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The Effect of Oligosaccharides on Ethylene Production in Mung Bean (*Vigna radiata* W.) Hypocotyl Segments.

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The physiological effects of oligogalacturonic acid (OGA: D. P. ≥6-7), a product of acid hydrolysis of polygalacturonic acid (PGA), on ethylene biosynthesis in mung bean (*Vigna radiata* W.) hypocotyl segments was studied. Among PGA, OGA and monogalacturonic acid (MGA), only OGA stimulated ethylene production in mung bean hypocotyl segments, and the most effective concentration of OGA was 50 μg/mL. Time course data indicated that this stimulation effect of OGA appeared after 90 min incubation period and continued until 24 h. When indol-3-acetic acid (IAA) and 1-aminocyclopropane-1-carboxylic acid (ACC) were treated with OGA to investigate the mechanism of OGA on ethylene production, they did not show synergistic effects on ethylene production. The stimulation of ethylene production by OGA was due to the increase of *in vivo* ACC synthase activity, but OGA treatment had no effect of *in vivo* ACC oxidase activity. The effect of aminoethoxy vinyl glycine (AVG) and Co^{2+}, the inhibitor of ethylene synthesis, was diminished a little by the OGA, but the treatment of Ca^{2+}, known to increase ACC, with OGA did not increase the ethylene production. this effect seems to be specific for Ca^{2+} because other divalent cation, Mg^{2+}, did not show the inhibition of OGA-induced ethylene production. It is possible that the OGA adopts a different signal transduction pathway to the ethylene biosynthesis.

Keywords : oligogalacturonic acid, ethylene, auxin, ACC synthase, calcium ion

Plant cell walls are complexes of carbohydrates, protein, lignins, water and incrusting substances such as cutin, suberin, and certain inorganic compounds depending on plant species, cell type, and even neighboring cells. Developments events and exposure to any of number of abiotic and biotic stresses further increase this compositional and structural variation. Moreover, the dynamic developments, environmental sensing and signaling, plant defense, intercellular communication, and selective exchange interfaces are reflected in these variations. Xyloglucan oligosaccharides (XG_9), also known as hemicellulose, is known to inhibit plant growth. In particular, the elongation of pea induced with 2,4-D is inhibited by a mass of 10^{-9} M hemicellulose (McDougal and Fry, 1988: 1990). Unlike XG_9, OGA derived from pectin by endopolygalacturonase regulates a number of physiological phenomena including inhibition of pea stem elongation induced by auxin (Branca *et al.*, 1988), inhibition of root formation and floral development in tobacco (Kiem Tran Thanhvan *et al.*, 1985; Eberhard *et al.*, 1989), and induction ethylene biosynthesis during fruit ripening in tomato (Brecht and Huber, 1988; Campbell and Lavavitch, 1991b). Polysaccharides in the plant cell wall generate phytoalexin and proteinase inhibitor (PI) (Bishop *et al.*, 1981; Darvill and Alversheim, 1984). At this time, OGA derived form plant cell wall by hydrolysis induce biosynthesis of β-1,3-glucanase and chitinase which are able to degrade fungal cell wall (Ryan, 1987). Glycopeptide, another type of OGA which is degraded by invertase of yeast, induces the biosynthesis of ethylene and phenylalanine ammonia lyase (PAL) in tomato cells (Basse *et al.*, 1992). There is report which show that OGA changed ion flux such as membrane potential, K^+ efflux, Ca^{2+} influx and alkalization of medium (Mathieu *et al.*, 1991). The reports suggest the possibility that OGA is a signal molecule in cell membrane.

Ethylene is a plant growth regulator induced by various environmental stress factors such as wound-

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ing, mechanical damage, pathogene attack, cold, drought, UV irradiation, CO_2 at high concentration, decreased O_2 concentration and various chemical (Abeles, 1973; Boller and Kenede, 1980; Liverman, 1979; Pratt and Goeschl, 1969; Takahashi and Jaffe, 1984; Yang and Hoffman, 1984). Many reports indicate that ethylene activates such genes as PAL, 4-coumarase CoA liase (4-CL), chalcon synthasc (CHS), hydroxyproline-rich glycoprotein (HRGPs) concerning synthesis of phytoalexin, and that they take part in the plant defense (Cassb and Varnar, 1988; Ecker *et al.*, 1987). OGA has also been recognized as a material that stimulates the synthesis of ethylene. But, as to its effects on the ethylene biosynthetic pathway, no established opionon has been formulated. The aim of this research is to investigate the steps of the ethylene synthetic pathway on which OGA acts.

MATERIALS AND METHODS

Plant material and reagents

Mung bean (*Vigna radiata* W.) seeds, after soaking in flowing water for about 12 h, were planted in agar meida 0.5% (w/v) and had them germianted for 3 days in humid dark room at 26±1°C. 0.8 cm long segment just the lower part of the apical hook was used for experimental materials. D-galacturonic acid, indol-3-acetic acid (IAA), 1-amminocyclopropane-1-carboxylic acid (ACC), carbazole, D-glucurono-6,3-lactone, benzolyated dialysis tubing purchased form Sigma.

Isolation and assay of OGA

The method of Campbell *et al.* (1991a) was modified. After hydrolysis of 1% PGA dissolved in 0.5 N HCl by means of autoclave (at 121°C for 50 min), it was titrated with NaOH to pH 7.0. Among these hydrolytic products, unresolved polymers were filtered with Whatman filter paper GF/A and dialysized in distilled water for 24 h to remove those polymers with less than 5-6 D. P. (degree of polymerization). Then, we used the filtered products as OGA. The concentration of OGA was assayed by Carbazole assay (Blumenkrantz and Asboe-Hansen, 1973; Chaplin and Kennedy, 1986). D-glucurono-6,3-lactone is used as standard, and was detected at 525 nm in a spectrophotometer (Hitachi, U-2000).

Measurement of Ethylene Production

The hypocotyl segments were incubated in a 15 mL-vial containing 2 mL of 10 mM Mes/Tris, pH 6.8, 50 μg/mL chloramphenicol and other reagents in a dark room (26±1°C) for 22 h. 1 mL of gas in the vial was withdrawn from the vial with a hyperdermic syringe and ethylene was assayed on a gas chromatograph (Shimadzu GC-9A, Flame Ionized Detector Porapark Q Column 100-200 mesh 90°C, Air: 0.5 kg/cm^2, Hydrogen: 0.6 kg/cm^2, Carrier (N_2): 60 mL/min).

Assay of ACC Synthase

The homogenization and extraction of plant tissue for the assay of ACC synthase were performed as described by Yip *et al.* (1991). Hypocotyl segments were incubated in 10 mM Mes/Tris, pH 6.8, containing 50 μg/mL chloramphenicol with or without OGA (50 μg/mL) in dark room (26±1°C) for 22 h. Tissue were homogenized 100 mM Hepes-KOH buffer, pH 8.5, containing 4 mM DTT, 0.5 μM pyridoxal phosphate (PLP), 10 mM EDTA, 0.1 mM PMSF, 2 M NaOH using a pestle in a mortar. Homogenization buffer was used a ratio of 1 mL/g fresh weight of tissue. The homogenate was centrifuged at 25,000 g for 15 min. The supernatant was applied to a Sephadex G-25 column (2×11 cm, bed volume, 30 mL) previously equilibrated with 2 mM Hepes-KOH, pH 8.5, containing 0.5 mM DTT, 0.5 μM PLP, and 1 mM EDTA. The amount of ACC formed was determined by chemical conversion of ACC to ethylene followed by gas chromatography. One unit of enzyme converts 1 nmol of SAM to ACC per h at 30°C

To know what effect OGA has on the activity of ACC synthase *in vitro*, we purified partially the enzyme from the tissue not incubated with OGA, and then the activity of enzyme was compared in the reaction mixture with OGA or without OGA (50 μg/mL).

Assay of ACC Oxidase

The homogenization and extraction of plant tissue for the assay of ACC oxidase were performed as described by Fernandes-Maculet and Yang (1992). The hypocotyl segments were incubated in 10 mM Mes/Tris, pH 6.8, containing 50 μg/mL chloramphenicol with or without OGA (50 μg/mL) in a dark room (26±1°C) for 22 h. In order to inhibit the activity of ACC synthase, the tissue was incubated for 2 h in Mes/Tris buffer containing 10^{-5} M AVG, and then

0.5 g of tissue were incubated in 2 mL of a medium containing 2% (w/v) sucrose, 1 mM $CaCl_2$, 50 mM Mes/Tris, pH 6.2, and 2 mM ACC for 1 h, and ethylene production was measured by gas chromatography.

RESULTS AND DISCUSSION

Recently, it has been reported that among the polysaccharides components in primary cell wall of plants, especially OGA, the hydrolysis product of hemicellulose and pectin plays various roles in signal transduction of plant defense system. So, we investigated which one of galacturonic acids with various D. P. such as MGA, OGA, and PGA was specific to ethylene synthesis (Fig. 1). Among the PGA, OGA, and MGA derived from the component of cell wall, MGA and PGA had no influence on the ethylene biosynthesis, but OGA alone appeared to be effective in ethylene biosynthesis. The most effective concentration of OGA was 50 μg/mL. Time course data indicated that this sitimulation effect of OGA appeared after 90 min incubation period and continued until 24 h (Fig. 2).

Auxin is well known to stimulate the synthesis of ACC synthase which converts SAM into ACC. To find out if the stimulative effect of ethylene biosynthesis by OGA was a physiological phenomenon related to auxin-induced ethylene biosynthesis. The hypocotyl segments were incubated in Mes/Tris buffer containing IAA from 10^{-7} M to 10^{-3} M with or without 50 μg/mL OGA for 22 h (Fig. 3). Ethylene production increased rapidly form 0.82 nL to 1.4 nl as IAA concentration increase, which the treatment with OGA showed no synergisitc effect in ethylene syn-

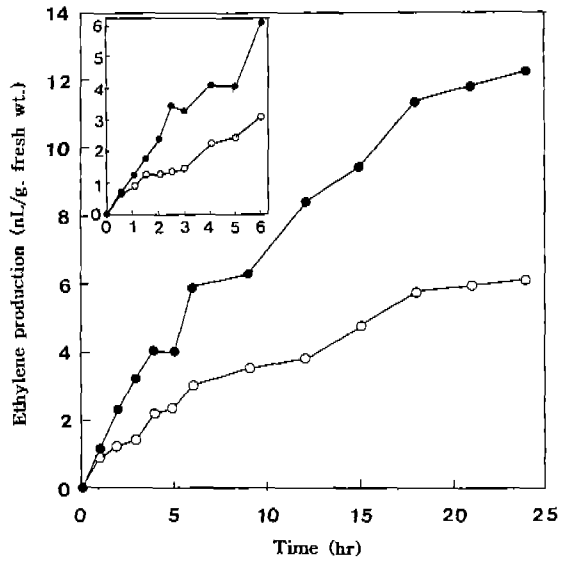

Fig. 2. Time course of ethylene production in mung beans hypocotyl segments with (●—●) or without (○—○) of 50 μg/mL OGA.

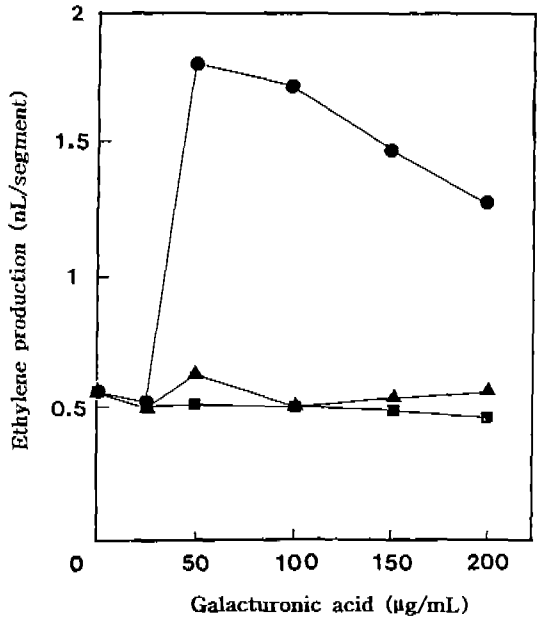

Fig. 1. Effect of galacturonic acids on ethylene production in mung beans hypocotyl segments (▲—▲: Monogalacturonic acid, ●—●: Oligalacturonic acid, ■—■: Polygalacturonic acid).

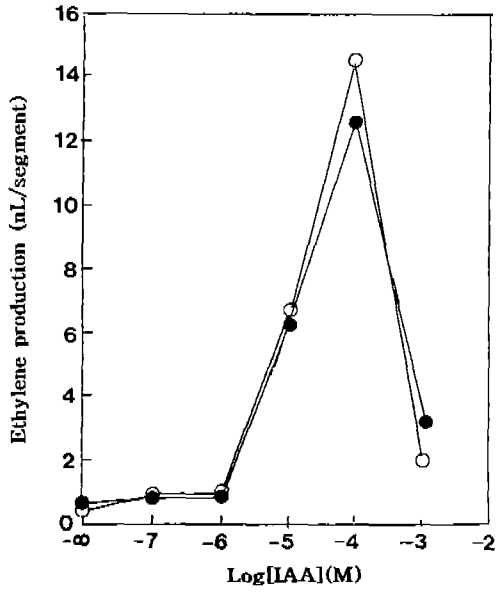

Fig. 3. Effect of OGA on IAA-induced ethylene production in mung beans hypocotyl segments. Segments were incubated with IAA in the presence of (●—●) or in the absence of OGA (50 μg/mL; ○—○) for 22 h.

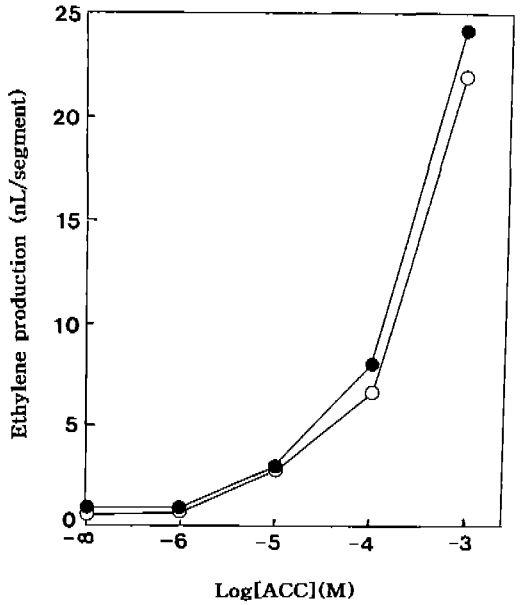

Fig. 4. Effect of OGA on ACC-induced ethylene production in mung beans hypocotyl segments. Segments were incubated with ACC in the presence of (●—●) or in the absence of OGA (50 μg/mL; ○—○) for 22 h.

thesis than IAA only. ACC, a precursor of ethylene, was treated at the every concentration level form 10^{-6} M to 10^{-3} M with or without 50 μg/mL OGA for 22 h (Fig. 4). As IAA, ACC had no influence on the ethylene biosynthesis by OGA. The results suggested that the ethylene synthesis pathway by OGA was different form that induced by IAA or ACC.

In order to confirm whether OGA had an influence on both ACC synthase and ACC oxidase *in vivo*, enzymes were partially purified from hypocotyl segments pretreated with or without 50 μg/mL OGA for 22 h. The activity of ACC synthase derived from tissue treated with OGA increased by 21-87% as the concentration SAM increases than that in absence of OGA (Table 1). But in case of the activity of ACC oxidase, OGA had a inhibitive effect by approximately 10-17% (Table 2). Thus we assumed that the stimulative effect of ethylene biosynthesis by OGA increased ACC synthase activity and in result, the amount of ACC increasesd enough to induce the ethylene biosynthesis. In the past, it was reported that the activity of ACC synthase, the concentration of ACC, and the production of ethylene was increase by wounding of the pericarp in unripened tomato fruits, and there were closely related among them (Yu and Yang, 1980). So, it is possbile for OGA to participate in plant defense system through stimulation of ethylene biosynthesis. In the

Table 1. Effect of OGA *in vivo* ACC synthase activity from mung beans hypocotyl segments. The activity of partial purified enzyme was measured after the 22 h incubation with or without OGA (50 μg/mL)

SAM (μM)	Specific activity (nmole/mg protein)		% of control
	Control	OGA	
1	0.63±0.16	0.76±0.23	121
3	1.26±0.27	2.28±0.19	181
5	2.23±0.31	4.24±0.38	190
10	2.73±0.41	4.96±0.31	182

*One unit of enzyme is the amount that liberates the formation of 1 nmole of ethylence catalyzing 10 μM SAM as substrate per 30 min under 30°C

Table 2. Effect of OGA on *in vivo* ACC oxidase activity in mung beans hypocotyl segments. The activity of ACC synthase was inhibited by 10^{-5} M AVG after the incubation with or without OGA (50 μg/mL) for 22 h

Time (min)	C_2H_4 production (nL/segment)		% of control
	Control	OGA	
30	1.12±0.17	0.93±0.09	83
60	1.56±0.13	1.40±0.12	90

Table 3. Effect of OGA on *in vitro* ACC synthase from mung beans hypocotyl segments. The activity of partial purified enzyme was measured with or without OGA (50 μg/mL) in the reaction mixture

SAM (μM)	Specific activity (nmole/mg protein)		% of control
	Control	OGA	
1	0.66±0.08	0.71±0.16	108
3	1.17±0.08	1.06±0.08	91
5	2.53±0.28	2.54±0.29	100
10	2.71±0.18	2.61±0.28	96

*One unit of enzyme is the amount that liberates the formation of 1 nmole of ethylene catalyzing 10 μM SAM as substrate per 30 min under 30°C.

mean time, to find out *in vitro* effect of OGA on the activity of ACC synthase, it was partially purified from tissue not treated with OGA (Table 3). *In vitro* activity of it, unlike Table 1, appeared to be about 91-108% of that in control, which meant there was no remarkable distinction. This indicated that OGA had no direct effect on the ACC synthase activity. In addition of above results, the phosphorylation of 34 kDa protein was stimulated by treatment of PGA derived form pectin in the cell wall of tomatoes and potatoes *in vitro*. But the concentration of PGA was lower than that of α-1,4-D-polyglucuronic acid (PGU) actually promoted the phosphorylation of protein, while β-1, 4-D-plymanuronic acid (PMU) had scarcely any effect on it at all. When the tomato

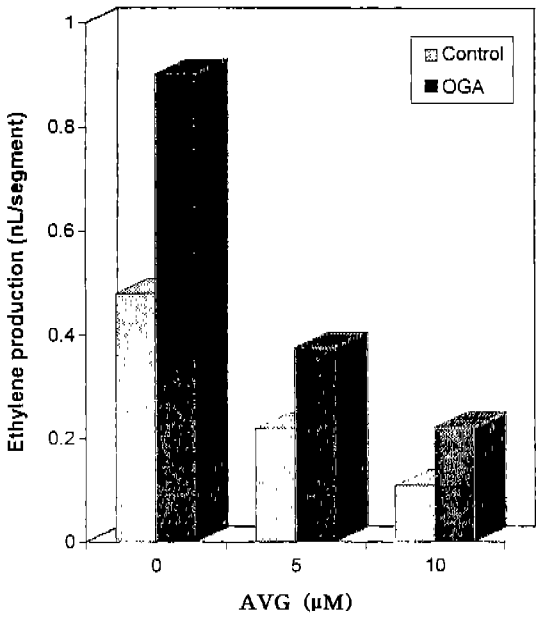

Fig. 5. Effect of AVG on OGA mediated ethylene production in mung beans hypocotyl segments incubated for 22 h.

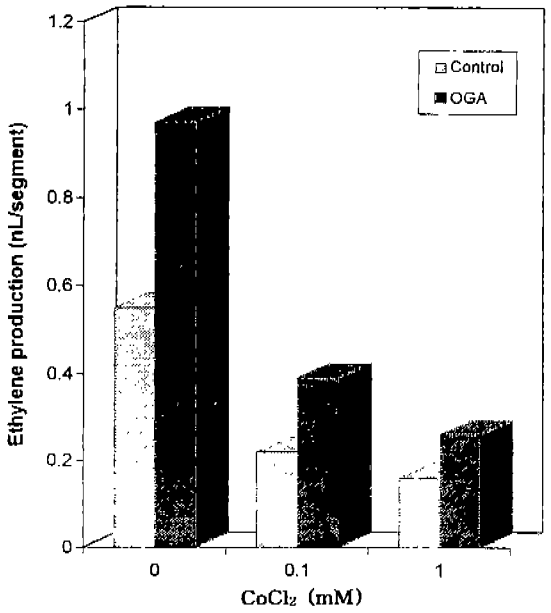

Fig. 6. Effect of $CoCl_2$ on OGA mediated ethylene production in mung beans hypocotyl segments incubated for 22 h.

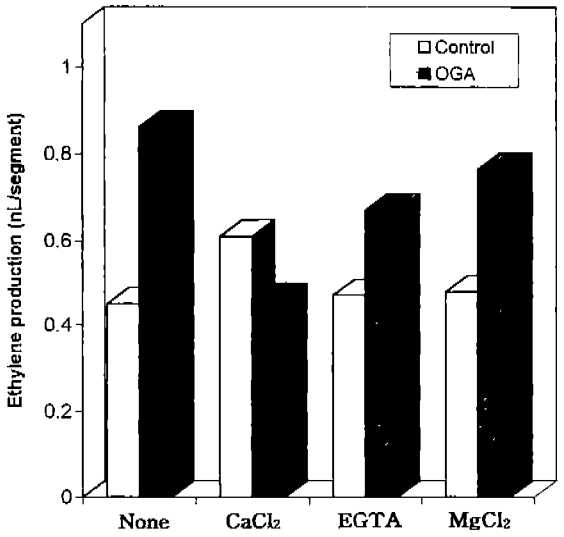

Fig. 7. Effect of $CaCl_2$, EGTA and $MgCl_2$ on OGA mediated ethylene production in mung beans hypocotyl segments incubated for 22 h (The concentration : 1 mM).

leaves was treated with PGA, PGU and PMU respectively, it was found the stimulation PI activity by PGA to be approximately twice as high as that of PGU, while in the case of PMU the activity was very weak (Farmer *et al.*, 1989; 1991). This results and reports indicate together that it is possible for OGA to act on the specific protein in plasma membrane and induce many physiological response including ethylene biosynthesis.

As the results above showed, OGA stimulated the ethylene biosynthesis in means of affecting ACC synthase, so the effect of OGA on the ethylene biosynthesis was examined in presence of inhibitors. Aminoethoxy vinyl glycine (AVG) is well known to inhibit ACC synthse activity; likewise, Co^{2+} is known to inhibit the conversion of ACC to ethylene. As in Fig. 5. cotreatment of AVG with OGA rather increased the ethylene synthesis compared to the treatment only of AVG. Co^{2+}, another inhibitor act on at the level of ACC oxidase, showed the same pattern as AVG treatment (Fig. 6).

Calcium ions that largely accumulate in the cell wall/cell membrane is reported to increase ACC synthesis. Also, the minimum D. P. size of PGA required to activate plant defense response is almost same as the size of PGA bound strongly with Ca^{2+} (Ryan, 1987; Farmer *et al.*, 1991). When OGA was treated with Ca^{2+}, ethylene synthesis rather decreased in comparison with the treatment of only I mM Ca^{2+} (Fig. 7). That meant that OGA might be interact with Ca^{2+}, to decrease the ethylene production. Because pectin in primary cell wall linked each other with the help of Ca^{2+}, it is known to isolate by Ca^{2+}-chelating agents EGTA. The EGTA treatment with OGA showed the same pattern of ethylene biosynthesis as the treatment of only OGA. Also, the treatment of other divalent cation, Mg^{2+}, confirmed that the interaction between OGA and Ca^{2+} was specific for ethy-

lene synthesis. This results indicated that physiological response including ethylene synthesis was reglulated by means of the interaction OGA with Ca^{2+}.

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