Accumulation of 9- and 13-KODEs in response to jasmonic acid treatment and pathogenic infection in rice

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The inducible metabolites in rice leaves treated with 1 mM jasmonic acid (JA) were analyzed using HPLC. We detected an increase in the levels of two compounds, 1 and 2. Based on the comparison with mass spectra and chromatographic behavior with authentic compounds, 1 and 2 were identified as 13-oxooctadeca-9,11-dienoic acid (13-KODE) and 9-oxooctadeca-10,12-dienoic acid (9-KODE), respectively, which have not been detected in rice to date. The accumulation of these compounds was also induced by an infection by Bipolaris oryzae. Treatment of rice leaves with KODEs induced the accumulation of defensive secondary metabolites, sakuranetin, naringenin, and serotonin, suggesting that KODEs may play a role in the elicitation of defense responses. The compounds that have an α,β-unsaturated carbonyl group similar to KODEs did not reproduce the response of accumulation of defensive secondary metabolites, suggesting that additional structural factors such as long hydrophobic carbon chain are needed to elicit defense responses. © Pesticide Science Society of Japan

Keywords: rice, jasmonic acid, oxylipin, KODE, phytoalexin, Bipolaris oryzae.

Introduction

Oxylipins are oxygenated fatty acids that play both physiological and ecological roles and are widely distributed in aerobic organisms within the animal, fungus, and plant kingdoms. The biosynthesis of oxylipins involves dioxygen-dependent oxidation catalyzed by lipoxygenases (LOXs). In plants, the precursors of oxylipins are polyunsaturated fatty acids, such as linoleic and linolenic acids. They are first converted to hydroperoxides by the action of 9- and 13-LOXs and are then metabolized to various oxylipins.

The 13-LOX pathway leads to the formation of jasmonic acid (JA), which is the most extensively investigated oxylipin in plants. JA is regarded as a plant hormone because it affects various aspects of plant development and is distributed universally within higher plants.1–3) The actions of JA on plant development include inhibition of root growth,4) stimulation of fruit maturation,5) and acceleration of leaf senescence.6) JA also plays pivotal roles in the response to biological stresses.7,8) In rice (Oryza sativa), JA has been considered a central player in signal transduction from the recognition of pathogen infection to the biosynthesis of defensive metabolites, such as terpenoid and flavonoid phytoalexins,9,10) serotonin, and phenylamides.11,12) The active form of JA is the amide with L-isoleucine,13,14) which is formed by jasmonate resistance 1 (JAR1).15) 12-Oxo-phytodienoic acid (12-OPDA), an intermediate compound in jasmonic acid biosynthesis, also serves as a signal mediator in an overlapping but distinct way from JA.16,17)

The 9-LOX pathway has also been indicated to be involved in the defense responses of plants, particularly in local defense reactions such as hypersensitive responses. Enhanced expression of a 9-LOX gene was shown to be essential for tobacco (Nicotiana tabacum) plants in establishing incompatibility with Phytophthora parasitica.18) In addition, it has been demonstrated that in the hypersensitive response induced by the avirulent pathogen Pseudomonas syringae pv. syringae, both 9-LOX and reactive oxygen species operated concurrently.19) In pepper (Capsicum annuum), expression of CaLOX1, which encodes a 9-LOX protein, was induced in leaves during Xanthomonas campestris pv. vesicatoria infection.20) 9-LOXs have been suggested to positively regulate defense and cell death responses to microbial pathogens.

The oxylipins involved in the plant responses to biotic stresses have been increasingly found. Treatment of Arabidopsis leaves...
with 9-hydroxyoctadecatrienoic acid (9-HOT) provoked the accumulation of callose, as well as the production of ROS, indicating a role of this oxylipin in defense responses.\(^{21}\) Colnelenic and colnelenic acids are unstable divinyl fatty acids that are considered to be synthesized from linoleic and linolenic acids, respectively, by oxidative reactions by a 9-LOX. These compounds were shown to be pathogen inducible and inhibitory to *Phytophthora infestans* in potato (*Solanum tuberosum*) leaves and thus were suggested to function as phytoalexins.\(^{22}\) In maize (*Zea mays*) leaves, southern leaf blight (*Cochliobolus heterostrophus*) infection resulted in the localized accumulation of 10-oxo-11-phytodienoic acid (10-OPDA), 10-oxo-11-phytoenoic acid (10-OPEA), and a series of related 14- and 12-carbon metabolites.\(^{23}\) Because these compounds are toxic to both pathogens and plants, they play roles as both phytoalexins and signal molecules that induce cell death in plant tissues. Both 12-OPDA and 10-OPEA promote the transcription of defense genes.

Considering that these new bioactive oxylipins have been discovered in various plants, it is reasonable to hypothesize that there may still be uncharacterized oxylipins that are involved in plant defense responses. In the course of our analysis of inducible, hydrophobic metabolites in rice leaves treated with JA, we detected the accumulation of two oxylipins, 9-oxooctadeca-10,12-dienoic acid (9-KODE) and 13-oxooctadeca-9,11-dienoic acid (13-KODE). In view of the possibility that they may serve as signal molecules, we analyzed their accumulation in pathogen-infected leaves, as well as their ability to induce defense responses such as phytoalexin accumulation in rice. In addition, because the \(\alpha\), \(\beta\)-unsaturated carbonyl group is the characteristic chemical structure of 9- and 13-KODEs, we examined the biological activity of other compounds that have an \(\alpha\), \(\beta\)-unsaturated carbonyl group.

**Materials and Methods**

1. **General**
   LC-PDA-MS measurements were performed using a Quatro Micro API mass spectrometer (Waters, Milford, MA, USA) connected to an Acquity UPLC system (Waters). HPLC analysis was performed using a 10 Awp HPLC system (Shimadzu, Kyoto, Japan).

2. **Plant materials and pathogen**
   Rice (*O. sativa* cv. Nipponbare) seeds were sown on a 1:1 mixture of vermiculite (Shoei Sangyo, Okayama, Japan) and artificial compost for rice (Grin Grow, Okayama, Japan) and incubated at 28°C with a 16 hr/8 hr light/dark (LD) cycle for two weeks. *Bipolaris oryzae* (MAFF 305067) was provided by the NIAS Genebank (http://www.gene.affrc.go.jp/index_en.php). *B. oryzae* inoculation was performed as previously described by Ishihara et al. (2008).\(^{15}\)

3. **Chemicals**
   JA was prepared by alkaline hydrolysis of methyl jasmonate (Tokyo Chemical Industry, Tokyo, Japan). KODEs were pur chased from Funakoshi, Tokyo. Saturated and unsaturated alkyl aldehydes, linoleic and linolenic acids, \(\beta\)-ionone, and 2,4-hexadienal were obtained from Wako Pure Chemical Industries (Osaka, Japan), and 3-hepten-2-one was obtained from Tokyo Chemical Industry.

4. **Characterization of 1 and 2**
   For the characterization of 1 and 2, rice leaves (110 g) from 14-day-old seedlings were floated in a 1 mM solution of JA in water containing 0.25% Tween20. After a 72 hr incubation, the leaves were extracted with 10 volumes of methanol for 24 hr. The extract was concentrated and subjected to silica gel (Daisogel IR-60-63-210, 70 g, Osaka Soda, Osaka, Japan) column chromatography. The compounds were eluted with mixtures of acetone and hexane (0:10, 1:9, 2:8, v/v, 500 mL each). The 2:8 fraction was subjected to HPLC and LC-PDA-MS analysis. The leaf extract treated with 0.25% Tween20 solution without JA was fractionated in the same way and subjected to HPLC analysis as a control.

5. **Treatment of rice leaves with JA, KODEs, and compounds with an \(\alpha\), \(\beta\)-unsaturated carbonyl group**
   The third leaves of 14-day-old rice seedlings were cut into segments 1-cm long and floated in a 1 mM JA solution (containing 0.25% Tween 20).

9- and 13-KODEs were dissolved in 0.25% Tween20 (Wako Pure Chemical Industries), and 5 \(\mu\)L droplets of the solution were placed on the wounded sites made by the tip of a micropipet on the third leaves of 14-day-old seedlings. The leaves were treated in the same way, with the compounds with the \(\alpha\), \(\beta\)-unsaturated carbonyl group at 1 mM. A solution of 0.25% Tween20 was used as a control. The seedlings were placed in an airtight bag for 24 hr to maintain high humidity. After removing the bag, the seedlings were further incubated for 48 hr at 28°C with a 16 hr/8 hr LD cycle.

6. **Analysis of metabolites**
   To determine the concentrations of KODEs in JA-treated and *B. oryzae*-inoculated leaves, the leaves were extracted by immersing leaf segments in methanol for 24 hr. The extract was subjected to HPLC analysis. The HPLC conditions were as follows: column: Mightysil RP18-GP 150-4.6 (3 \(\mu\)m) (Kanto Kagaku, Tokyo, Japan); column temperature: 40°C; solvents: 0.1% trifluoroacetic acid in water (A) and acetonitrile (B); gradient: 50–100% within 40 min; flow rate: 0.6 mL/min; detection 280 nm.

For the analysis of sakuranetin, naringenin, and serotonin, leaves were excised from seedlings, cut into small pieces, and immersed in 80% methanol for 24 hr. The extracts were subjected to HPLC analysis for serotonin and to LC-MS/MS analysis for sakuranetin and naringenin. The HPLC conditions for the analysis of serotonin were the same as those for KODEs, except that a gradient of 5–70% B/(A+B) within 90 min was applied. The LC conditions for sakuranetin and naringenin were as follows: column: ACQUITY UPLC BEH C18 2.1×50 \(\mu\)m (1.7 mm) (Waters); column temperature: 40°C; solvents: 0.1% formic acid...
in water (A) and 0.1% formic acid in acetonitrile (B); gradient: 25–100% B/(A+B) within 15 min; flow rate: 0.2 mL/min. Mass transitions for sakuranetin and naringenin were from m/z 287.1 to 167.0 and from m/z 273.0 to 152.9, respectively. The cone voltage and collision energy for both compounds were optimized to 34.0 V and 28.00 eV, respectively, using authentic compounds.

**Results**

1. **Induced accumulation of KODEs in JA-treated rice leaves**

To discover new oxylipins in rice, we treated rice leaves with JA, as it is known to be a strong inducer of defense responses in plants.8–11) The leaf segments were floated in a 1 mM JA solution and then extracted with methanol after a 72 hr incubation. The extract was concentrated 10 times and subjected to HPLC analysis. Increases in the peak areas corresponding to compounds 1 and 2, in addition to known phytoalexins, were detected in the extract from JA-treated leaves (Fig. 1).

We prepared a methanol extract of the rice leaves treated with JA in a large scale and fractionated the extract using silica gel column chromatography. Compounds 1 and 2 were eluted in a 20% acetone fraction, and then the fraction was analyzed via LC-PDA-MS. The negative ESI MS indicated that their molecular weights were both 294, while the UV-Vis spectra showed that their \( \lambda_{max} \)s were both 278.7 nm. We performed a database search, assuming that 1 and 2 were oxylipins, and speculated that 1 and 2 were KODEs based on the spectra. We then compared the retention times on HPLC analysis, UV spectra, negative ESI MS, and product ion spectra of 1 and 2 with authentic compounds (Figs. 2 and 3). The chromatographic behavior and spectra of 1 and 2 were identical to those of authentic 13-oxooctadeca-9,11-dienoic acid (13-KODE) and 9-oxooctadeca-10,12-dienoic acid (9-KODE), respectively. On the basis of these findings, we concluded that 1 and 2 are 13-KODE and 9-KODE, respectively. The chemical structures of KODEs are depicted in Fig. 4.

The kinetics of the accumulation of KODEs in rice leaves treated with 1 mM JA were then investigated (Fig. 5). The concentration of 9-KODE reached its maximum 72 hr after treatment, while that of 13-KODE reached its maximum 24 hr after treatment. The maximum concentrations of 9- and 13-KODEs were about 10 and 9.3 times larger, respectively, than those measured in leaves at 0 hr after treatment. The concentration of 9-KODE had increased 4.2-fold in the control leaves 72 hr after treatment, while that of 13-KODE had increased 4.9-fold 48 hr after treatment.

**Fig. 1.** Chromatograms of extracts from the leaves treated with 1 mM jasmonic acid and intact leaves. The leaves were extracted by 10 volumes of methanol. The 5 mL extracts were concentrated to 1 mL and subjected to HPLC analysis. Compounds 1 and 2, which were eluted immediately after *ent*-10-oxodepressin \(^{46}\), were further purified.

**Fig. 2.** Comparison of chromatograms in the HPLC analysis (A, E), UV-Vis spectra (B, F), negative ESI MS (C, G), and product ion spectra (D, H) between 1 (A–D) and 13-KODE (E–H).
2. Accumulation of 9- and 13-KODEs in leaves infected with B. oryzae
To test whether the accumulation of KODEs is induced by an infection with pathogens, we analyzed rice leaves infected by B. oryzae. As shown in Fig. 6, an infection with B. oryzae induced KODE accumulation. The concentrations of 9- and 13-KODEs in the inoculated leaves were 4.1 and 8.4 times higher than those measured in mock-inoculated leaves.

3. Induction of defensive secondary metabolite accumulation by treatment with KODEs
To examine the effects of treatment with 9- and 13-KODEs on the accumulation of defensive secondary metabolites of rice, we placed droplets of 1 mM KODE solutions covering the wounded sites made by a tip of a micropipet on the third leaves of 14-day-old rice seedlings. The leaves treated with KODEs showed an accumulation of brown materials at the wounded sites after a 72 hr incubation while the accumulation was absent on the control leaves treated with distilled water. The leaves were then extracted using 80% methanol, and the accumulation of sakuranetin, naringenin, and serotonin was analyzed (Fig. 7). Sakuranetin is the flavonoid phytoalexin of rice, while naringenin is its immediate precursor. Serotonin is a secondary metabolite inducible in response to a pathogen attack in rice and has been indicated to be incorporated into cell walls as a brown material to form a physical barrier against pathogens.

Treatment with 9- and 13-KODEs elicited the accumulation of these compounds, and their concentrations increased in a dose-dependent manner. The respective concentrations of

![Fig. 3. Comparison of chromatograms in the HPLC analysis (A, E), UV-Vis spectra (B, F), negative ESI MS (C, G), and product ion spectra (D, H) between 2 (A-D) and 9-KODE (E-H).](https://example.com/fig3)

![Fig. 4. Chemical structures of 9-oxooctadeca-10,12-dienoic acid and 13-oxooctadeca-9,11-dienoic acid.](https://example.com/fig4)

![Fig. 5. Accumulation of 9-oxooctadeca-10,12-dienoic acid (9-KODE, A) and 13-oxooctadeca-9,11-dienoic acid (13-KODE, B) in leaves treated with 1 mM jasmonic acid (JA). The third leaves of 14-day-old seedlings were floated in 1 mM JA solution in 0.25% Tween20 (closed bars) and in distilled water containing 0.25% Tween20 as a control (open bars). Leaves before treatment were also extracted (gray bars). Data are presented as the means of three replicates. Error bars indicate standard deviations. Asterisks indicate statistically significant differences from the control (*p<0.05, **p<0.01, Student’s t-test).](https://example.com/fig5)
sakuranetin, naringenin, and serotonin in leaves treated with 9-KODE at 1 mM were 49.1, 21.2, and 23.0 times higher than those measured in control leaves. Similarly, treatment with 13-KODE induced 19.9-, 6.4- and 10.5-fold increases in concentrations of sakuranetin, naringenin, and serotonin, respectively, in comparison with those in the control leaves. The effect of 9-KODE on the concentrations of these compounds was stronger than that of 13-KODE.

Upon induction of these compounds, we analyzed the activity of other compounds that have an α, β-carbonyl group in common with KODEs. We treated rice leaves with 1 mM solutions of various compounds, including aldehydes with three to ten carbon atoms possessing double bonds at the α position of the aldehyde group (abbreviated as C3:1 to C10:1 in Fig. 8), and propanal, hexanal, and decanal (C3, C6, and C10 in Fig. 8, respectively). We also included β-ionone, 2,4-hexadienal, and 3-hepten-2-one in the assay because they also have α, β-unsaturated carbonyl groups. While KODEs effectively induced sakuranetin accumulation, the other compounds were almost inactive. This tendency was also observed for the induction of naringenin, although C10, β-ionone, 2,4-hexadienal, and 3-hepten-2-one induced naringenin accumulation. In the case of serotonin accumulation, KODEs showed strong activity, but C3:1, 2,4-hexadienal, and 3-hepten-2-one induced serotonin at similar levels to those induced by 13-KODE.

**Discussion**

In the present study, we found that the treatment of rice leaves with JA induced the accumulation of 9- and 13-KODEs. Their accumulation was also induced by *B. oryzae* infection. Furthermore, the exogenous application of KODEs to rice leaves provoked the accumulation of defensive secondary metabolites, including sakuranetin, naringenin, and serotonin. The induced accumulation of sakuranetin has been demonstrated in leaves treated with JA and in leaves infected by pathogens such as *B. oryzae* and *Magnaporthe grisea*. The accumulation of serotonin has also been reported to be induced by methyl jasmonate treatment and infection by *B. oryzae*. Collectively, these findings suggest that the induced accumulation of KODEs might be involved in the signal transduction that leads to the accumulation of defensive secondary metabolites. As biotic and abiotic stresses generally induce diterpenoid and flavonoid phytoalexins simultaneously, KODEs likely activate both biosynthetic pathways, although we need to examine the effects of KODE treatment on the accumulation of diterpenoid phytoalexins.

It is of interest to note that LOX-generated 9- and 13-hydroperoxides have been shown to affect plant cell viability and may function in localized cell death during the hypersensitive reaction in tobacco leaves, because these hydroperoxides are possible precursors for KODEs. 13-Hydroperoxide and 13-hydroxide of both linoleic and linolenic acids have also been shown to accumulate after inoculation with *Pyricularia oryzae*. These compounds were effective to induce the production of momilactone A. It is likely that these oxygenated fatty acids as well as KODEs that we found in the present study, serve as the damage-associated molecular pattern that activates the defense responses in rice leaves.

KODEs have been detected in animals and are estimated to comprise approximately 20% of the oxylipins esterified to rabbit reticulocyte plasma membranes. The presence of KODE in plant tissues was reported in *Arabidopsis* and the soybean (*Glycine max*). KODEs can be produced directly and efficiently by purified lipoxygenases, but the enzymatic dehydroge-

![Fig. 6. Accumulation of 9-octadeca-10,12-dienoic acid (9-KODE, A) and 13-octadeca-9,11-dienoic acid (13-KODE, B) in leaves inoculated with *Bipolaris oryzae*. Droplets of a suspension of *B. oryzae* conidia (10^6 conidia/mL) in 0.25% Tween20 were placed on leaves (inoculation, closed bars). As a control, a solution without conidia was used (mock, open bars). Leaves without any treatment were also extracted (intact, gray bars). After a 72-h incubation, leaves were extracted with methanol. Data are presented as the means of three replicates. Error bars indicate standard deviations. Asterisks indicate statistically significant differences (*p*<0.05, **p*<0.01, Tukey–Kramer test).](image1)

![Fig. 7. Accumulation of sakuranetin (A), naringenin (B), and serotonin (C) in leaves treated with 9-octadeca-10,12-dienoic acid (9-KODE) and 13-octadeca-9,11-dienoic acid (13-KODE). Droplets of KODE solutions at 0.1, 0.3, and 1.0 mM in 0.25% Tween20 were placed on the third leaves of 14-day-old seedlings. Droplets of a solution without KODE were placed on leaves as a control. After a 72 hr incubation, leaves were extracted with 80% methanol. Data are presented as the means of three replicates. Error bars indicate standard deviations. Asterisks indicate statistically significant differences from the control (*p*<0.05, **p*<0.01, Dunnett’s test).](image2)
leaves resulted in cell death and expression of the glutathione
bonyl compounds, such as acrolein and methyl vinyl ketone, as
duction of LOX expression is a response frequently observed in
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hernia), changes in metabolites in seeds germinating in the pres-
itions. The exogenous application of KODEs to
sis
leaves led to the accumulation of KODEs at high concentra-
Knowledge of KODEs in plants is limited. It has been shown
fection of Pseudomonas syringae pv. tomato in Arabidop-
sis
les leads to the accumulation of KODEs at high concentra-
The exogenous application of KODEs to Arabidopsis
les resulted in cell death and expression of the glutathione
S-transferase gene (GST1). GST1 is known to be strongly in-
duced in pathogenesis as well as by oxidative stress. Therefore, the accumulation of KODE was considered to be intimately associated with the expression of stress responses. In the soybean, changes in metabolites in seeds germinating in the presence of Aspergillus niger, A. oryzae, Rhizopus oligosporus, and white rice yeast (A. niger wry) were investigated. Microbially stressed germination of soybeans resulted in the generation of 9- and 13-KODEs and their respective glyceryl esters in addition to glyceollins, a known phytoalexin of the soybean. The induction of LOX expression is a response frequently observed in plants attacked by pathogenic microorganisms and KODEs may be the direct products of the LOX reaction from lino-
lenic acid. The accumulation of KODEs may be a general response of plants to a pathogen attack, although it appears to have escaped detailed attention to date.

The ability of KODEs to serve as acceptors in the Michael addition has been suggested to be related to the induction of gene expression. The application of α, β- unsaturated carbonyl compounds, such as acrolein and methyl vinyl ketone, as well as KODEs, to Arabidopsis leaves induced the expression of GST1. The induction of stress responses by treatments with α, β-unsaturated carbonyl compounds has been reported in many other plant species.

Fig. 8. Accumulation of sakuranetin (A), naringenin (B), and serotonin (C) in leaves treated with various compounds at 1 mM. Droplets of solutions of various compounds in 0.25% Tween20 solution were placed on the third leaves of 14-day-old seedlings. Droplets of 0.25% Tween20 solution were placed on leaves as a control. Abbreviations C3:1 to C10:1 indicate aldehydes with 3 to 10 carbons with a double bond at the α position, while C3, C6, and C10 indicate propanal, hexanal, and decanal, respectively. After a 72 hr incubation, leaves were extracted with 80% methanol. Data are presented as the means of three replicates. Error bars indicate statistically significant differences from the control (*p<0.05, **p<0.01, Dunnett’s test).

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