*Journal of Plant Biology*, September 2002, 45(3) : 148-153

# Brassinosteroids Affect Ethylene Production in the Primary Roots of Maize (*Zea mays* L.)

**Sun Hwa Lim[1], Soo Chul Chang[2], June Seung Lee[3], Seong-Ki Kim[4], and Soon Young Kim[1]***

[1]Department of Biological Sciences, Andong National University, Andong 760-749, Korea
[2]Center for Cell Signaling Research, Ewha Woman's University, Seoul 120-750, Korea
[3]Department of Biological Science, Ewha Woman's University, Seoul 120-750, Korea
[4]Department of Life Science, Chung-Ang University, Seoul 156-756, Korea

**To better understand the physiological roles of brassinosteroids (BRs) in the primary roots of maize, we examined their effect on ethylene production. Exogenously applied brassinolide (BL; $10^{-9}$ to $10^{-7}$ M) incrementally increased the level ethylene in a dose-dependent manner. This BL-induced production was enhanced in the presence of IAA, thereby implying a synergistic effect between BR and IAA. At $10^{-7}$ M BL, the level of free ACC was increased, but that of conjugated ACC was diminished. Moreover, greater concentrations of BL proportionally increased ACC oxidase activity. In contrast, higher levels of IAA increased the endogenous content of conjugated ACC as well as ACC synthase activity. Based on these results, we conclude that BR activates ethylene production mainly via ACC oxidase, and interacts with IAA to produce ethylene. However, the functional site for ethylene production is different for each hormone.**

*Keywords*: ACC oxidase, ACC synthase, brassinosteroid, ethylene production, maize root

Brassinolide (BL), a known regulator of plant growth and development, was first isolated from *Brassica napus* pollen by Grove et al. (1979). This compound shows remarkable biological activity in the bean second-internode assay. BL has a steroidal structure and apparently hormonal properties. A number of other steroidal compounds related to BL have now been isolated from a variety of plant tissues, and are referred to collectively as brassinosteroids (BRs). Because these BRs are found throughout the plant kingdom, regulating growth and development at low concentrations, they are considered a sixth class of plant hormones (Sasse, 1990; Sakurai and Fujioka, 1993; Yokota, 1997).

BRs stimulate hypocotyl elongation (Wang et al., 1993), induce epinasty (Schlagnhaufer and Arteca, 1985), and increase pollen-tube elongation (Hewitt et al., 1985). In addition, they have been shown to inhibit root elongation in *Arabidopsis* (Clouse et al., 1993), while stimulating both root elongation and adventitious rooting in wheat (Braun and Wild, 1984). These effects might be correlated with the action of ethylene, a gaseous plant hormone that plays an important role in many physiological phenomena such as epinasty, senescence and ripening, and the inhibition of elongation in plant organs.

Yang and Hoffman (1984) have described the pathway of ethylene production from methionine via two major intermediates, s-adenosylmethionine (AdoMet) and ACC. Two enzymes, ACC synthase (ACS) and ACC oxidase (ACO), control this process, with ACS regulating the formation of ACC from AdoMet, and ACO being involved in the conversion step of ACC to ethylene.

BR has been shown to stimulate ethylene production in mung bean hypocotyls (Arteca et al., 1983), and to induce an epinastic response in tomato plants (Schlagnhaufer and Arteca, 1985). This epinasty is inhibited by the addition of aminooxyacetic acid and cobalt ions, known inhibitors of ethylene production. Arteca and Bachman (1987) demonstrated that treatment of mung bean hypocotyls with 2 μM BR to increase ethylene levels was as effective as applying 10 μM IAA. Moreover, Arteca et al. (1988) found that the synergistic effect between auxin and BR was mediated by ACC synthase. When combined with auxin, BR also had caused a similar response in the bending of the second leaf lamina in rice (Arteca and Bachman, 1987). In another study, BRs increased the IAA level by about 50% within 3 h in squash hypocotyl segments (Eun et al., 1989). Low concentrations of BRs (e.g., 2 nM) significantly increased the fresh weight of the segments. However, Eun et al. (1989) did not suggest that this BR-mediated increase in IAA might involve ethylene stimulation.

Arteca et al. (1985) reported that the hydroxyl

*Corresponding author; fax +82-54-841-1627
e-mail kimsy@andong.ac.kr

groups of C-22 and C-23 in BR are necessary for activating ethylene production in etiolated mung bean hypocotyl segments. Furthermore, the change from an α- to a β-conformation on C-22, C-23, and C-24 reduced ethylene activity at lower BR concentrations. However, at higher concentrations, these conformational changes did not differ. Therefore, Arteca et al. (1985) proposed that all the tested BRs could stimulate ethylene production synergistically with IAA at lower concentrations, and that the BRs might require endogenous auxin for this activity.

The question remains whether BRs require IAA for exerting an influence on maize roots. Most research has been focused on their effects in the hypocotyls or stems. Evidence exists for a BR role in a plant's physiological response when ethylene production is controlled simultaneously. Kim et al. (2000) have shown that BRs stimulate a gravitropic response in the presence of auxin in maize roots. However, no evidence has been found for the regulation of ethylene production in roots. Therefore, in this study, we investigated the effect of BR in the primary roots of maize, as well as any synergistic effect with auxin. In addition, we focused on the action of BRs in the ethylene production pathway by measuring the activities of ACC synthase and ACC oxidase in the primary roots.

## MATERIAL AND METHODS

### Plant Material

Maize seeds (*Zea mays* L. Golden x Bantam 70) were soaked overnight in tap water, then germinated upright between wet paper towels in opaque plastic trays. The trays were kept in a dark growth chamber at 27°C. We used two-day-old seedlings with primary roots that were about 1.5 to 2.0 cm long.

### Measurement of Ethylene Production

Ethylene production was measured in silicon-capped vials that contained 1 mL of potassium phosphate buffer (0.05 M; pH 6.8) and 40, 1.0-cm root segments. A gas chromatograph (Donam System, DS 6200, Korea) was used. The vials were held in the dark at 27°C in a shaking incubator.

### Assay of ACC Oxidase (ACO) Activity

The analysis of ACC oxidase activity was carried out in-vivo, following a modified method of Wang and Woodson (1989). Forty root segments were placed in a potassium phosphate buffer (0.05 M; pH 6.8) containing 0.1 mM aminoethoxyvinylglycine (AVG), with or without BR. After incubation, the segments were washed with distilled water, then infiltrated with 1 mM ACC in the dark for 2 h at 27°C. After washing the segments, ACO activity was determined by measuring their ethylene production for 1 h in distilled water.

### Assay of ACC Synthase (ACS) Activity

The assay method for ACS activity was modified from that of Woeste et al. (1999). Root segments (4 g) were ground on ice with 8 mL of 250 mM potassium phosphate buffer (pH 8.0) that contained 10 μM pyridoxal phosphate, 1 mM EDTA, 2 mM PMSF, and 5 mM DTT. Samples were kept for 4 min on ice, then centrifuged at 15,000*g* for 15 min. The supernatant (1 mL) was incubated for 1 h with 5 mM AdoMet (100 μL) at 22°C. Ethylene production measured from this mixture was used for calculating ACS activity, using a blend of 100 μL of 20 mM $HgCl_2$ and 100 μL of NaOH/NaOCl that was incubated on ice for 10 min (Lizada and Yang, 1979).

### Measurement of Free and Conjugated ACC

Both free and conjugated ACC amounts were measured by following a modified method of Petruzzelli et al. (2000). Root segments (8 g) were ground on ice with 15 mL of 80% (v/v) ethanol. The extract was then centrifuged at 12,000*g* for 15 min, and the supernatant was dried in a centrifugal evaporator. The residue was then dissolved in 1 mL of distilled water. Total ACC content was measured according to a modified method of Lizada and Yang (1979).The content of conjugated ACC was determined by incubating the residue solution for 3 h in 2 M HCl at 100°C, followed by neutralization with saturated NaOH and centrifugation. The amount of conjugated ACC was then defined as the difference between the free and the total ACC contents.

## RESULTS AND DISCUSSION

BRs have been shown to increase auxin-induced ethylene production in mung bean hypocotyls or stems (Arteca et al., 1983; Arteca and Bachman, 1987), but no reports had been made of their effects on root tissue. In our study of the primary roots of maize, we treated the segments with levels of BL ranging from

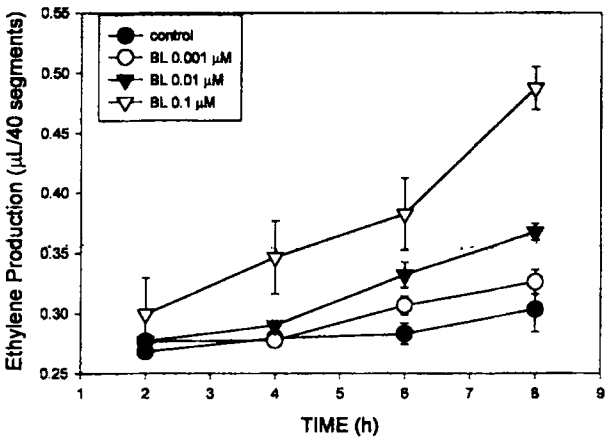


**Figure 1.** Kinetics of ethylene production from primary root segments of maize in the presence of various concentrations of BL (0.001 μM - 0.1 μM). Ethylene production was measured as described in Materials and Methods. Symbols are mean values ± SE from five independent experiments.

0.001 μM to 0.1 μM (Fig. 1). At the highest concentration, ethylene production was stimulated to about 60% of that measured from the control following an 8-h incubation period. Stimulation at lower concentrations began after only 4 h of incubation. In the 8-h incubation experiment, the amount of ethylene produced was in dose-dependent.

Although we found no evidence that BRs alone induced ethylene production in the roots, we do suggest, based on these results, that BL could regulate production in the absence of auxin. Kim et al. (2000) have reported that BL alone is involved in the gravitropism of maize roots. In fact, gravicurvature was increased, especially when tissues were treated with 0.1 μM BL. The degree of increase in curvature was dependent on BL concentration, an observation similar to what we observed with ethylene production. Likewise, Schlagnhaufer and Arteca (1985) have demonstrated that BR-induced epinasty in tomato plants is regulated by treatment with inhibitors of ethylene biosynthesis. Such physiological phenomena as gravitropism and epinasty are correlated with the action of ethylene (Abeles et al., 1992). Therefore, some physiological actions of BL (Schlagnhaufer and Arteca, 1985; Kim et al., 2000) might be mediated with ethylene production.

Nonetheless, BL can also induce some biological activities in the presence of auxins, perhaps synergistically (Arteca and Bachman, 1987; Yi et al., 1999). For example, BL may be involved in IAA-mediated gravitropism in maize roots. Kim et al. (2000) showed that

**Table 1.** The synergistic effect of IAA on BL-induced ethylene production in maize root segments. Roots were incubated with or without IAA in various concentrations of BL. Ethylene production was measured after 8 h incubation. Values are percentage (%) of control, and are the means of five replications ± SE.

| | Concentrations of BL (μM) | | | |
|---|---|---|---|---|
| | 0 | 0.001 | 0.01 | 0.1 |
| Control | 100 ± 0.2 | 110 ± 0.1 | 123 ± 0.1 | 163 ± 0.2 |
| 0.1 mM IAA | 198 ± 0.4 | 237 ± 0.5 | 277 ± 0.8 | 315 ± 0.2 |

the influence of BL on gravitropic curvature was significantly increased with IAA; this response was decreased by treatment with an antagonist of auxin activity. We also noted that auxin stimulated ethylene production in our primary root segments (data not shown). As shown in Table 1, separate treatments with 0.1 μM BL and 0.1 mM IAA increased production by up to 63% and 98%, respectively. However, when 0.1 mM IAA and 0.1 μM BL were applied simultaneously to the root segments, the level of production increased by 215%, rather than by only a hypothetical 161% (63% + 98%). That is, the combined effect of the two hormones was greater than the sum of the individual BL and IAA effects. This enhancement was also observed when either 1 nM BL (10% + 98% vs. 137%) or 10 nM BL (23% + 98% vs. 177%) was applied to the roots in the presence of IAA. Therefore, we suggest that BL and IAA act synergistically on ethylene production in maize roots.

Auxin can stimulate the manufacture of ethylene during the conversion step of AdoMet to ACC, which is regulated by ACS (Abeles et al., 1992). This results in an increase of endogenous ACS in the plant tissue. We examined the effect of BL on this enzymatic process to better understand its action in the ethylene production pathway. ACS activity was stimulated significantly by the treatment of 0.1 μM BL only after 8 h of incubation (Fig. 2). However, lower BL concentrations had no effect during this time frame. Indeed, BL-mediated formation of ACC from AdoMet increased after 8 h only at the 0.1-μM treatment level.

Schlagnhaufer et al. (1984) reported that the promotion of ethylene production by BRs in mung bean hypocotyls was caused by an increase in ACC. Furthermore, Arteca et al. (1988) suggested that the synergistic effect of BR with IAA on ethylene in mung bean hypocotyls was due to stimulation of ACS activity, although the kinetics differed between IAA and BR. That is, IAA rapidly increased ACS levels, while the effect of the BR exhibited a 12-h lag. Only the highest

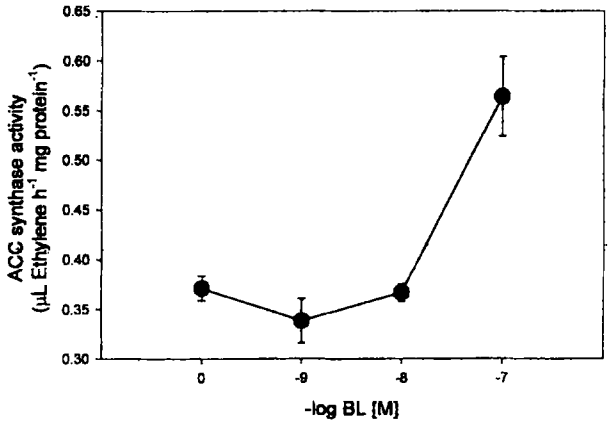


**Figure 2.** In-vitro ACS activity in the presence of various concentrations of BL (0.001 μM - 0.1 μM) in primary root segments of maize. ACS was assayed from crude extract of roots incubated for 8 h in various BL concentrations. Activity was defined as by the amount of ACC formed by converting it to ethylene, as described in Materials and Methods. Symbols are mean values ± SE from seven independent experiments.

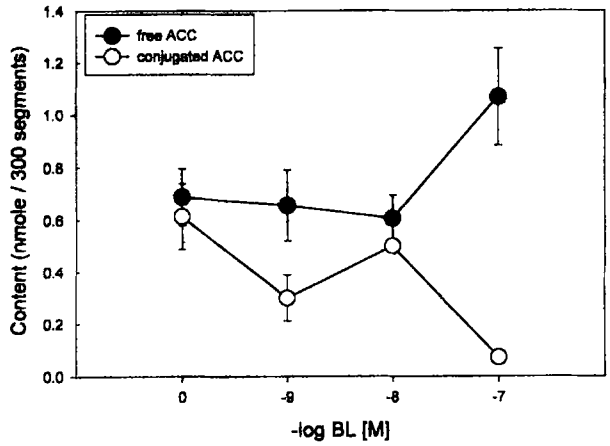


**Figure 3.** Effect of BL on free ACC and conjugated ACC in primary root segments of maize. Roots were incubated in various concentrations of BL for 8 h. Amounts of free and conjugated ACC were measured according to the amount of ACC converted to ethylene. Symbols are mean values ± SE from five independent experiments.

concentration of BL (0.1 μM) stimulated ACS activity after 8 h of incubation (Fig. 2). The focus of earlier ACS research was on hypocotyls rather than roots. Therefore, we suggest that the objective of future experiments should be to explain the regulation by BL of ACS activity in several plant tissue types and over various incubation periods.

Yi et al. (1999) have reported that BRs synergistically increased the transcript levels of IAA-induced *VR-ACS6* and *VR-ACS7* in the mung bean, and have suggested that expression of ACS is controlled by the multiple regulatory pathways of auxin and BR. Based on those results, we are currently conducting gene cloning experiments of the ACS and ACO genes in maize roots. Future research at the molecular level in roots should also help elucidate the role of BL in various other plant tissues. It is, of course, possible that the level of ACS is lower in roots than in hypocotyls.

We measured the contents of both free and conjugated ACC associated with various BL concentrations after 8 h of incubation (Fig. 3). Free ACC increased by 40% versus the control in the 0.1 μM BL-treated roots. Other concentrations of BL did not increase the free ACC contents as much as we had expected based on the results shown in Figure 2. Conjugated ACC content, although not decreasing, did not increase as much as the free ACC (Fig. 3). In several tissue types, ethylene production can be regulated to form conjugated ACC, an inactive form; its major form is 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC) (Amrhein et al., 1981; Peiser and Yang, 1998). Ethylene increases the level of malonyltransferase, which accounts for the autoinhibition of ethylene production (Liu et al., 1985). Likewise, Riov and Yang (1989) have demonstrated that IAA causes a steady increase in the MACC content of the mung bean during a transient increase in both ACC formation and ethylene production. Our decreasing content of conjugated ACC (Fig. 3) might indicate that the action of BL is not the same when combined with IAA. This means that BL might affect only the action of ACC formation, but not that of conjugated ACC.

The last step in ethylene production is the conversion of ACC to ethylene, which is mediated by ACO. Figure 4 shows the effect of BL on in-vivo ACO activity over an 8-h incubation period. This activity increased in proportion to the concentration of BL tested, a relationship similar to that seen between BL concentration and the level of ethylene production. Here, the ratio of enzymatic activity to BL concentration was 30% of the control at the 0.1-μM level. Based on these results, we suggest that the stimulation of ethylene production by BL might be due to the increase in ACO activity in maize root segments.

Kim et al. (2000) reported that the endogenous BR isolated from maize seedlings might be a castasterone (CS). Here, we tested the effect of CS on ethylene in root segments (Fig. 5), and found that production increased in a dose-dependent manner. However, this level of stimulation by CS was lower than that

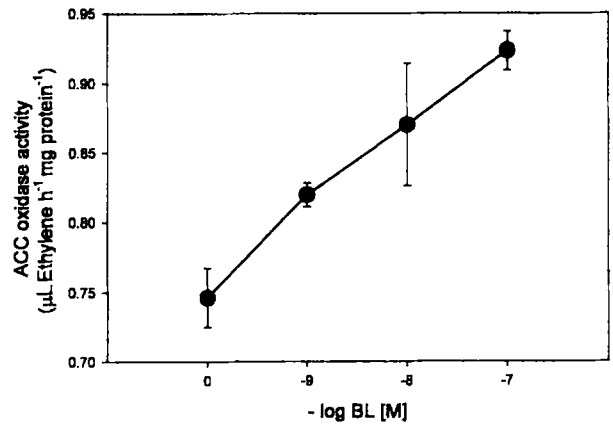


**Figure 4.** In-vivo ACO activity in the presence of various concentrations of BL (0.001 μM - 0.1 μM) in primary root segments of maize. ACO was assayed from intact root segments incubated for 8 h in 0.1 mM AVG with various concentrations of BL. Afterward, 1 mM ACC was infiltrated for 1 h. ACO activity was determined from the amount of ethylene production from these root segments. Symbols are mean values ± SE from five independent experiments.

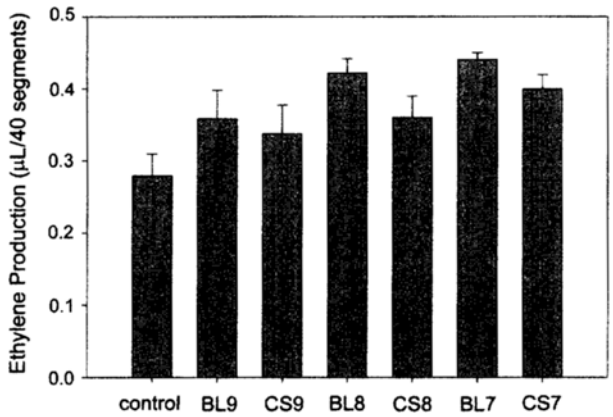


**Figure 5.** Effects of BL and CS on ethylene production in primary root segments of maize. Roots were incubated in potassium phosphate buffer (0.05 M, pH 6.8) containing various concentrations of BL or CS for 8 h; measurements of ethylene production are described in Materials and Methods. BL9, 0.001 μM BL; CS9, 0.001 μM; BL8, 0.01 μM BL; CS8, 0.01 μM CS; BL7, 0.1 μM BL; CS7, 0.1 μM CS. Symbols are mean values ± SE from three independent experiments.

induced by BL (Fig. 5). CS, a 6-keto derivative of BL, shows less biological activity than that of BL in BR bioassays. In addition, CS has been identified in some plants that do not contain BL (Kim, 1991), which suggests that it is either a precursor or a kind of active BL, depending on the species. In our study, exogenous applications of BL stimulated ethylene production more than did CS, especially at higher concentrations, e.g., 0.01 and 0.1 μM. We propose, therefore, that a structure-activity relationship for BR is necessary to promote ethylene production in maize roots. In addition, Kim et al. (2000) showed that BL might be the active form of BR in the primary roots of maize, even though only endogenous CS was found in that tissue. Perhaps, although BL may show stronger activity than CS, the the level of the former may be too low to be detected.

We conclude that BL alone can increase ethylene production in the primary roots of maize, a result of increased ACO activity. Likewise, ACS activity and the internal, free-ACC contents are affected only at a high concentration of BL (i.e., 0.1 μM). This BL-induced ethylene production is also increased synergistically with IAA.

## ACKNOWLEDGMENT

This study was supported by a grant from Andong National University to S.Y. Kim.

Received April 19, 2002; accepted July 2, 2002.

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