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Dose-Dependent Effects of Malformin A1 on IAA-Induced Ethylene Production in Mung Bean (*Vigna radiata* L.) Hypocotyl Segments

Soon Young Kim[2], A-young Cho[2], Kun Woo Kim[3], and Seung-Eun Oh[1]*

[1]Department of Biological Sciences, Konkuk University, Seoul 143-701, Korea
[2]Department of Biological Sciences, Andong National University, Gyeongbuk 760-749, Korea
[3]School of Bioresource Science, Andong National University, Gyeongbuk 760-749, Korea

Purified malformin A1 (cyclo-D-Cys-D-Cys-L-Val-D-Leu-L-Ile), a cyclicpentapeptide toxin from *Aspergillus niger*, was applied to the hypocotyl segments of mung bean (*Vigna radiata* L.) seedlings to investigate its role in regulating ethylene biosynthesis. Production of ethylene was induced by treating the plants with 0.1 mM indole-3-acetic acid (IAA). When 0.1 μM malformin A1 was then applied, ethylene production increased and the activities of two key enzymes for its biosynthesis, 1-aminocyclopropane-1-carboxylic acid (ACC)-synthase (ACS) and ACC-oxidase (ACO), were also stimulated. However, at levels of 1 or 10 μM malformin A1, both ethylene production and enzymatic activities were significantly reduced. In the case of ACO, *in vitro* activity was regulated by malformin A1, independent of ACS activity or the influence of IAA. Furthermore, the conjugate form of ACC, N-malonyl ACC, was significantly promoted by treatment with 0.1 μM malformin A1. These data suggest that malformin A1 can modulate ethylene production through diverse paths and that its effect depends on the concentration of the treatment administered.

Keywords: 1-aminocyclopropane-1-carboxylic acid, ACC-oxidase, ACC-synthase, IAA-induced ethylene production, N-malonyl ACC, malformin A1

The malformin complex, which comprises a small family of cyclicpentapeptides, is a natural metabolite synthesized from *A. niger*. This compound was first presented as a plant growth regulator that evoked the malformation of stems and petioles (Curtis, 1958; Postlethwait and Curtis, 1959). Further studies revealed that it also promotes ethylene-mediated physiological responses, such as the induction of root curvature (Izhar et al., 1969) and epinasty (Curtis, 1968), and the stimulation of abscission (Curtis, 1977). Besides being a positive effector, malformin also inhibits various ethylene-mediated process, e.g., hook retention and stunting of dark-grown plants (Curtis and John, 1975). Furthermore, malformin has an organ-specific effect of stimulating ethylene production in the seedlings and explants of *Phaseolus vulgaris* (Curtis, 1969) but not the roots of that species (Izhar et al., 1969). Because malformin is closely related to ethylene production, it has been suggested that these organ-specific growth disturbances may be mediated, in part, by malformin-modulated ethylene production (Curtis, 1968).

In higher plants, ethylene is synthesized from methionine through two known intermediates: S-adenosylmethionine (AdoMet) and 1-aminocyclopropane-1-carboxylic acid (ACC). ACC-synthase (ACS) and ACC-oxidase (ACO) are responsible for converting AdoMet to ACC and ACC to ethylene, respectively (Theologis, 1992; Kende, 1993). The ethylene precursor, ACC, is also converted to malonyl ACC (MACC), a conjugate form of ACC, by ACC N-malonyltransferase (Chick and Leung, 1997).

Although earlier research attempted to unravel the effects of malformin on plants (Curtis, 1958, 1968, 1984; Curtis and John, 1975), no further progress has been made on the mechanism for malformin functioning or its regulation of ethylene production specifically, those enzymes that participate in the biosynthetic pathway. Furthermore, most of those earlier studies were performed with malformin A complexes or crude malformin extracts.

Among the nine types of malformin that have been purified from malformin complexes (Kim et al., 1993a, b), malformin A1 (cyclo-D-Cys-D-Cys-L-Val-D-Leu-L-Ile) is the major component of the 'A' complex. It is also a potent physiological effector that induces root curvatures in corn and stimulates growth in mung bean hypocotyls (Kim, 1995). The objective of our study was to determine its effect on ethylene

*Corresponding author; fax +82-2-3436-5432
e-mail seunoh@konkuk.ac.kr

biosynthesis in mung bean hypocotyl segments. Here, we used purified malformin A1 rather than the malformin A complex. Our primary focus was on the role of ACS and ACO enzymes in ethylene production.

MATERIALS AND METHODS

Plant Material

Mung bean (*V. radiata* L.) seeds were soaked overnight in tap water and germinated for 2.5 d in a 0.5% agar plate at 27°C in the dark. We placed the germination plate in a box containing 0.5 mM of $KMnO_4$ solution in order to maintain an appropriate level of humidity and to absorb the ethylene produced during the germination process. Afterward, 1-cm hypocotyl segments were removed from these new seedlings.

Purification of Malformin A1 from Malformin A Complex

Purification of malformin A1 was performed according to the method of Kim et al. (1993a, b). Briefly, malformin A complex in a DMSO solution was prepared from *A. niger* strain 56-39 by the method of Takahashi and Curtis (1961). Malformin A1 was separated from the complex by HPLC (Waters 1525 binary HPLC pump, 2487 dual λ absorbance detector, 410 differential refractor meter; Waters, USA) equipped with ODS columns (Capcell Pak C_{18}, 18 × 250 mm; Shiseido, Japan) in a mixture of MeOH-water-TFA (60:40:0.5) at a flow rate of 2 mL min^{-1}. After HPLC separation, the peaks were identified by UV (254 nm) and RI detectors, and the isolated malformin A1 was confirmed by EIMS (JEOL JMS-DX303; JEOL, Japan). The fraction containing malformin A1 was then evaporated *in vacuo* and lyophilized to powder.

Measurement of Ethylene Production

Hypocotyl segments were placed in 25-mL vials and submerged in 0.05 M potassium phosphate buffer (pH 6.8) with or without 0.1 mM IAA and/or malformin A1 (0.0, 0.01, 0.1, 1.0, or 10 μM). The vials were sealed with silicon stoppers and incubated at 27°C for up to 8 h. Periodically, 1-mL gas samples were withdrawn and analyzed by gas chromatography (DS 6200; Donam Instrument, Korea), using an aluminum oxide column and a flame-ionization detector.

Assay of *in Vitro* ACC Synthase (ACS) Activity

ACS activity was assayed using the protocol described by Peck and Kende (1995), with some modifications. Two grams of hypocotyl tissue was homogenized on ice with 8 mL of buffer A (250 mM phosphate buffer pH 8.0, 10 μM pyridoxal phosphate, 1 mM EDTA, 2 mM PMSF, and 5 mM DTT). These samples were then centrifuged at 15,000*g* for 15 min, and the supernatant was re-spun at 15,000*g* for 15 min. The supernatant (1 mL) was added to a vial containing 100 μL of 5 mM AdoMet, and the mixture was incubated at 22°C for 1 h. The ACC that formed was assayed using the method of Lizada and Yang (1979).

Assay of *in Vitro* ACC-Oxidase (ACO) Activity

We measured ACO activity according to the technique of Mekhedov and Kende (1996). After 40 chemically treated hypocotyl segments were rinsed with distilled water and blotted dry, they were frozen in liquid nitrogen and ground to a fine powder. Each gram of hypocotyl sample was then extracted with 3 mL of extraction buffer [100 mM Tris-HCl pH 7.2, 30 mM Na-ascorbate, and 10% (w/v) glycerol] for 20 min at 4°C. The suspension was centrifuged at 5,000*g* for 15 min and the recovered supernatant was further centrifuged at 12,000*g* for 20 min. Protein content in the supernatant was determined with a protein assay reagent (Bio-Rad, USA) according to the manufacturer's instructions. A solution including 1 mg of protein was added to 2 mL of extraction buffer containing a final concentration of 4 mM ACC and 50 μM $FeSO_4$ in a 25-mL glass vial. Afterward, the vial was sealed and incubated in the dark at 30°C for 1 h while being gently shaken. Then, 1-mL gas samples were withdrawn and analyzed by gas chromatography.

Quantification of Free and Conjugated ACC

Two grams of hypocotyl segments was extracted in 10 mL of 70% ethanol (w/v 1:2) at 4°C. The sample was centrifuged at 15,000*g* for 15 min. After 1 mL of the supernatant was evaporated under vacuum, the residues were dissolved in 0.5 mL distilled water. The dissolved residue was transferred into an injection vial and 0.1 mL of 10 mM $HgCl_2$ and 0.3 mL of distilled water were added. The vials were then capped and 0.1 mL of a 1:2 mixture of cold saturated NaOH/NaOCl was injected with a syringe. After the reaction

mixtures were placed on ice for 1 h, ethylene production was measured. To determine the amount of conjugated ACC, 0.5 mL of the dissolved solution was hydrolyzed in 1 mL of 2 N HCl at 100°C for 3 h. Following neutralization with 1 N NaOH, the mixture was centrifuged at 10,000g for 5 min. The ACC contents were then determined as described above, with the amount of conjugated ACC being calculated as the difference between free and total ACC values.

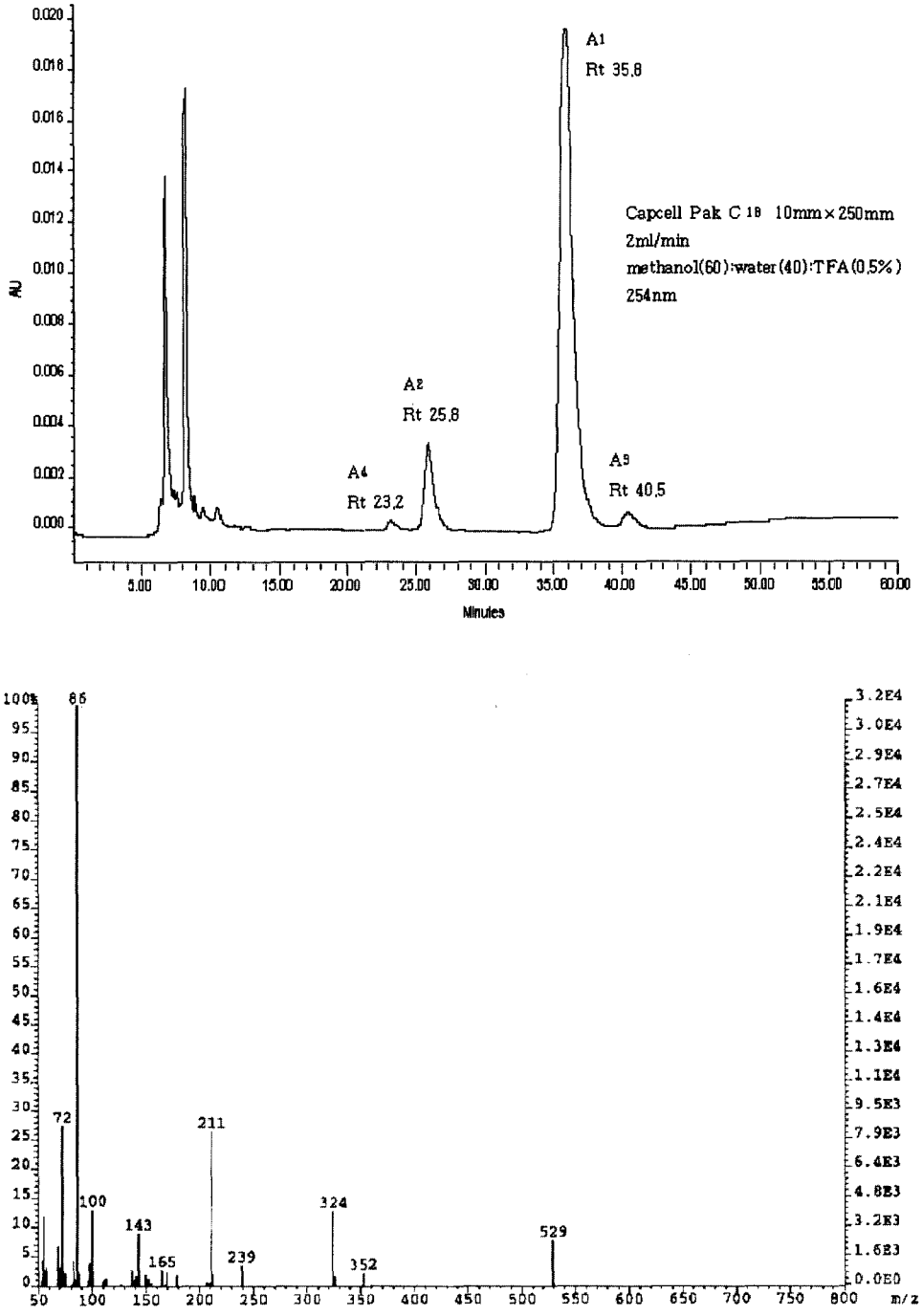

Figure 1. Separation of malformin A compounds from malformin A complex by HPLC (**A**), and EIMS analysis of the separated malformin A1 (**B**).

RESULTS

Purification of Malformin A1

During the HPLC separation of malformin A1, one major peak was distinguishable from three minor ones (Fig. 1A). We performed EIMS at 70 eV for that major peak (Fig. 1B), and obtained the following m/z (rel. int.): 529 [M](8), 352 (3.5), 324 (13), 239 (4), 211 (35.5), 165 (3), 143 (9), 100 (13), and 86 (100). Based on these results, we determined the compound's molecular mass to be 529 ($C_{23}H_{39}N_5O_5S_2$). These data from the HPLC and EIMS were consistent with those for malformin A1 isolated by Kim et al. (1993a). Therefore, we labeled this compound as malformin A1 and used it in further experiments.

Effect of Malformin A1 on IAA-Induced Ethylene Production

In a preliminary study, we were unable to detect any effect of malformin A1 on normal ethylene production in mung bean hypocotyl segments (data not shown). Therefore, we amplified ethylene biosynthesis by treating the tissues with indole-3-acetic acid (IAA), which is known to induce ethylene production in the hypocotyls of etiolated seedlings (Kang et al., 1971).

The degree of influence on 0.1 mM IAA-induced ethylene production depended on the amount of malformin A1 that was applied (Fig. 2). For example, a concentration of 0.1 μM increased ethylene levels by 50%, whereas higher malformin concentrations, i.e., 1 or 10 μM, significantly suppressed ethylene production by 30 to 80%.

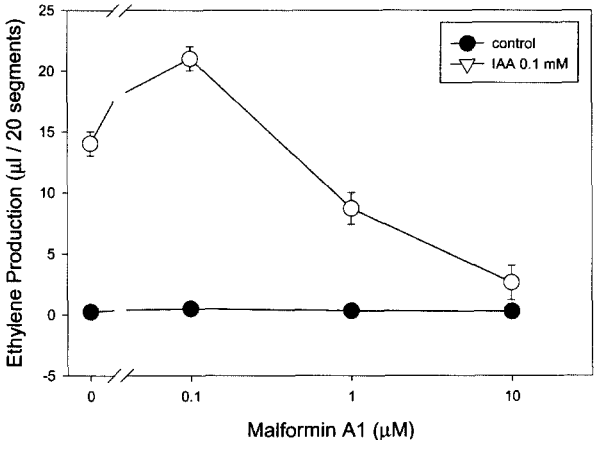

Figure 2. Effect of malformin A1 on IAA-induced ethylene production in mung bean. Twenty hypocotyl segments (1 cm long) excised from 2.5-d-old seedlings were incubated at 27°C for 8 h in potassium phosphate buffer that included malformin A1 with or without 0.1 mM of IAA. Results are mean values (±SE) for 7 replicates.

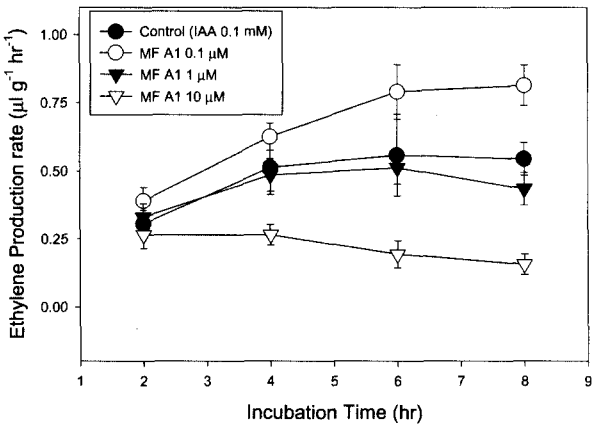

Figure 3. Time course assay of effect of malformin A1 on IAA-induced ethylene production in mung bean hypocotyl segments. Results are mean values (±SE) for 7 replicates.

To further examine the effect of malformin A1 treatment, we conducted a time course analysis with data collected at 2 h intervals (Fig. 3), but found no significant change, regardless of malformin A1 concentration, in the first 2 h of treatment. However, we did detect a 15% stimulatory effect of 0.1 μM malformin A1 on IAA-induced ethylene production after 4 h, as well as a 40% increase in ethylene content after 6 h, a level that was maintained for up to 8 h. In contrast, treatment with 10 μM malformin A1 inhibited ethylene production by 50% after 4 h. This suppression rate was further increased, by up to 70 to 75%, when the treatment was extended for up to 8 h.

Effect of Malformin A1 on ACS Activity

To understand how various concentrations of malformin A1 modulated IAA-induced ethylene production, we examined the conversion of AdoMet to ACC (Fig. 4). *In vitro* ACS activity in hypocotyl segments treated with 0.1 mM IAA rose steadily during the 8 h incubation, whereas a 4 h treatment with 0.1 μM malformin A1 increased ACS activity by 15%. However, that stimulatory effect by malformin disappeared after 8 h. In contrast, 1 or 10 μM malformin A1 inhibited activity over a treatment period of up to 8 h, with the suppression rate being up to 50% at the highest concentration of malformin.

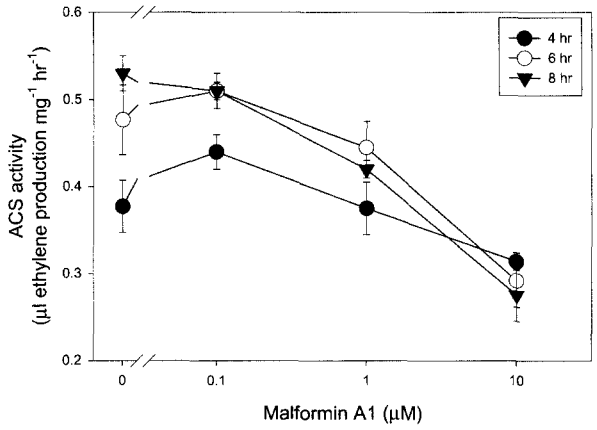

Figure 4. Effect of malformin A1 on *in vitro* ACC-synthase activity in mung bean. Two grams of hypocotyl segments (1 cm) excised from 2.5-d-old seedlings were used to determine *in-vitro* ACC-synthase (ACS) activity following 4, 6, or 8 h of incubation with malformin A1. Results are mean values (±SE) for 7 replicates.

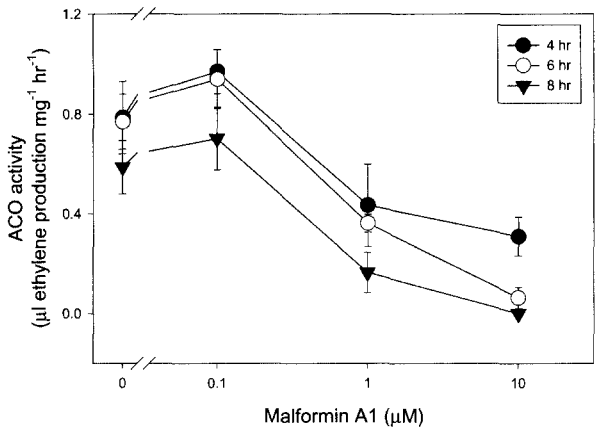

Figure 6. Effect of malformin A1 on *in vitro* ACC-oxidase (ACO) activity in mung bean in the presence of IAA. Forty hypocotyl segments were incubated in 0.05 M potassium phosphate buffer (pH 6.8) that included 0.1 mM IAA, without or with malformin A1. Results are mean values (±SE) for 10 replicates.

Effect of Malformin A1 on Free and Conjugated ACC Contents

We measured levels of free and conjugated ACC to determine whether they were influenced by malformin A1 (Fig. 5). Although ACS activity was promoted by 0.1 µM malformin A1 (Fig. 4), the level of free ACC following such treatment was lower than for the control. When hypocotyl segments were treated with 0.1 or 10 µM malformin A1, the amount of free ACC was reduced by 25% or 50%, respectively. To examine whether the level of conjugated ACC was affected by malformin A1 treatment, we focused on the level of malonyl-ACC (MACC), a major conjugated form of ACC. Here, MACC levels increased by 70% in the presence of 0.1 M malformin A1, but decreased by 70% at the 10 µM concentration.

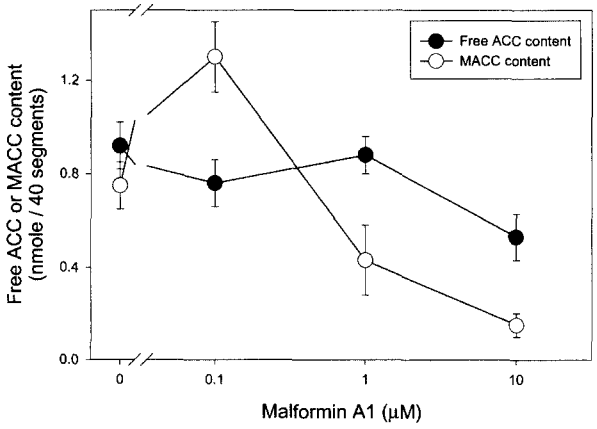

Figure 5. Effect of malformin A1 on free and conjugated ACC (MACC) contents in mung bean. Two grams of hypocotyl segments (1 cm) excised from 2.5-d-old seedlings were incubated for 8 h in 0.05 M potassium phosphate buffer (pH 6.8) that included malformin A1. Results are mean values (±SE) for 10 replicates.

Effect of Malformin A1 on ACO Activity

We analyzed a time course study of the effect of malformin A1 on ACO (Fig. 6). In contrast to ACS, *in vitro* ACO activity was reduced in proportion to the length of the IAA treatment. However, the addition of 0.1 µM malformin A1 stimulated ACO activity by 30%, a level that was maintained for 4 to 6 h. However, this stimulatory effect was reduced by up to 15% after 8 h of treatment. In contrast, a 10 µM concentration of malformin A1 inhibited ACO activity by 55% and 95% following 4 h and 8 h treatments, respectively. Therefore, we have demonstrated that malformin A1 can modulate ACO activity, depending on its concentration, and that the level of free ACC can be reduced by accelerating the conversion of ACC to ethylene.

DISCUSSION

In this study of mung bean hypocotyls, we examined how malformin A1, a fungal toxin cyclicpentapeptide purified from the malformin A complex in *A. niger*, regulates the enzymes that participate in auxin-induced ethylene production. We identified two remarkable properties for malformin A1. First, its

experimental concentration is an important determinant of performance (Fig. 2, 3, 4, 6). Although 0.01 μM malformin A1 displayed a tendency to stimulate ethylene production and ACS and ACO activities, those effects were insignificant (data not shown). However, quantification assays consistently showed that a slightly higher concentration (i.e., 0.1 μM) was effective in achieving measurable results. In contrast, high concentrations (1 and 10 μM) inhibited all those activities.

We might speculate that this inhibitory effect was the result of sheer toxicity from a high dose of chemicals. However, Curtis (1975) has successfully used 10 μM malformin to explore the interaction of the malformin A complex with phytochrome in stem segments of *P. vulgaris* seedlings. That same concentration has also proven effective on the interaction of malformin with other growth regulators in the stems of 14-d-old mung bean seedlings (Curtis, 1984). Likewise, in the primary roots of maize plants, 10 μM of malformin A1 stimulates both ethylene production and ACO activity (Hong, 2004). Finally, we have also recently found that *in vitro* ACO activity, which is suppressed by 10 μM malformin A1, can again be recovered to some degree by treatment with a Ca^{2+} ionophore or an inhibitor of serine/threonine phosphatase (data not shown). Therefore, based on all these data, the inhibitory effects observed with 1 and 10 μM of malformin A1 should be considered physiological responses.

The second property of malformin A1 pertains to its multi-functional capacity. For example, in our etiolated hypocotyl segments, the same cyclicpentapeptide regulated at least three steps: ACS and ACC activities as well as the conversion of ACC into malonyl ACC, as catalyzed by ACC N-malonyltransferase (Fig. 3, 4, 5). In contrast, Hong (2004) has reported that the effect of malformin A1 on ethylene production is limited just to ACO activity in maize primary roots.

The effect of malformin A1 on ethylene production was detectable when biosynthesis was amplified by treatment with IAA (Fig. 2). Meanwhile, 1 and 10 μM malformin A1 inhibited activity when a relatively low IAA concentration (e.g., 10 μM) was used (data not shown), whereas 0.1 μM malformin A1 was an effective stimulant at higher concentrations of IAA (e.g., 0.1 mM). In mung bean and pea seedlings, auxin promotes ethylene production by inducing or activating ACS (Yoshii and Imaseki, 1982; Peck and Kende, 1995). In addition, the amount of *VR-ACS1* and *VR-ACS6* transcripts encoding mung bean ACS is increased after IAA treatment (Yoon et al., 1997; Kim et al., 2001). Here, we observed a significant stimulatory influence by IAA on ACS activity following 6 hr of treatment with that hormone (Fig. 4).

We might speculate here on a possible interaction between malformin A1 and IAA in terms of ethylene production, as has previously been reported with regard to the malformin A complex. Curtis and Fellenberg (1972) have demonstrated that IAA antagonizes malformin-induced inhibition of rooting on etiolated stem segments of *P. vulgaris*. Furthermore, they have found that malformin inhibits IAA-induced swelling at the bases of those segments, which they explain as malformin possibly promoting the efflux of IAA. In our study, variations in the amount of IAA after malformin A1 treatment could have been a causal factor in modulating ethylene production. However, we currently have no data to support the theory that malformin A1 effects IAA efflux in this species.

The stimulatory effect of 0.1 μM malformin A1 was more sustained with ACO than with ACS, but even the activity of the former was reduced when IAA treatment was extended (Fig. 4 and 6). ACO activity and transcript are increased by exogenous ethylene in both apple and mung bean plants (Dong et al., 1992; Kim and Yang, 1994; Kim et al., 1997), which implies that the promotion of ACO activity after malformin A1 treatment might result from the feedback stimulation of ethylene production promoted by ACS induction. Therefore, further exploration is needed to determine whether ACO is regulated by malformin A1 independent of ACS activity. We also analyzed *in vitro* ACO activity under conditions where ACS activity was excluded (Fig. 7) and found that the former was reduced in proportion to the extent of the IAA treatment (Fig. 6).

Kim et al. (2001) have reported that IAA inhibits ethylene-promoted *VR-ACO1* expression and *in vivo* ACO activity. Nevertheless, our treatment with IAA greatly induced ethylene production, suggesting the need to examine the effect of malformin at the exclusion of IAA's influence on ACO. To eliminate the effects of ACS activity on ethylene production and IAA on ACO activity, we also incubated hypocotyl segments in an IAA-free solution containing AVG, an inhibitor of ACS. In this case, *in vitro* ACO activity increased further depending on the length of the incubation period (Fig. 7). Furthermore, a 10 to 15% stimulatory effect by 0.1 μM malformin A1 was maintained during 8 h of treatment. In contrast, 10 μM malformin A1 inhibited ACO activity by 80%. These

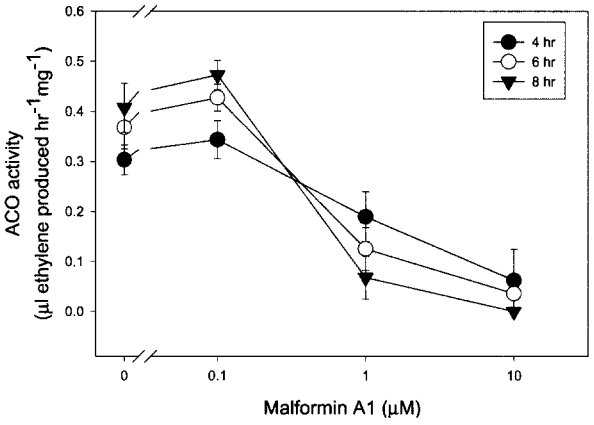

Figure 7. Effect of malformin A1 on *in vitro* ACC-oxidase (ACO) activity in mung bean in the absence of IAA. Forty hypocotyl segments were incubated in 0.05 M potassium phosphate buffer (pH 6.8) that included 0.1 mM AVG, without or with malformin A1. Results are mean values (±SE) for 10 replicates.

results imply that malformin A1 can regulate ACO activity independent of ACS activity. We also examined the effect of malformin A1 on ACC-induced ethylene production in the presence of AVG and 1 mM ACC after 8 h of incubation. Whereas 10 μM malformin A1 suppressed 40 to 50% of the ethylene production, 0.1 μM malformin A1 promoted its biosynthesis by 20 to 25%. Therefore, we have confirmed that malformin A1 does regulate ACO activity, independent of ACS, in proportion to the treatment concentration used.

The amount of conjugated malonyl-ACC (MACC) was significantly increased by 0.1 μM malformin A1 (Fig. 5). Chick and Leung (1997) have shown that synthesized ACC converts to MACC through ACC N-malonyltransferase in mung bean samples. We also detected a high MACC content, which probably resulted from the promotion of ACS activity by treatment with 0.1 μM malformin A1, while the conversion rate of ACC to MACC by ACC N-malonyltransferase remained constant. However, we cannot exclude the possibility that malformin A1 directly regulated the activity of ACC N-malonyltransferase and increased the level of MACC.

Ciarlante and Curtis (1976) have reported the localization of malformin-binding proteins in cell walls of the primary leaves of *P. vulgaris*. Although proteins that bind malformin molecules should not be receptors, we cannot exclude the possible presence of malformin A1 receptor(s) in mung bean hypocotyl segments that induce various responses. Moreover, with regard to the multiple functions of malformin A1 in ethylene biosynthesis, further investigation is required to determine whether a unique receptor induces all those activities by malformin A1 or if some receptors induce each specific function. Through such research, we could drastically increase our understanding of how the cyclicpentapeptide malformin A1 from *A. niger* regulates ethylene production by triggering physiological responses in its host plants.

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