Phytoalexin production elicited by exogenously applied jasmonic acid in rice leaves (*Oryza sativa* L.) is under the control of cytokinins and ascorbic acid

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Abstract Jasmonic acid (JA) has been shown to be a signaling compound which elicits the production of secondary metabolites including phytoalexins in plants. It has been shown that the phytoalexin production is elicited by exogenously applied JA in rice leaves. We now show that this phytoalexin production by exogenously applied JA is significantly counteracted by cytokinins, kinetin and zeatin. Kinetin and zeatin also inhibit the induction of naringenin-7-*O*-methyltransferase (a key enzyme in rice phytoalexin production) by JA. A natural free radical scavenger, ascorbic acid (AsA) shows both counteractive and enhancing effects on JA-inducible phytoalexin production, depending on its concentration. This effect of AsA suggests that active oxygen species (AOS) may play important roles in phytoalexin production by JA in rice leaves.

Key words: Jasmonic acid; Kinetin; Phytoalexin; Ascorbic acid; Rice plant

1. Introduction

It has been shown that jasmonic acid (JA, Fig. 1) and its related compounds play important roles as the signaling molecules that induce proteins and secondary metabolites in the defense systems in plants [1–5]. One of the most important defensive systems in plants against pathogen attack is the production of antimicrobial compounds called phytoalexins [6]. We have been focussing attention on the flavonoid phytoalexin sakuranetin (Fig. 1) because of its high antimicrobial activity and a large amount of accumulation in rice leaves [6]. We have recently shown that sakuranetin production is mediated by endogenous production of JA in stressed rice leaves and have also shown that sakuranetin production is strongly elicited by exogenously applied JA [7]. Thus JA functions as a cellular signaling molecule for the phytoalexin production in rice leaves [7]. However, mechanisms underlying JA induced elicitation of sakuranetin production in rice leaves are still unknown. Recent reports indicate that the defensive reactions elicited by exogenously applied JA occur relatively late [1,4]. In rice leaves, sakuranetin production begins 24 h after treatment with JA and requires 48 h for a significant sakuranetin accumulation [7]. In our previous report, we suggested that the further metabolism of JA into its amino acid conjugates would be needed to furnish the signal transduction pathway [5], but this conversion of JA cannot fully explain such a considerable time lag of 48 h for the sakuranetin production in rice leaves. This implies that treatment of rice leaves with JA may not lead to sakuranetin production directly. The aim of this present study is to investigate the mechanism(s) by which exogenously applied JA elicits phytoalexin production in rice leaves. This study first describes the finding that plant hormones, kinetin and zeatin counteract the JA-inducible sakuranetin production in rice leaves. Second, tiron, a synthetic free radical scavenger and ascorbic acid, a natural free radical scavenger also counteract the JA-inducible sakuranetin production. Third, sakuranetin production by JA is significantly enhanced by high concentration of ascorbic acid.

2. Materials and methods

2.1. Chemicals

JA was prepared by de-esterification of methyl jasmonate (Harmann and Reimer, Holzminden, Germany). Sakuranetin was obtained by the selective methylation of naringenin (Aldrich) as described [5]. Kinetin, zeatin and adenine were purchased from Sigma Chemical Co., St. Louis, MO. Tiron (1,2-dihydroxy-3,5-benzene disulfonic acid, disodium salt, monohydrate) and ascorbic acid were purchased from Wako Pure Chemical Industries Ltd., Tokyo Japan.

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 and quantified by HPLC as described [5]. Naringenin 7-*O*-methyltransferase (NOMT) was purified from stressed rice leaves and the NOMT assay in crude leaf homogenate was performed as described previously [8,9].

3. Results and discussion

3.1. The effect of cytokinin on phytoalexin production by JA

Exogenously applied JA shows a variety of physiological effects in plants besides phytoalexin production. When oat leaves are treated with JA, senescence promotion is observed [10]. It should be noted that the senescence promotion by JA is counteracted by a plant hormone, kinetin [10]. Thus, it is possible that kinetin might be counteracting the phytoalexin production by JA in rice leaves. Our results confirm this possibility and a strong counteractive effect of kinetin against the sakuranetin production by JA is observed (Fig. 2I). Kinetin strongly counteracts the activity of JA at a considerable low concentration of 7.8 μM. Moreover, it is observed that the extent of counteraction is concentration dependent. We investigate the activity of other kinetin related compounds, zeatin and adenine against JA (Fig. 2II). A natural cytokinin, zeatin also shows strong counteractive effect against JA, whereas

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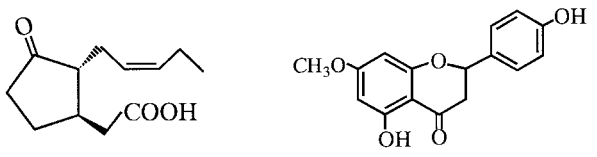

jasmonic acid (JA) sakuranetin

Fig. 1. Structures of JA and rice phytoalexin sakuranetin.

adenine has little effect. Adenine has the common purine skeleton among these compounds, but adenine has no cytokinin like activity. Our results are in good agreement with previous results where adenine is completely inactive in inhibiting browning and phytoalexin accumulation [11]. This result shows that the hormonal cytokinin activity is important for the counteractive effect against JA. The counteractive effect of kinetin is investigated in detail by a time course experiment (Fig. 3). It is shown that low concentration of kinetin suppresses the large accumulation of sakuranetin. As previously described, sakuranetin production is delayed to approximately 48 h after exogenous treatment with JA. Thus, we speculate that this delay might be as a result of the counteractive effect of endogenous cytokinin against exogenously applied JA.

It has been shown that kinetin inhibits a hypersensitive response (HR), one of the plant defensive reactions, when hyphal wall component (HWC) is used as an elicitor [11]. It was also shown that phytoalexin production by the HWC elicitor is inhibited by kinetin in potato tubers [11]. The HWC elicitor has been used as a model for the interaction between plants and pathogen in a rapid localized cell death. On the contrary, JA has been proposed as a systemic secondary signal that follows the rapid localized responses and leads to defensive gene expression [2]. Thus our results show that this systemic signal, JA in rice leaves might be also under the control of kinetin from our present results of exogenously applied JA.

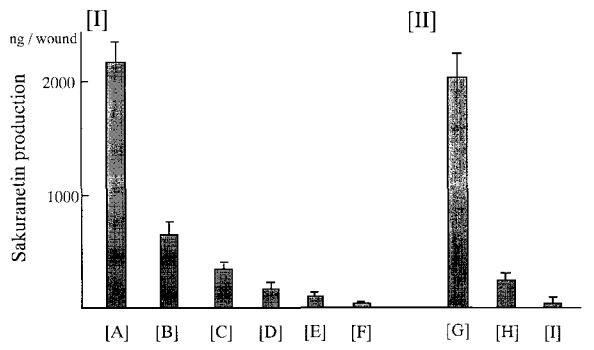

Fig. 2. I: Counteractive effect of kinetin on the sakuranetin production in rice leaves by JA. [A] = JA (250 μM); [B] = JA (250 μM) + kinetin (7.8 μM); [C] = JA (250 μM) + kinetin (31 μM); [D] = JA (250 μM) + kinetin (62 μM); [E] = JA (250 μM) + kinetin (125 μM); [F] = kinetin (250 μM). Sakuranetin was quantified 72 h after the treatment of JA. II: Counteraction of adenine and zeatin on the sakuranetin production in rice leaves. [G] = JA (250 μM) + adenine (250 μM); [H] = JA (250 μM) + zeatin (250 μM); [I] = control (water).

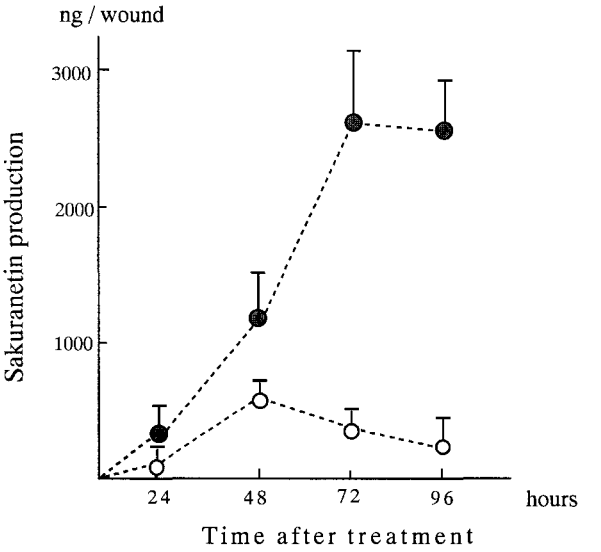

Fig. 3. Counteractive effect of kinetin on the time-dependent production of sakuranetin by JA. ●, JA (250 μM); ○, JA (250 μM) + kinetin (5 μM).

3.2. *Effect of free radical scavengers on phytoalexin production by JA*

Kinetin has been suggested to be an effective free radical scavenger [12], and has been suggested to inhibit HR by scavenging the free radicals in HWC elicited potato tubers [11]. Assuming that kinetin counteracts the JA-inducible sakuranetin production in rice leaves based on the above effect, then it is possible that known free radical scavengers might also show counteractive effect against JA. Thus, counteractive effects of

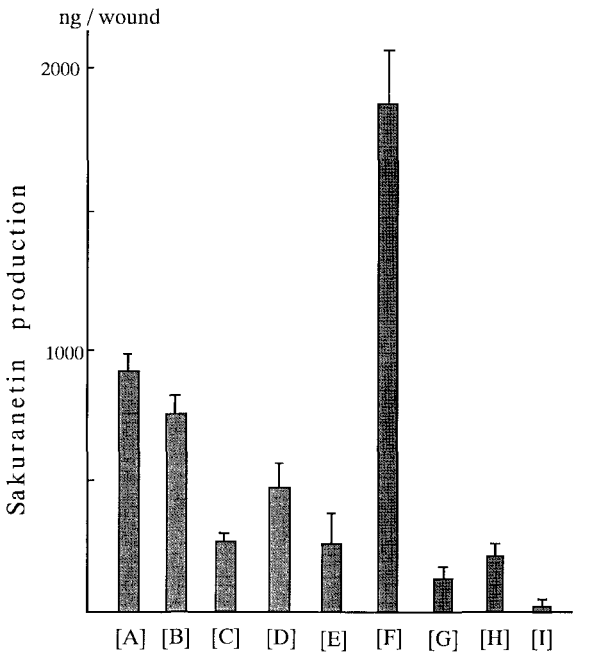

Fig. 4. Effect of tiron and AsA on the sakuranetin production by JA. Sakuranetin was quantified 48 h after the treatment of JA. [A] = JA (250 μM); [B] = JA (250 μM) + tiron (10 mM); [C] = JA (250 μM) + tiron (100 mM); [D] = JA (250 μM) + AsA (250 μM); [E] = JA (250 μM) + AsA (500 μM); [F] = JA (250 μM) + AsA (5 mM); [G] = AsA (5 mM); [H] = JA (250 μM) + AsA (5 mM) + kinetin (250 μM); [I] = control (water).

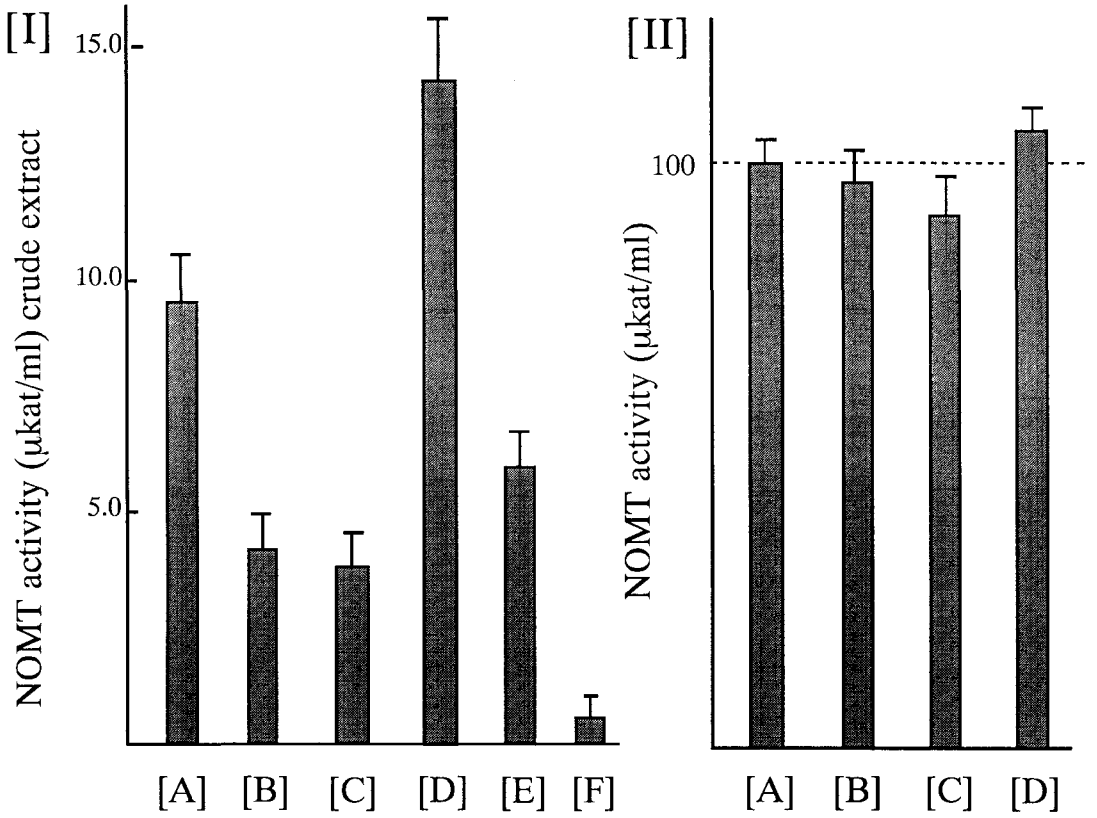

Fig. 5. I: Counteractive effect of cytokinin and enhancing effect of AsA on the NOMT induction by JA. [A] = JA (250 μM); [B] = JA (250 μM) + kinetin (250 μM); [C] = JA (250 μM) + zeatin (250 μM); [D] = JA (250 μM) + AsA (5 mM); [E] = JA (250 μM) + AsA (250 μM) + kinetin (250 μM); [F] = control (water). II: Direct effect of cytokinin and AsA on purified NOMT activity. Enzyme activity of NOMT is calculated as relative activity to positive control [A]. [A] = purified NOMT, [B] = [A] + kinetin (500 μM); [C] = [A] + zeatin (500 μM); [D] = [A] + AsA (5 mM).

tiron and a natural free radical scavenger, ascorbic acid (AsA) on the JA-inducible sakuranetin production are investigated (Fig. 4). Tiron is a synthetic catechol analogue that is widely used in physiological and phytopathological field as a free radical scavenger [13,14], and shows a counteractive effect at concentrations of 100 mM. It is well known that AsA is an effective scavenger of superoxide anion and hydroxy radicals [15,16]. As shown in Fig. 4, AsA counteracts the JA-inducible sakuranetin production at low concentrations of 0.25 and 0.5 mM. These results suggest that the elicitation by exogenously applied JA might be associated with the process that is inhibited by radical scavengers.

It should be noted that 5 mM of AsA significantly enhances the JA-inducible sakuranetin production, although AsA itself has no effect. These results show that the exogenously applied AsA accelerates JA-inducible sakuranetin production by the interaction with JA. One possible interpretation of above results is that the generation of active oxygen species (AOS) might be connected with the JA-inducible sakuranetin production. Because AsA is known to work as a potent AOS generator by accelerating Fenton reaction [17,18], and the resulting generation of AOS (e.g. hydroxy radicals and superoxides) by AsA might accelerate the JA-inducible sakuranetin production. Previous studies on HWC and oligosaccharide elicitation suggest that AOS are important in activating plant defense systems including phytoalexin production [14,19,20]. It is speculated that AOS might also play important roles in phytoalexin production by exogenously applied JA in plants.

The effect of AsA on phytoalexin production by JA is counteracted by kinetin as shown in Fig. 4. This significant counteraction by kinetin will give a clue in future studying for an understanding of the elicitation mechanism by JA in plants.

3.3. *Effect of cytokinin and AsA on NOMT*

We have already shown that NOMT is a key enzyme in the sakuranetin biosynthesis and NOMT activity is induced by JA in rice leaves [8]. Fig. 5I shows the effects of kinetin, its related compounds and AsA on NOMT induction by JA. The extent of inhibitory effect by kinetin and zeatin are in good agreement with the reduction of sakuranetin production. On the other hand, AsA (5 mM) enhances both NOMT induction (Fig. 5) and the resulting sakuranetin production (Fig. 4). Fig. 5II shows the direct effect of above compounds on purified NOMT activity. Pure NOMT is obtained from JA-treated rice leaves [9] and enzyme activity is measured by the previous method [8,9]. Kinetin, zeatin and AsA show no direct effect on NOMT activity suggesting that these compounds do not interact with NOMT itself, but interact with pathway(s) to NOMT induction and effecting subsequent sakuranetin production in rice leaves.

It has been reported that the production of many important secondary metabolites are elicited by exogenously applied JA [1–5], but the elicitation mechanism by JA is still unknown. Our finding that cytokinin and AsA are connected with the elicitation by exogenously applied JA is worthwhile in future study for the understanding of secondary metabolites production in plants.

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