al., 2019a, 2019b). These results indicated that barley possesses multiplicity of phytoalexin to prevent pathogen infection.

The multiplicity of phytoalexin production has been well known in rice and maize. Approximately twenty compounds have been identified as phytoalexins in rice, and they are classified into diterpenoids, flavonoids, and phenylamides (Yamane, 2013; Park et al., 2013; Morimoto et al., 2018). In maize, infection with pathogens induced the accumulation of kauralexins (Schmelz et al., 2011) and zealexins (Huffaker et al., 2011), which belong to diterpenoids and sesquiterpenoids, respectively. Phytoalexins with different structural characteristics likely demonstrate different antimicrobial spectra. Indeed, the rice flavonoid phytoalexins, sakuranetin and naringenin, showed distinct antimicrobial activities against fungal and bacterial pathogens. Sakuranetin showed strong inhibitory activity against fungal pathogens, while naringenin exhibited the activity against bacterial pathogens, but exerted almost no inhibitory effect on fungal pathogens (Murata et al., 2020).

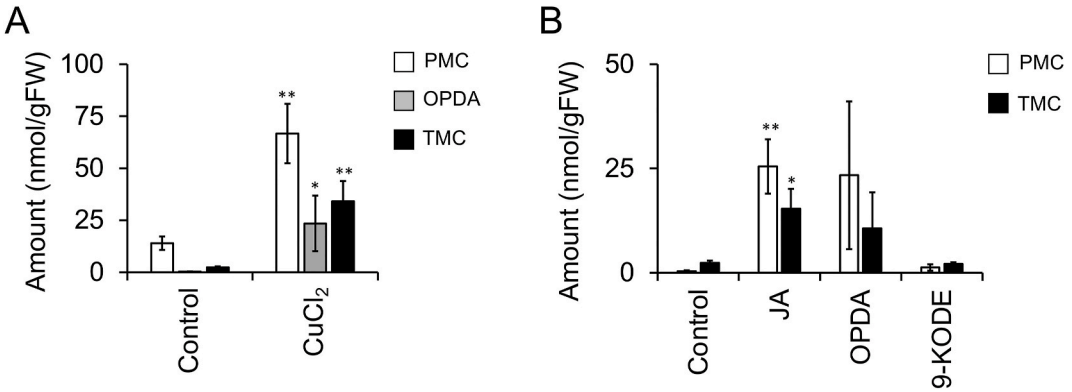


Fig. 4. Accumulation of TMC, PMC, and OPDA in barley leaves treated with 0.5 mM $CuCl_2$ (A) and oxylipins (B)
For the treatment of $CuCl_2$, the third leaves of barley seedlings were floated in a 0.5 mM $CuCl_2$ solution containing 0.25% Tween20, and in distilled water containing 0.25% Tween20 as a control for 72 h. For treatments with oxylipins, droplets of 1 mM solutions of JA, OPDA, and 9-KODE containing 0.25% Tween20 were placed on leaves and incubated for 72 h. Droplets of distilled water containing 0.25% Tween20 were placed on leaves as a control. Data represent mean and standard deviation values obtained from three independent samples (3 plant/sample). Asterisks indicate a statistically significant difference from the value of the control (*$p < 0.05$; **$p < 0.01$; Student's *t*-test).

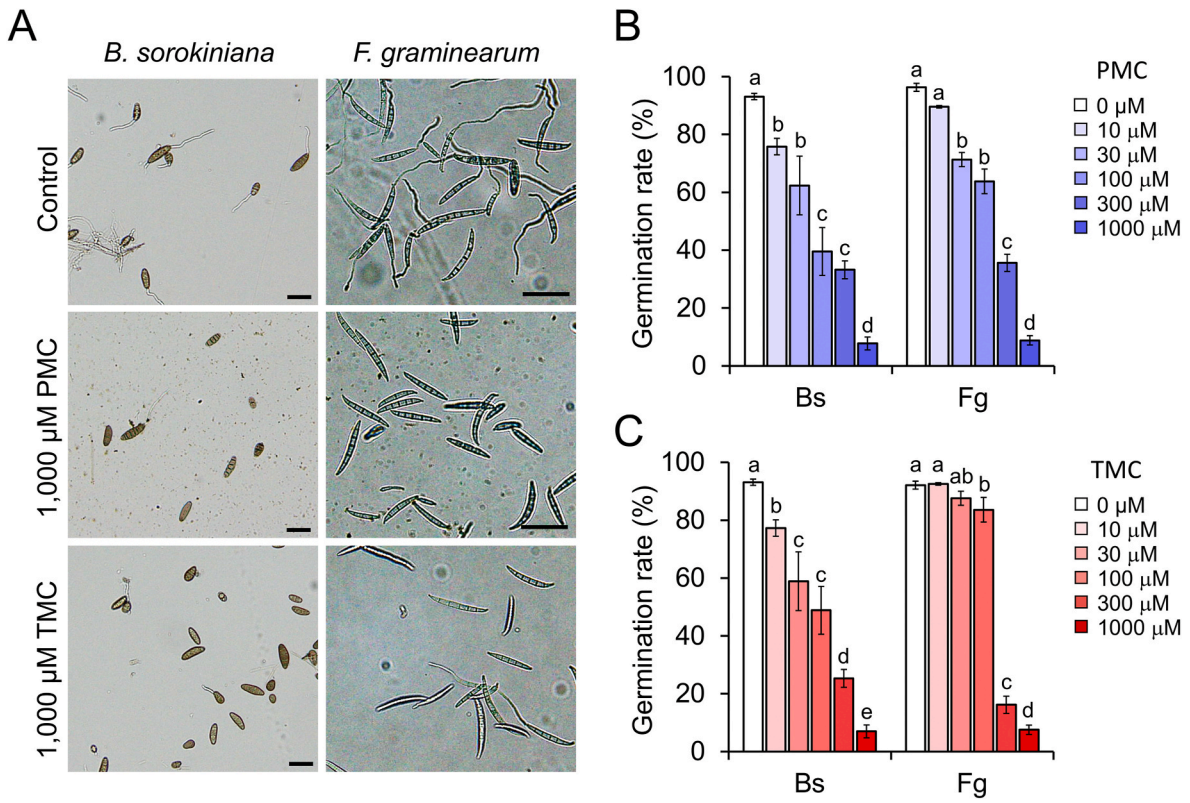


Fig. 5. Antifungal activities of PMC and TMC.
The images show the appearance of the conidia of *F. graminearum* (Fg) and *B. sorokiniana* (Bs) when treated with distilled water as a control and with 1000 μM PMC and TMC solutions (A). The conidia of Fg and Bs were incubated with PMC (B) and TMC (C) at various concentrations between 0 and 1000 μM. Scale bar represents 50 μm (A). Germination rates in the PMC and TMC solutions were determined after a 6-h incubation for Fg and after an 8-h incubation for Bs. Data represent mean and standard deviation values obtained from three independent samples. Letters (a–e) represent significant differences at $p < 0.05$, as measured using the Tukey-Kramer test.

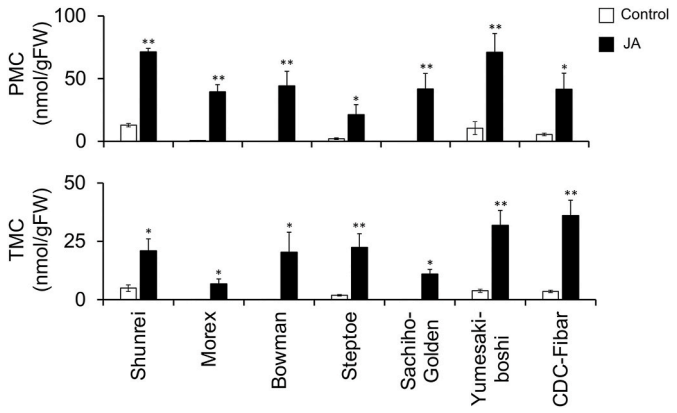


Fig. 6. Accumulation of PMC and TMC in JA-treated leaves of various barley cultivars. Leaf segments were treated with 0.5 M JA solution for 72 h by floating method. Distilled water containing 0.25% Tween20 was used as a control. Data represent mean and standard deviation values obtained from three independent samples (3 plant/sample). Asterisks indicate a statistically significant difference from the value of the control (*$p < 0.05$; **$p < 0.01$; Student's *t*-test).

They are structurally different only in the substituent (methoxyl or hydroxyl group) at the 7-position of the flavanone skeleton. It is thus reasonable to assume that the production of multiple phytoalexins is a strategy for plants to counter a wide array of pathogenic microorganisms.

The distribution of PMC and TMC was limited in cultivated barley, and these compounds were not detected in wheat, rice, other *Hordeum* species, or the wild relative of cultivated barley, *H. vulgare* ssp. *spontaneum*. Thus, the biosynthesis of methoxychalcones may have been acquired during the domestication process of barley. However, barley has been suggested to have been domesticated from *H. vulgare* ssp. *spontaneum* at least twice, and there is presumably a considerable natural variation in the specialized metabolism of *H. vulgare* subsp. *spontaneum* (Pourkheirandish et al., 2015). Analysis of methoxychalcones in various strains of *H. vulgare* subsp. *spontaneum* is needed to address the evolutionary origin of their biosynthetic pathway.

The expression of the chalcone synthase gene *HvCHS2* (accession no. Q96562) was upregulated in powdery mildew-infected and UV-treated barley leaves (Christensen et al., 1998a), although inducible flavonoids themselves have not been identified. HvCHS2 showed a preference for caffeoyl- and feruloyl-CoAs as substrates over coumaroyl- and cinnamoyl-CoAs. Thus, if this enzyme is involved in the biosynthesis of methoxychalcones, 2′,3,4,4′,6′-pentahydroxychalcone or 2′,4,4′,6′-tetrahydroxy-3-methoxychalcone—which are formed from caffeoyl- and feruloyl-CoAs, respectively—are the first intermediates in their biosynthetic pathway.

Two methyltransferases that accept flavonoids as substates have been characterized in barley. Christensen et al. (1998b) reported the expression of a flavonoid 7-*O*-methyltransferase gene (*OMT*, accession no. X77467.1) was upregulated in barley leaves in response to powdery mildew infection. Furthermore, when this OMT-favored apigenin served as a substrate, genkwanin (7-*O*-methylapigenin) was generated. Zhou et al. (2008) characterized the barley flavone methyltransferase-like gene (*HvOMT1*, accession no. BI956358). HvOMT1 preferred tricetin (5,7,3′,4′,5′-pentahydroxyflavone) as a substrate and generated its 3′, 5′-dimethyl derivative, tricin, as the major product. These flavone *O*-methyltransferases demonstrated clear regiospecificity in terms of

methylation position. It is probable that multiple methyltransferases with different substrate- and regio-specificities are involved in the biosynthesis of PMC and TMC to ensure completion of the methylation reactions. Feeding experiments using labeled precursors as well as analyses of the substrate- and regio-specificities of inducible methyltransferases and chalcone synthases, are needed to elucidate the precise methylation order in the biosynthesis of TMC and PMC.

Induced accumulation of OPDA, JA, and JA-Ile in response to exogenous stimuli, such as osmotic stress and pathogen infection, has been demonstrated in barley (Kramell et al., 2000; Kumaraswamy et al., 2011). In addition to these compounds, we found that 9- and 13-KODEs accumulated in pathogen-infected barley leaves (Fig. 4). However, OPDA, JA, and JA-Ile accumulated earlier than the 9- and 13-KODEs. Furthermore, treatments of barley leaves with JA and OPDA induced PMC and TMC accumulation, but treatment with 9-KODE did not. This indicated that these oxylipins play different roles at different times during the disease response in barley.

JA and OPDA are signal molecules involved in stress responses and trigger the accumulation of defensive metabolites in plants (Schmelz et al., 2011; Huffaker et al., 2011; Murata et al., 2020; Ube et al., 2019a). In barley, PMC and TMC were induced in the JA-treated leaves, whereas triticamides were not induced by JA treatment. Ishihara et al. (2002) reported that 6‴-feruloylsaponarin and 6‴-sinapoylsaponarin accumulated in JA-treated leaves, but not in leaves treated with other plant hormones in barley. The signaling role of JA in promoting sakuranetin production was clearly demonstrated by rice mutants lacking the JA-signaling pathway, wherein sakuranetin did not accumulate in response to pathogen attack (Ogawa et al., 2017). In *Petroselinum crispum* (Mill.) fuss cell culture, the accumulation of flavonoid phytoalexin apiin was induced by OPDA treatment (Dittrich et al., 1992). These findings suggest that the JA-mediated signaling pathway is closely associated with the activation of the flavonoid biosynthetic pathway.

The functions of KODEs remain unclear, although their accumulation in several plant species has been reported (Nishiguchi et al., 2018; Vollenweider et al., 2000; Feng et al., 2007; Gao et al., 2007). In rice, the treatment of leaves with KODEs caused the accumulation of defensive metabolites sakuranetin and serotonin (Nishiguchi et al., 2018). In *Arabidopsis thaliana*, it has been shown that infection of *Pseudomonas syringae* pv. *tomato* Van Hall led to high concentrations of KODE accumulation in the leaves (Vollenweider et al., 2000). The exogenous application of KODEs to Arabidopsis leaves resulted in cell death and the expression of the glutathione *S*-transferase gene (*GST1*). *GST1* expression is known to occur in pathogenesis and is caused by oxidative stress (Jabs et al., 1996; Mauch and Dudler, 1993). Therefore, KODE accumulation was considered to be intimately related to the expression of stress responses. The accumulation of KODEs may be a general response of plants towards a pathogen attack; however, the function of KODEs in barley requires elucidation.

4. Conclusion

PMC (**1**), OPDA (**2**), and TMC (**3**) accumulation in barley was induced in response to pathogen attack. Based on PMC and TMC antifungal activity, we proposed that these two methoxychalcones serve as chalcone-type phytoalexins. Since indole amine derivatives (Ishihara et al., 2017) and triticamides A–C (Ube et al., 2019a,b) have been previously reported as phytoalexins, barley plants utilize multiple classes of phytoalexin as a chemical defense barrier. Treatments of barley leaves with JA and OPDA promoted the accumulation of PMC and TMC, suggesting that chalcone phytoalexin biosynthesis was regulated by oxylipins.

5. Experimental

5.1. Instrumentation

^{1}H and 2D (^{1}H–^{1}H COSY) NMR spectra were recorded using the Avance II instrument (Bruker, Billerica, MA, USA). HR-MS was measured using the Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). ESI-MS measurements were performed using the Quattro Micro API mass spectrometer (Waters, Milford, MA, USA) connected to the Acquity UPLC system (Waters). HPLC was performed using the 10 A HPLC system (Shimadzu, Kyoto, Japan).

5.2. Plant materials and pathogenic fungi

Barley (*Hordeum vulgare* L. 'Shunrei' (Poaceae)] and wheat [*Triticum aestivum* L. 'Norin 61' (Poaceae)] were purchased from JA Inaba (Tottori, Japan) and Tsurushin Shubyo (Kyoto, Japan), respectively. Seeds of rice [*Oryza sativa* L. 'Nipponbare' (Poaceae)] were acquired from the stock of the Faculty of Agriculture at Tottori University (Tottori, Japan). The seeds of these species were immersed in distilled water for one night, sowed on a 1:1 (v/v) mixture of vermiculite (Shoei Sangyo, Okayama, Japan) and culture soil (Iris Ohyama, Sendai, Japan), and incubated at 25 °C with 14-h light/10-h dark cycles for 3 weeks.

H. vulgare L. subsp. *spontaneum* (C. Koch.) Thell., *H. murinum* subsp. *glaucum* (Steudel.) Tzvelev., *H. marinum* subsp. *marinum* Hudson, *H. chilense* Roem. & Schult., *H. brachyantherum* Nevski. subsp. *californicum* (Cov. & Steb.) Both. & et al., *H. bogdanii* Wil., *H. flexuosum* Steud., *H. pusillum* Nutt. (these nine species are all diploids), *H. murinum* subsp. *leporinum* (Link.) Arcang., and *H. jubatum* L. (both species are tetraploids) were obtained from the stocks of the Institute of Plant Science and Resources, Okayama University (Kurashiki, Okayama, Japan). The taxonomy of the *Hordeum* species is in accordance with the description reported by von Bothmer et al. (1995).

B. sorokiniana (Sacc. ex Sorok.) Shoem. (OB-25-1), the causal agent of spot blotch of the poaceous species, was a stock from the Laboratory of Natural Product Chemistry, Faculty of Agriculture, Tottori University. The causal agent of *Fusarium* head blight, *F. graminearum* Schw. (H-3), was a stock from the National Agriculture and Food Research Organization. *B. sorokiniana* and *F. graminearum* were inoculated on V8 agar plates and cultured for 1 week and 2 weeks, respectively, at 25 °C under a black light bulb (BLB: FL15BL-B, Hitachi, Tokyo) to obtain conidia.

5.3. Chemicals

JA was prepared by alkaline hydrolysis of methyl jasmonate (Tokyo Chemical Industry, Tokyo, Japan). JA-Ile, 9-KODE, and 13-KODE were purchased from Funakoshi (Tokyo). OPDA was purified from injured barley leaves. Aerial parts of barley leaves (213 g) were ground in liquid N_2 with a mortar and pestle. The ground residue was incubated at 30 °C for 6 h and immersed in 2.1 L of 80% methanol for 24 h. The extract was evaporated *in vacuo* and partitioned with water and EtOAc. The EtOAc layer was evaporated to dryness and dissolved in 50% methanol. This solution was subjected to Sep-Pak Plus C18 environmental (Waters) and eluted with 50%, 60%, 70%, 80%, 90%, and then 100% methanol in water. These fractions were subjected to LC-MS analysis to monitor the elution of OPDA. The fractions containing OPDA (60% and 70% methanol fractions) were combined, concentrated, and subjected to preparative HPLC. The HPLC conditions were as follows: column, Cosmosil 5C_{18}-AR-II (Nacalai Tesque, Kyoto), 20 I.D. × 250 mm; solvents, 0.1% formic acid water (A) and methanol (B); elution, 75% isocratic B/(A + B); flow rate, 7.0 mL/min; detection, 220 nm; column temperature, 40 °C. OPDA was eluted at 26.2 min, and the fraction corresponding to it was collected and concentrated to dryness to yield OPDA (1.7 mg). The identity of the compound was confirmed by comparing the ^{1}H NMR spectra with reported data (Nonaka et al., 2010). The preparation of triticamides A–C was conducted as described in a previous study (Ube

et al. 2019a, 2019b).

5.4. Treatment of barley leaves with chemicals

We used the following two methods to treat leaves with compounds: floating method and droplet method. In the floating method, leaf segments were floated in a solution of compounds containing 0.25% Tween20 and incubated for 72 h at 25 °C with 14-h light/10-h dark cycles. In the droplet method, droplets (5 μL) of a solution of compounds containing 0.25% Tween20 were placed on a barley leaf at 1.0-cm intervals. The seedlings were incubated for 72 h at 25 °C with 14-h light/10-h dark cycles.

5.5. Inoculation of pathogens

The conidia of *B. sorokiniana* were inoculated to the third leaves of 3-week-old barley seedlings. Droplets (5 μL) of the conidial suspension (5×10^5 conidia/mL in distilled water containing 0.25% Tween 20) were placed on a barley leaf at 1.0-cm intervals. Droplets of distilled water containing 0.25% Tween 20 were also placed on the leaves as a control. Intact leaves without any treatments were also extracted. The inoculated seedlings were kept in a moist, air-tight bag for 24 h, and then removed from the bag and further incubated at 25 °C with 14-h light/10-h dark cycles. The metabolites in the leaves were extracted at 0, 6, 12, 24, 48, and 72 h after inoculation.

5.6. Analyses of oxylipins and methoxylchalcones

In all analyses, we extracted metabolites in the leaves by immersing the leaves in 80% methanol for 24 h. The volume of 80% methanol was fixed at a ratio of 10 mL/g FW leaves. The concentrations of metabolites in the leaves were calculated under the assumption that the concentrations in the leaves were 10 times higher than those in the extracts.

For the detection of metabolites induced by $CuCl_2$ treatment, the extracts of $CuCl_2$-treated leaves were analyzed by LC-MS (Quattro Micro API mass spectrometer connected with Aquity UPLC, Waters). The LC conditions were as follows: column, Acquity UPLC BEH C18 (Waters), 2.1 mm × 50 mm (1.7 μm); flow rate, 0.2 mL/min; column temperature, 40 °C; solvents, 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B); gradient, 5%–100% A/(A + B) within 10 min. The MS scan conditions were as follows: capillary voltage, 3 kV; cone voltage, 20 V; source temperature, 120 °C; desolvation temperature, 350 °C; desolvation gas flow, 600 L/h; cone gas flow, 50 L/h; *m/z* range, 150–1000.

Compounds **1**–**3** and KODEs were analyzed using LC-MS/MS (Quattro Micro API mass spectrometer connected with Aquity UPLC, Waters) coupled with multiple reaction monitoring (MRM) methods. The LC conditions were as follows: column, Acquity UPLC BEH C18 (Waters), 2.1 mm × 50 mm (1.7 μm); flow rate, 0.2 mL/min; column temperature, 40 °C; solvents, 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B); gradient for OPDA, 9-KODE, and 13-KODE, 40%–80% A/(A + B) within 5 min; gradient for PMC and TMC, 5%–70% A/(A + B) within 10 min. The MRM conditions were optimized using authentic compounds (Table S1). The amounts of compounds were determined using external standards.

The amounts of JA and JA-Ile were determined by LC-MS/MS analysis in the MRM mode with the Shimadzu LCMS-8040 system (Shimadzu). The HPLC conditions were as follows: column, Inertsil ODS-4 5 μm (GL Sciences, Tokyo, Japan), 4.6 mm × 150 mm; column temperature, 40 °C; solvent, water (A) and acetonitrile (B); gradient, 10% B/(A + B) (0–2 min), 10%–100% (2–12 min), 100% (12–13 min); flow rate, 0.4 mL/min. ESI-MS/MS analysis in the MRM mode was performed in the negative mode. For quantitative analysis of these compounds, ESI-MS/MS analysis in the MRM mode was performed in the negative mode for JA and JA-Ile, and the MRM conditions were optimized using authentic compounds (Table S2). Authentic JA and JA-Ile were used as external standards to determine the contents of compounds.

5.7. Isolation of compounds **1** and **3**

Aerial parts (485 g) of barley seedlings were immersed in a 0.1 mM $CuCl_2$ solution and incubated at 23 °C with 14-h light/10-h dark cycles. After a 72-h incubation, metabolites in the aerial parts were extracted with 80% methanol for 24 h, and the obtained extract was evaporated *in vacuo*. The residue was partitioned with water and EtOAc, and the EtOAc layer was evaporated to dryness. The EtOAc extract was subjected to column chromatography using silica gel (Daisogel IR-60-63/210, Osaka Soda, Osaka, Japan). The column was eluted with a mixture of acetone and hexane. The concentration of acetone was increased from 0% to 100% in increments of 10%. Compounds **1** and **3** were detected in the 30% acetone fraction, which was then subjected to ODS column chromatography (Cosmosil $75C_{18}$-PREP, Nacalai Tesque). The column was eluted with 50%, 60%, 70%, and 80% methanol in water (150 mL each). Compounds **1** and **3** were eluted in 60% and 70% methanol fractions. These fractions were combined, evaporated to a small volume, and subjected to preparative HPLC under the following conditions: column, Cosmosil $5C_{18}$-AR-II (Nacalai Tesque), 10 I.D. × 250 mm; solvents, water (A) and acetonitrile (B); elution, 60% B/(A + B); flow rate, 3.0 mL/min; detection, 280 nm; column temperature, 40 °C. Compounds **1** and **3** were eluted at 14.2 and 17.8 min, respectively.

Compound **1** (2′,3,4,4′,6′-pentamethoxychalcone), 1.2 mg; HR-MS (positive ESI): *m/z* 359.1487 $[M+H]^+$ (calcd. for $C_{20}H_{23}O_6$, *m/z* 359.1494); UV–Vis (acetonitrile-water containing 0.1% formic acid): λ_{max} 223 and 340 nm; ^{1}H NMR data are shown in Table 1.

Compound **3** (2′-hydroxy-3,4,4′,6′-tetramethoxychalcone), 1.8 mg; HR-MS (positive ESI): *m/z* 345.1331 $[M+H]^+$ (calcd. for $C_{19}H_{21}O_6$, *m/z* 345.1338); UV–Vis (acetonitrile-water containing 0.1% formic acid): λ_{max} 222 and 375 nm; ^{1}H NMR data are shown in Table 1.

5.8. Syntheses of **1** and **3**

Compound **1** was synthesized according to the methods described by Chu et al. (2004). A mixture of 2,4,6-trimethoxyacetophenone (210 mg, 1.0 mmol) and 3,4-dimethoxybenzaldehyde (191 mg, 1.15 mmol) in 20 mL of anhydrous EtOH was stirred at room temperature for 20 min. A solution of 50% KOH (40 mL) was added to the mixture and the mixture was stirred at room temperature for 3 h. The resulting solution was neutralized by the addition of 4 M HCl and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, filtered, and the solvent was removed under reduced pressure. The residue was subjected to silica gel column chromatography (Daisogel IR-60-63-210). The compounds were eluted with EtOAc/*n*-hexane (3:7, v/v), and the fraction volume was set to 5 mL. The fractions containing **1** were combined and concentrated to give the product (353 mg, 99% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 3.77 (6H, s, OMe × 2), 3.86, 3.90, and 3.91 (3H each, s, OMe × 3), 6.17 (2H, s, H–3,5), 6.84 (1H, d, J = 16.0 Hz, H-7), 6.86 (1H, d, J = 8.0 Hz, H-5′), 7.07 (1H, s, H-2′), 7.09 (1H, d, J = 8.0 Hz, H-6′), and 7.29 (1H, d, J = 16.0 Hz, H-8).

Compound **3** was synthesized from 2-hydroxy-4,6-dimethoxyacetophenone (98 mg, 0.5 mmol) and 3,4-dimethoxybenzaldehyde (96 mg, 0.58 mmol) using the same method utilized for the synthesis of **1**. After extraction with CH_2Cl_2, the residue of **3** was recrystallized from EtOH and purified **3** was obtained (54.0 mg, 31% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 3.83, 3.90, 3.92, 3.93 (3H each, s, OMe × 4), 5.95 (1H, d, J = 1.8 Hz, H-5), 6.10 (1H, d, J = 1.8 Hz, H-3), 6.89 (1H, d, J = 8.0 Hz, H-5′), 7.21 (1H, d, J = 8.0 Hz, H-6′), 7.25 (1H, s, H-2′), 7.75 (1H, d, J = 15.5 Hz, H-8), 7.79 (1H, d, J = 15.5 Hz, H-7), and 14.40 (1H, bs, –OH).

5.9. Antifungal activity

The antifungal activities of PMC and TMC for *B. sorokiniana* and *F. graminearum* were evaluated as described by Ube et al. (2019a). The

conidial germination assay was performed in 10, 30, 100, 300, and 1000 μM solutions of PMC and TMC.

Declaration of competing interest

The authors declare no conflicts of interest associated with this manuscript.

Acknowledgments

This work was supported by the Japan Society of the Promotion of the Science (JSPS) KAKENHI (Grant Number 18J11919 to N.U.) and by the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) as part of a joint research program implemented at the Institute of Plant Science and Resources, Okayama University, Japan, and the Arid Land Research Center, Tottori University, Japan.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2020.112650.

References

Alméras, E., Stolz, S., Vollenweider, S., Reymond, P., Mène-Saffrané, L., Farmer, E.E., 2003. Reactive electrophile species activate defense gene expression in Arabidopsis. Plant J. 34, 205–216. https://doi.org/10.1046/j.1365-313x.2003.01718.x.

Chamarthi, S.K., Kumar, K., Gunnaiah, R., Kushalappa, A.C., Dion, Y., Choo, T.M., 2014. Identification of fusarium head blight resistance related metabolites specific to doubled-haploid lines in barley. Eur. J. Plant Pathol. 138, 67–78. https://doi.org/10.1007/s10658-013-0302-8.

Christensen, A.B., Gregersen, P.L., Schröder, J., Collinge, D.B., 1998a. A chalcone synthase with an unusual substrate preference is expressed in barley leaves in response to UV light and pathogen attack. Plant Mol. Biol. 37, 849–857. https://doi.org/10.1023/A:1006031822141.

Christensen, A.B., Gregersen, P.L., Olsen, C.E., Collinge, D.B., 1998b. A flavonoid 7-*O*-methyltransferase is expressed in barley leaves in response to pathogen attack. Plant Mol. Biol. 36, 219–227. https://doi.org/10.1023/a:1005985609313.

Chu, H.W., Wu, H.T., Lee, Y.J., 2004. Regioselective hydroxylation of 2-hydroxychalcones by dimethyldioxirane towards polymethoxylated flavonoids. Tetrahedron 60, 2647–2655. https://doi.org/10.1016/j.tet.2004.01.023.

Detsi, A., Majdalani, M., Kontogiorgis, C.A., Hadjipavlou-Litina, D., Kefalas, P., 2009. Natural and synthetic 2'-hydroxy-chalcones and aurones: synthesis, characterization and evaluation of the antioxidant and soybean lipoxygenase inhibitory activity. Bioorg. Med. Chem. 17, 8073–8085. https://doi.org/10.1016/j.bmc.2009.10.002.

Dittrich, H., Kutchan, T.M., Zenk, M.H., 1992. The jasmonate precursor, 12-oxo-phytodienoic acid. Induces phytoalexin synthesis in *Petroselinum crispum* cell cultures. FEBS Lett. 309, 33–36. https://doi.org/10.1016/0014-5793(92)80733-W.

Farmer, E.E., Alméras, E., Krishnamurthy, V., 2003. Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. Curr. Opin. Plant Biol. 6, 372–378. https://doi.org/10.1016/s1369-5266(03)00045-1.

Feng, S., Saw, C.L., Lee, Y.K., Huang, D., 2007. Fungal-stressed germination of black soybeans leads to generation of oxooctadecadienoic acids in addition to glyceollins. J. Agric. Food Chem. 55, 8589–8595. https://doi.org/10.1021/jf0716735.

Gao, X., Shim, W.B., Göbel, C., Kunze, S., Feussner, I., Meeley, R., Balint-Kurti, P., Kolomiets, M., 2007. Disruption of a maize 9-lipoxygenase results in increased resistance to fungal pathogens and reduced levels of contamination with mycotoxin fumonisin. Mol. Plant Microbe Interact. 20, 922–933. https://doi.org/10.1094/MPMI-20-8-0922.

Hain, R., Reif, H.J., Krause, E., Langebartels, R., Kindl, H., Vornam, B., Wiese, W., Schmelzer, E., Schreier, P.H., Stöcker, R.H., Stenzel, K., 1993. Disease resistance results from foreign phytoalexin expression in a novel plant. Nature 361, 153–156. https://doi.org/10.1038/361153a0.

Hasegawa, M., Mitsuhara, I., Seo, S., Okada, K., Yamane, H., Iwai, T., Oha-shi, Y., 2014. Analysis on blast fungus-responsive characters of a flavonoid phytoalexin sakuranetin; Accumulation in infected rice leaves, antifungal activity and detoxification by fungus. Molecules 19, 11404–11418. https://doi.org/10.3390/molecules190811404.

Horie, K., Inoue, Y., Sakai, M., Yao, Q., Tanimoto, Y., Koga, J., Toshima, H., Hasegawa, M., 2015. Identification of UV-induced diterpenes including a new diterpene phytoalexin, phytocassane F, from rice leaves by complementary GC/MS and LC/MS approaches. J. Agric. Food Chem. 63, 4050–4059. https://doi.org/10.1021/acs.jafc.5b00785.

Huffaker, A., Kaplan, F., Vaughan, M.M., Dafoe, N.J., Ni, X.Z., Rocca, J.R., Alborn, H.T., Teal, P.E.A., Schmelz, E.A., 2011. Novel acidic sesquiterpenoids constitute a dominant class of pathogen-induced phytoalexins in maize. Plant Physiol 156, 2082–2097. https://doi.org/10.1104/pp.111.179457.

Ibraheem, F., Gaffoor, I., Chopra, S., 2010. Flavonoid phytoalexin-dependent resistance to anthracnose leaf blight requires a functional yellow seed1 in Sorghum bicolor. Genetics 184, 915–926. https://doi.org/10.1534/genetics.109.111831.

Ishihara, A., Ogura, Y., Tebayashi, S., Iwamura, H., 2002. Jasmonate-induced changes in flavonoid metabolism in barley (*Hordeum vulgare*) leaves. Biosci. Biotechnol. Biochem. 66, 2176–2182. https://doi.org/10.1271/bbb.66.2176.

Ishihara, A., Kumeda, R., Hayashi, N., Yagi, Y., Sakaguchi, N., Kokubo, Y., Ube, N., Tebayashi, S., Ueno, K., 2017. Induced accumulation of tyramine, serotonin, and related amines in response to *Bipolaris sorokiniana* infection in barley. Biosci. Biotechnol. Biochem. 81, 1090–1098. https://doi.org/10.1080/09168451.2017.1290520.

Iwase, Y., Takahashi, M., Takemura, Y., Ju-ichi, M., Ito, C., Furukawa, H., Yano, M., 2001. Isolation and identification of two new flavanones and a chalcone from *Citrus kinokuni*. Chem. Pharm. Bull. 49, 1356–1358. https://doi.org/10.1248/cpb.49.1356.

Jabs, T., Dietrich, R.A., Dangl, J.L., 1996. Initiation of runaway cell death in an Arabidopsis mutant by extracellular superoxide. Science 273, 1853–1856. https://doi.org/10.1126/science.273.5283.1853.

Kato, H., Kodama, O., Akatsuka, T., 1994. Oryzalexin F, a diterpene phytoalexin from UV-irradiated rice leaves. Phytochemistry 36, 299–301. https://doi.org/10.1016/S0031-9422(00)97064-X.

Kodama, O., Miyakawa, J., Akatsuka, Y., Kiyosawa, S., 1992. Sakuranetin, a flavanone phytoalexin from ultraviolet-irradiated rice leaves. Phytochemistry 31, 3807–3809. https://doi.org/10.1016/S0031-9422(00)97532-0.

Kramell, R., Miersch, O., Atzorn, R., Parthier, B., Wasternack, C., 2000. Octadecanoid-derived alteration of gene expression and the "oxylipin signature" in stressed barley leaves. Implications for different signaling pathways. Plant Physiol 123, 177–188. https://doi.org/10.1104/pp.123.1.177.

Kumaraswamy, G.K., Bollina, V., Kushalappa, A.C., Choo, T.M., Dion, Y., Rioux, S., Mamer, O., Faubert, D., 2011. Metabolomics technology to phenotype resistance in barley against *Gibberella zeae*. Eur. J. Plant Pathol. 130, 29–43. https://doi.org/10.1007/s10658-010-9729-3.

Li, S., Lo, C.Y., Ho, C.T., 2006. Hydroxylated polymethoxyflavones and methylated flavonoids in sweet orange (*Citrus sinensis*) peel. J. Agric. Food Chem. 54, 4176–4185. https://doi.org/10.1021/jf060234n.

Li, Y.L., Li, J., Wang, N.L., Yao, X.S., 2008. Flavonoids and a new polyacetylene from Bidens parviflora Willd. Molecules 13, 1931–1941. https://doi.org/10.3390/molecules13081931.

Mauch, F., Dudler, R., 1993. Differential induction of distinct glutathione-S-transferases of wheat by xenobiotics and by pathogen attack. Plant Physiol 102, 1193–1201. https://doi.org/10.1104/pp.102.4.1193.

Morimoto, N., Ueno, K., Teraishi, M., Okumoto, Y., Mori, N., Ishihara, A., 2018. Induced phenylamide accumulation in response to pathogen infection and hormone treatment in rice (*Oryza sativa*). Biosci. Biotechnol. Biochem. 82, 407–416. https://doi.org/10.1080/09168451.2018.1429889.

Murata, K., Kitano, T., Yoshimoto, R., Takata, R., Ube, N., Ueno, K., Ueno, M., Yabuta, Y., Teraishi, M., Holland, C.K., Jander, G., Okumoto, Y., Mori, N., Ishihara, A., 2020. Natural variation in the expression and catalytic activity of a naringenin 7-*O*-methyltransferase influences antifungal defenses in diverse rice cultivars. Plant J. 101, 1103–1117. https://doi.org/10.1111/tpj.14577.

Nicholson, R.L., Kollipara, S.S., Vincent, J.R., Lyons, P.C., Cadena-Gomez, G., 1987. Phytoalexin synthesis by the sorghum mesocotyl in response to infection by pathogenic and nonpathogenic fungi. Proc. Natl. Acad. Sci. U.S.A. 84, 5520–5524. https://doi.org/10.1073/pnas.84.16.5520.

Nishiguchi, S., Murata, K., Ube, N., Ueno, K., Tebayashi, S., Teraishi, M., Okumoto, Y., Mori, N., Ishihara, A., 2018. Accumulation of 9- and 13-KODEs in response to jasmonic acid treatment and pathogenic infection in rice. J. Pestic. Sci. 43, 191–197. https://doi.org/10.1584/jpestics.D18-022.

Nojiri, H., Sugimori, M., Yamane, H., Nishimura, Y., Yamada, A., Shibuya, N., Kodama, O., Murofushi, N., Omori, T., 1996. Involvement of jasmonic acid in elicitor-induced phytoalexin production in suspension-cultured rice cells. Plant Physiol 110, 387–392. https://doi.org/10.1104/pp.110.2.387.

Nonaka, H., Ogawa, N., Maeda, N., Wang, Y.G., Kobayashi, Y., 2010. Stereoselective synthesis of epi-jasmonic acid, tuberonic acid, and 12-oxo-PDA. Org. Biomol. Chem. 8, 5212–5223. https://doi.org/10.1039/C0OB00218F.

Ogawa, S., Miyamoto, K., Nemoto, K., Sawasaki, T., Yamane, H., Nojiri, H., Okada, K., 2017. OsMYC2, an essential factor for JA-inductive sakuranetin production in rice, interacts with MYC2-like proteins that enhance its transactivation ability. Sci. Rep. 7, 40175. https://doi.org/10.1038/srep40175.

Oikawa, A., Ishihara, A., Hasegawa, M., Kodama, O., Iwamura, H., 2001. Induced accumulation of 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside (HDMBOA-Glc) in maize leaves. Phytochemistry 56, 669–675. https://doi.org/10.1016/s0031-9422(00)00494-5.

Park, H.L., Lee, S.W., Jung, K.H., Hahn, T.R., Cho, M.H., 2013. Transcriptomic analysis of UV-treated rice leaves reveals UV-induced phytoalexin biosynthetic pathways and their regulatory networks in rice. Phytochemistry 96, 57–71. https://doi.org/10.1016/j.phytochem.2013.08.012.

Pourkheirandish, M., Hensel, G., Kilian, B., Senthil, N., Chen, G., Sameri, M., Azhaguvel, P., Sakuma, S., Dhanagond, S., Sharma, R., Mascher, M., Himmelbach, A., Gottwald, S., Nair, S.K., Tagiri, A., Yukuhiro, F., Nagamura, Y., Kanamori, H., Matsumoto, T., Willcox, G., Middleton, C.P., Wicker, T., Walther, A., Waugh, R., Fincher, G.B., Stein, N., Kumlehn, J., Sato, K., Komatsuda, T., Komatsuda, T., 2015. Evolution of the grain dispersal system in barley. Cell 162, 527–539. https://doi.org/10.1016/j.cell.2015.07.002.

Rakwal, R., Tamogami, S., Kodama, O., 1996. Role of jasmonic acid as a signaling molecule in copper chloride-elicited rice phytoalexin production. Biosci. Biotechnol. Biochem. 60, 1046–1048. https://doi.org/10.1271/bbb.60.1046.

Reuber, S., Bornman, J.F., Weissenböck, G., 1996. A flavonoid mutant of barley (*Hordeum vulgare* L.) exhibits increased sensitivity to UV-B radiation in the primary leaf. Plant Cell Environ. 19, 593–601. https://doi.org/10.1111/j.1365-3040.1996.tb00393.x.

Schmelz, E.A., Kaplan, F., Huffaker, A., Dafoe, N.J., Vaughan, M.M., Ni, X.Z., Rocca, J.R., Alborn, H.T., Teal, P.E., 2011. Identity, regulation, and activity of inducible diterpenoid phytoalexins in maize. Proc. Natl. Acad. Sci. U.S.A. 108, 5455–5460. https://doi.org/10.1073/pnas.1014714108.

Snyder, B.A., Nicholson, R.L., 1990. Synthesis of phytoalexins in sorghum as a site specific response to fungal ingress. Science 248, 1637–1639. https://doi.org/10.1126/science.248.4963.1637.

Tanaka, T., Iinuma, M., Yuki, K., Fujii, Y., Mizuno, M., 1992. Flavonoids in root bark of *Pongamia pinnata*. Phytochemistry 31, 993–998. https://doi.org/10.1016/0031-9422(92)80055-J.

Thomma, B.P.H.J., Nelissen, I., Eggermont, K., Broekaert, W.F., 1999. Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. Plant J. 19, 163–171. https://doi.org/10.1046/j.1365-313X.1999.00513.x.

Ube, N., Harada, D., Katsuyama, Y., Osaki-Oka, K., Tonooka, T., Ueno, K., Taketa, S., Ishihara, A., 2019a. Identification of phenylamide phytoalexins and characterization of inducible phenylamide metabolism in wheat. Phytochemistry 167, 112098. https://doi.org/10.1016/j.phytochem.2019.112098.

Ube, N., Yabuta, Y., Tohnooka, T., Ueno, K., Taketa, S., Ishihara, A., 2019b. Biosynthesis of phenylamide phytoalexins in pathogen-infected barley. Int. J. Mol. Sci. 20, 5541. https://doi.org/10.3390/ijms20225541.

Vollenweider, S., Weber, H., Stolz, S., Chételat, A., Farmer, E.E., 2000. Fatty acid ketodienes and fatty acid ketotrienes: michael addition acceptors that accumulate in wounded and diseased Arabidopsis leaves. Plant J. 24, 467–476. https://doi.org/10.1111/j.1365-313X.2000.00897.x.

von Bothmer, R., Jacobsen, N., Baden, C., Jorgensen, R.B., Linde-Laursen, I., 1995. An ecogeographical study of the genus Hordeum. In: Systematic and Ecogeographical Studies on Crop Genepools 7, second ed. International Plant Genetic Resources Institute, Rome.

Wakuta, S., Suzuki, E., Saburi, W., Matsuura, H., Nabeta, K., Imai, R., Matsui, H., 2011. OsJAR1 and OsJAR2 are jasmonyl-L-isoleucine synthases involved in wound- and pathogen-induced jasmonic acid signalling. Biochem. Biophys. Res. Commun. 409, 634–639. https://doi.org/10.1016/j.bbrc.2011.05.055.

Wasternack, C., Hause, B., 2013. Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. Ann. Bot. 111, 1021–1058. https://doi.org/10.1093/aob/mct067.

Winkel-Shirley, B., 2001. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol 126, 485–493. https://doi.org/10.1104/pp.126.2.485.

Yamane, H., 2013. Biosynthesis of phytoalexins and regulatory mechanisms of it in rice. Biosci. Biotechnol. Biochem. 77, 1141–1148. https://doi.org/10.1271/bbb.130109.

Zhou, J.M., Fukushi, Y., Wollenweber, E., Ibrahim, R.K., 2008. Characterization of two *dd*-methyltransferase-like genes in barley and maize. Pharm. Biol. 46, 26–34. https://doi.org/10.1080/13880200701729745.