# Phytoalexin production by amino acid conjugates of jasmonic acid through induction of naringenin-7-*O*-methyltransferase, a key enzyme on phytoalexin biosynthesis in rice (*Oryza sativa* L.)

Shigeru Tamogami, Randeep Rakwal, Osamu Kodama*

*Laboratory of Phytochemical Ecology, School of Agriculture, Ibaraki University, 3-21-1 Chuo Ami, Ibaraki 300-03, Japan*

Received 28 October 1996; revised version received 6 December 1996

**Abstract Amino acid conjugates of jasmonic acid are found to elicit production of the flavonoid phytoalexin, sakuranetin in rice leaves. The elicitation is shown to arise from induction of naringenin 7-*O*-methyltransferase, a key enzyme of sakuranetin biosynthesis. The (−)-phenylalanine conjugate, one of the active compounds, is characterized by high activity for both sakuranetin and enzyme induction and low phytotoxicity against rice growth. Both (+)-enantiomers of the conjugates and free amino acids do not show any activity. The amino acid conjugate of jasmonic acid is speculated to be the later component in the signaling transduction chain in stressed rice plants.**

*Key words:* Phytoalexin; Sakuranetin; Naringenin; Rice plant; Jasmonic acid; Jasmonoyl phenylalanine

## 1. Introduction

Jasmonic acid (JA, **1**) has been shown to be a signaling compound which elicits the production of second metabolites including defensive metabolites in plants [1,2]. One of the most important defensive metabolites synthesized in plants against pathogen attack is the accumulation of antimicrobial compounds called phytoalexins. We have been studying rice plant phytoalexins and have characterized some antimicrobial compounds including oryzalexins [3] and sakuranetin (**10**) [4]. Among these rice plant phytoalexins, the flavonoid compound sakuranetin (**10**) is considered to be the most important because of its high antimicrobial activity and large accumulation in and around infection sites [4]. Our recent results show that this phytoalexin production is mediated by an endogenous production of JA (**1**), and that the phytoalexin production is strongly elicited by exogenously applied JA (**1**) in rice leaves [5].

Up to now, many JA related compounds have been isolated from various plants, for example dihydro-JA (DHJA, **2**), 7-iso-cucurbic acid (7-iso-CA, **3**), tuberonic acid, 12-oxo-phytodienoic acid (12-oxo-PDA), JA amino acid conjugates and JA glucosyl ether [6]. Recent results [7,8] show that 12-oxo-PDA, an important biosynthetic precursor of JA, is also active in second metabolite production in cell suspension cultures, and that JA amino acid conjugates are implied to work as intermediates in the octadecanoid signaling pathway leading to production of volatile [9].

These results indicate that the elicitation process is not only controlled by JA (**1**) and emphasize the importance of the activity by JA related compounds.

Although the role of JA (**1**) in secondary metabolite production including phytoalexin accumulation has been established, there is relatively less information on events after the rapid release of JA (**1**) in the stressed plant. To investigate the true component that concerns most closely the rice phytoalexin production among JA related compounds including its metabolites, is one of the most important subjects for a deeper understanding of rice plant defense mechanisms.

Here, we describe the activities of JA related compounds including JA amino acid conjugates (Figs. 1 and 2) on phytoalexin production in rice leaves, and also describe the inducing activity of these compounds for the naringenin 7-*O*-methyltransferase (NOMT), a key enzyme of phytoalexin biosynthesis in rice plants [10].

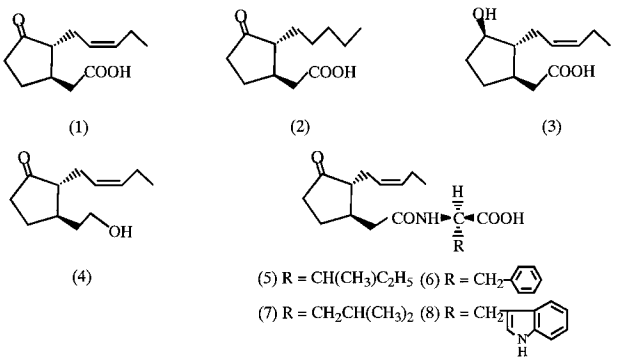
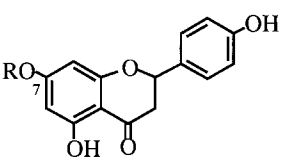



Fig. 1. Structures of JA (**1**) and its related compounds, DHJA (**2**), 7-iso-CA (**3**), alcohol derivative of JA (**4**), (−)-isoleucine conjugate (**5**), (−)-phenylalanine conjugate (**6**), (−)-leucine conjugate (**7**) and (−)-tryptophan conjugate (**8**).

## 2. Materials and methods

### 2.1. Chemicals

JA (**1**) was prepared by de-esterification of methyl jasmonate (Harmann and Reimer, Holzminden, Germany) and successive enantiomeric separation was done as described [11]. DHJA (**2**) was synthesized from 2-pentyl-2-cyclopenten-1-one according to a previously described method [12]. 7-Iso-CA (**3**) was prepared from JA as described [13]. The alcohol derivative (**4**) [14] was prepared from methyl jasmonate in three steps. Amino acid conjugates of JA, *N*-[(−)-jasmo-




R = H ; naringenin (9)
R = $CH_3$; sakuranetin (10)

Fig. 2. Structures of naringenin (**9**) and sakuranetin (**10**).

*Corresponding author. Fax: (81) (298) 88-0081.

0014-5793/97/$17.00 © 1997 Federation of European Biochemical Societies. All rights reserved.
*PII* S0014-5793(96)01482-2

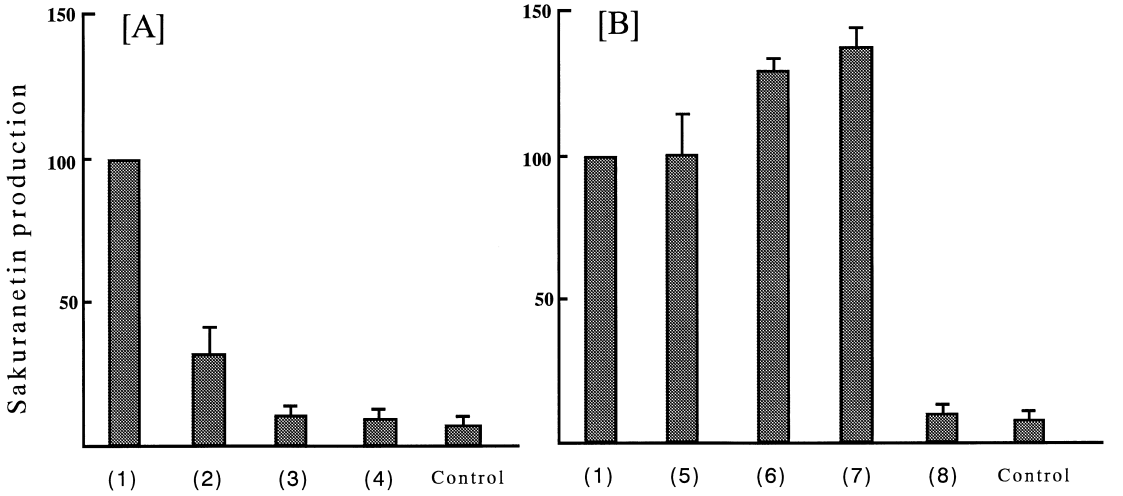



Fig. 3. Sakuranetin (**10**) production in rice leaves by JA (**1**) and its related compounds at 72 h after treatment. Sakuranetin production is calculated as relative activity to JA (**1**) (JA = 100). (A) Activities of JA (**1**) and its analogues DHJA (**2**), 7-iso-CA (**3**), alcohol derivative of JA (**4**). Compounds tested are racemic mixtures. (B) Activities of (−)-JA (**1**) and amino acid conjugates of JA [(−)-isoleucine conjugate (**5**), (−)-phenylalanine conjugate (**6**), (−)-leucine conjugate (**7**) and (−)-tryptophan conjugate (**8**)]. Compounds in A and B were tested at 500 μM.

noyl]-*S*-isoleucine (**5**), *N*-[(−)-jasmonoyl]-*S*-phenylalanine (**6**), *N*-[(−)-jasmonoyl]-*S*-leucine (**7**), *N*-[(−)-jasmonoyl]-*S*-tryptophan (**8**) and corresponding (+)-enantiomers were synthesized according to a previously described method [15]. Sakuranetin (**10**) was obtained by the selective methylation of naringenin (**9**, Aldrich) as described [16].

*2.2. Plant material*

Rice plants (*Oryza sativa* L. Nipponbare) were cultivated and used as described previously [5].

*2.3. Elicitation for sakuranetin and induction of NOMT*

Droplets (20 μl) of a test solution were applied to press injured spots on rice leaves. After an appropriate incubation period, sakuranetin was extracted as described [5]. After purifying by Sep-Pak Light Silica Cartridge (Waters), sakuranetin was analyzed by HPLC (column, LichroCART, RP-C18, Merck; solvent, methanol: water = 6:4; detection, UV at 285 nm). The retention time of sakuranetin (**10**) was 8.0 min as elutant at a flow rate of 1.0 ml/min. The NOMT assay was performed as described previously [10].

*2.4. Bioassay for phytotoxic activity against shoot elongation of rice*

Samples in acetone were put on filter paper (5.5 cm diameter) in a petri dish. After the acetone had evaporated, 3 ml of water was added. Four germinated seeds of rice (*Oryza sativa* L. Nipponbare) were placed on the filter paper and kept at 25°C under fluorescent light. After 7 days, the shoot lengths of the rice plant were measured and the inhibition percentages were calculated against control.

## 3. Results

*3.1. Induction of sakuranetin and NOMT with JA related compounds.*

The amounts of sakuranetin (**10**) in rice leaves treated with JA (**1**) and JA related compounds, are summarized in Fig. 3A. Other than JA, only DHJA (**2**) elicits sakuranetin production, i.e. the carboxylic acid group and carbonyl group turn out to be crucial for the activity and the olefinic double bond in the side chain is needed for high activity in JA (**1**). These results lead to the conclusion that structural variation is strictly limited in JA (*1*). In contrast to these low activities of JA related compounds (**2–4**), the amino acid conjugates (**5–7**) show very high activities (Fig. 3B). It is notable that the phenylalanine

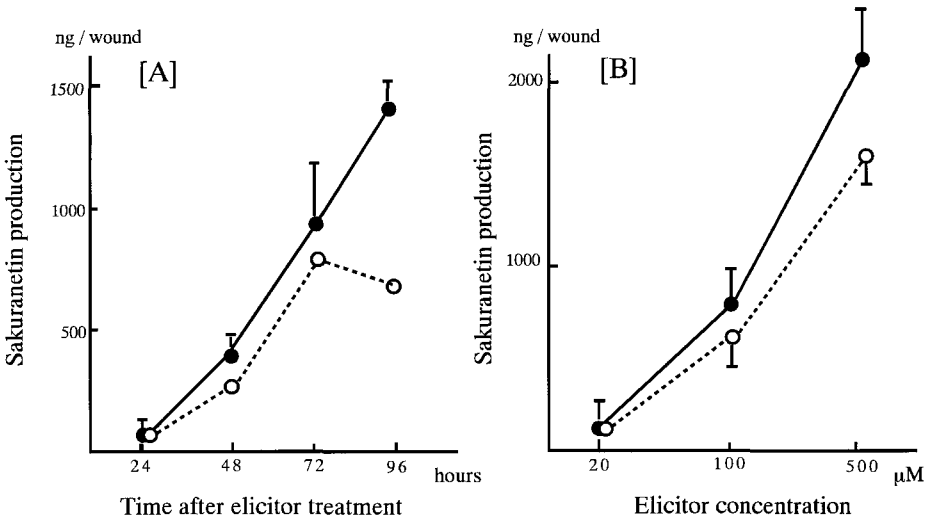



Fig. 4. Time (A) and dose (B) dependent production of sakuranetin (**10**) in rice leaves by (−)-JA (**1**) (○) and (−)-phenylalanine conjugate (**6**) (●). In A, compounds were tested at 500 μM.

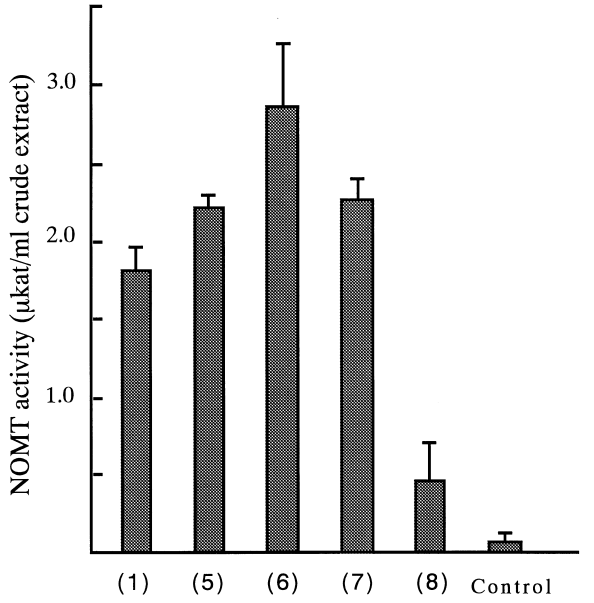



Fig. 5. Induction of NOMT in rice leaves by (−)-JA (**1**) and amino acid conjugates of JA [(−)-isoleucine conjugate (**5**), (−)-phenylalanine conjugate (**6**), (−)-leucine conjugate (**7**) and (−)-tryptophan conjugate (**8**)]. Compounds were tested at 500 μM.

conjugate (**6**) and leucine conjugate (**7**) show significantly higher activities (about 35% increase) in sakuranetin (**10**) production than JA (**1**). The production of sakuranetin (**10**) by the phenylalanine conjugate (**6**) is time and dose dependent, and its increasing curve of phenylalanine conjugate (**6**) is almost identical with that of JA (**1**) as previously observed [5] (Fig. 4), and the phenylalanine conjugate (**6**) shows higher activity than JA (**1**). The threshold concentration was ca. 20 μM for the production of sakuranetin (**10**) (44 ng/wound, 3 times as much as control) and the accumulation can be identified about 24 h after elicitation. The (+)-enantiomers of the conjugates of JA and free amino acids (Phe, Ile, Leu and Trp) do not show activities (data not shown).

We have already shown that NOMT is a key enzyme in the biosynthesis of sakuranetin (**10**), because this enzyme catalyzes the conversion of the non-antifungal naringenin (**9**) into the highly antifungal sakuranetin (**10**) [4,10]. As shown in Fig. 5, the isoleucine (**5**), phenylalanine (**6**) and leucine (**7**) conjugates result in an increase of NOMT induction as by JA (**1**), while the tryptophan conjugate (**8**) results in a considerably lower induction. It is emphasized that these conjugates, especially the phenylalanine conjugate (**6**), induce NOMT more strongly than JA. This observation on NOMT induction is in agreement with that of sakuranetin (**10**) production. This accordance clearly suggests that these amino acid conjugates exhibit the same abilities as signaling compound in the elicitation process as shown by JA (**1**).

### *3.2. Phytotoxicities of amino acid conjugates of JA*

It is well known that exogenously applied JA (**1**) causes not only phytoalexin production but also a variety of physiological reactions, e.g. growth inhibition [17]. Among the JA amino acid conjugates, the isoleucine (**5**) and phenylalanine conjugates (**6**) turned out to be less toxic as compared to JA (**1**), but the leucine conjugate (**7**) is as toxic as JA (**1**) against rice shoot growth (Fig. 6). Considering the results on sakuranetin production, conjugates **5** and **6** prove to be selective only for the elicitation activity.

## 4. Discussion

Although the release and increase of JA (**1**) occurs early and rapidly in the stressed plant, its observed defense reactions were relatively late, suggesting that further metabolism for the conversion of JA (**1**) was needed to furnish the signal transduction pathway [1,7,9,18,19]. A similar time lag between the rapid increase of JA (**1**) and the following slow reaction was observed in phytoalexin production in rice leaves, i.e. a rapid increase of JA (**1**) within 6 h after the addition of an elicitor, followed by accumulation of sakuranetin (**10**) after 24 h [5,20]. The observation that JA (**1**) returns to normal levels after a rapid increase indicates that JA (**1**) is either rapidly metabolized to an inactivated form or converted to the other functional compounds in the signal transduction chain. Up to now, some metabolites of JA have been found in plants, e.g. amino acid conjugates and glucosyl ether, but their physiological importance has been explored less [6]. Thus we synthesized four varieties of JA amino acid conjugates which are found in plants [6], and investigated their abilities on the elicitation mechanism in rice plants.

Our present results reveal the importance of amino acid conjugates of JA on phytoalexin production in rice plants, and demonstrated that these conjugates induce NOMT, a key enzyme in phytoalexin biosynthesis. Besides the induction of NOMT, more than 10 proteins are induced by JA (**1**) which are also induced by the phenylalanine conjugate (**6**) in rice leaves, which indicates the potential of the amino acid conjugates in regulating gene expression (Rakwal et al., in preparation). These results are consistent with the recent speculation that JA amino acid conjugates may work as important intermediates in the signaling pathway on the production of volatiles [9]. In contrast to these active conjugates, the tryptophan conjugate (**8**) shows less activity for sakuranetin produc-

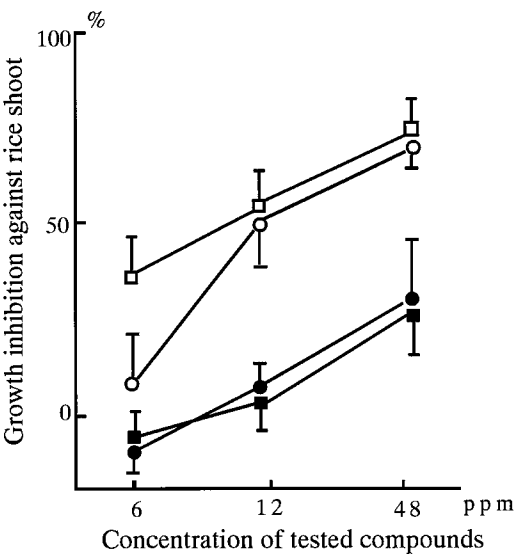



Fig. 6. Phytotoxic activities of (−)-JA (**1**) (○) and amino acid conjugates of JA [(−)-isoleucine conjugate (**5**) (■), (−)-phenylalanine conjugate (**6**) (●), (−)-leucine conjugate (**7**) (□)] against rice shoot growth.

tion and NOMT induction, suggesting that conjugate **8** may be an inactivated or stored form of JA (**1**) in rice plants.

From the results on the inhibitory activities of amino acid conjugates on rice plant growth, phytotoxicity turns out to be dependent on the variety of amino acid. The phytotoxicity of the isoleucine (**5**) and phenylalanine conjugates (**6**) is very small and this result contrasts with the high elicitation activity in sakuranetin production of these conjugates. As JA (**1**) is a small and highly flexible molecule, it will interact with more than one receptor and produce a variety of biological responses, i.e. phytotoxicity, when it is applied exogenously to the plant. In contrast, the isoleucine (**5**) and phenylalanine conjugates (**6**) may fix their shape into a more rigid and suitable conformation to fit only the target receptor site that leads to the sakuranetin production more readily.

Although the phenylalanine conjugate (**6**) is even more polar than JA (**1**) [15] and seems less absorbable into the plant cell by crossing the fatty cell membranes than JA (**1**), it shows significantly higher activity for NOMT induction than JA (**1**) and leads to sakuranetin production as early as JA (**1**) after the treatment. These results strongly suggest that amino acid conjugates of JA have to be taken into consideration as the next functional compounds in the signal transduction chain in rice plants, although JA (**1**) is an exact signaling compound in the early transduction chain in plants.

Our recent results and previous results [8,9] which suggest the importance of the precursors and metabolites of JA enable us to propose an inclusive understanding that these JA related compounds may be working as a cluster of functional compounds in the signal transduction chain in plants. Further synthesis and physiological investigations on the amino acid conjugates of JA will give not only compounds with higher elicitation activity, but also information about their integral role in the signal transduction chain in plants.

In conclusion, our results demonstrate that (i) amino acid conjugates of JA (**1**) are able to elicit phytoalexin accumulation, (ii) these conjugates are also able to induce NOMT, a key enzyme in phytoalexin production, (iii) especially the phenylalanine conjugate (**6**) shows considerably higher activity than JA on NOMT induction, (iv) the variety of amino acid is highly specific to these activities, and suggest that these conjugates may play an important role in the signaling transduction system in stressed rice plants.

*Acknowledgements:* This work has been supported by CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Corporation.

## References

[1] Gundlach, H., Müller, M.J., Kuchan, T.M. and Zenk, M.H. (1992) Proc. Natl. Acad. Sci. USA 89, 2389–2393.
[2] Farmer, E.E., Johonson, R.R. and Ryan, C.A. (1992) Plant Physiol. 98, 995–1002.
[3] Tamogami, S., Mitani, M., Kodama, O. and Akatsuka, T. (1993) Tetrahedron 49, 2025–2032, and references therein.
[4] Kodama, O., Miyakawa, J., Akatsuka, T. and Kiyosawa, S. (1992) Phytochemistry 31, 3807–3809.
[5] Rakwal, R., Tamogami, S. and Kodama, O. (1996) Biosci. Biotech. Biochem. 60, 1046–1048.
[6] Hamberg, M. and Gardner, W. (1992) Biochim. Biophys. Acta 1165, 1–18, and references therein.
[7] Blechert, S., Brodschelm, W., Hölder, S., Kammerer, L., Kutchan, T.M., Muller, M.J., Xia, Z.-Q. and Zenk, M.H. (1995) Proc. Natl. Acad. Sci. USA 92, 4099–4105.
[8] Dittrich, H., Kutchan, T.M. and Zenk, M.H. (1992) FEBS Lett. 309, 33–36.
[9] Krumm, T., Bandemer, K. and Boland, W. (1995) FEBS Lett. 377, 523–529.
[10] Rakwal, R., Hasegawa, M. and Kodama, O. (1996) Bichem. Biophys. Res. Commun. 222, 732–735.
[11] Kamell, R., Schneider, G., Schmidt, J., Sembdner, G. and Schreiber, K. (1990) Z. Naturforsch. B Chem. Sci. 45, 377–81.
[12] Büchi, G. and Egger, B. (1971) J. Org. Chem. 36, 2021–2023.
[13] Yamane, H., Sugawara, J., Suzuki, Y., Shimamura, E. and Takahashi, N. (1980) Agric. Biol. Chem. 44, 2857–2864.
[14] Ishikawa, A., Yoshihara, T. and Nakamura, K. (1994) Biosci. Biotech. Biochem. 58, 544–547.
[15] Kramell, R., Schmidt, G., Schneider., Sembdner, G. and Schreiber, K. (1988) Tetrahedron 44, 5791–5807.
[16] Aida, Y., Tamogami, S. and Kodama, O. (1996) Biosci. Biotech. Biochem. 60, 1495–1496.
[17] Gross, D. and Parthier, B. (1994) J. Plant Growth Regul. 13, 93–114, and references therein.
[18] Weiler, E., Kuchan, T.M., Gorba, T., Brodschelm., Niesel, U. and Bublitz, F. (1994) FEBS Lett. 345, 9–13.
[19] Hopke, J., Donath, J., Blechert, S. and Boland, W. (1994) FEBS Lett. 352, 146–150.
[20] Nojiri, H., Sugimori, M., Yamane, H., Nishimura, Y., Yamada, A., Shibuya, N., Kodama, O., Murofushi, N. and Omori, T. (1996) Plant Physiol. 110, 387–392.