Involvement of Brassinosteroids in the Gravitropic Response of Primary Root of Maize[1]

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Exogenously applied brassinolide (BL, 10^{-9}–10^{-5} M) increased gravitropic curvature in maize (*Zea mays*) primary roots. The BL-enhanced gravitropic curvature was clearly promoted in the presence of indole-3-acetic acid (IAA, 10^{-10}–10^{-8} M), indicating that BL is interactive with IAA during the gravitropic response. The interactive effect between BL and IAA was completely diminished by treatment of *p*-chlorophenoxy isobutric acid, an auxin action antagonist. The activation of the gravitropic response by BL in the absence and in the presence of IAA was nullified by application of 2,3,5-triiodobenzoic acid, a polar auxin transport inhibitor. The data indicate that brassinosteroids (BRs) might be involved in auxin-mediated processes for the gravitropic response. Gas chromotography-selected ion-monitoring analysis revealed that maize primary roots contained approximately 0.3 ng g^{-1} fresh weight castasterone as an endogenous BR. Exogenously applied castasterone also increased the gravitropic response of maize roots in an IAA-dependent manner. This study provides the first evidence, to our knowledge, for occurrence and gravitropic activity of BRs in plant roots.

Since brassinolide (BL) has been identified as a plant growth promoting substance in rape pollen (Grove et al., 1979), over 40 members of related steroids, collectively named as brassinosteroids (BRs), have been characterized in the entire plant kingdom (Kim, 1991; Fujioka, 1999). Early studies investigated possible physiological roles of BRs by exogenous application. The results of those studies suggested that BRs might be involved in the regulation of cell elongation and division, leaf bending, reproductive and vascular development, membrane polarization and proton pump, source/sink definition, and modulation of stress (for review, see Yokota and Takahashi, 1986; Mandava, 1988; Sakurai and Fujioka, 1993; Arteca, 1995; Sasse, 1997, 1999; Yokota, 1997; Clouse and Sasse, 1998). Recent studies using BR-deficient Arabidopsis, pea, and tomato mutants revealed that BR-deficiency caused abnormal pleiotropic developments such as reduced shoot elongation (dwarfism), reduced fertility, delayed senescence, and altered vasculature. These mutants can be rescued only by application of BRs (for review, see Yokota, 1997; Clouse and Sasse, 1998; Clouse and Feldmann, 1999). Thus, BRs are now regarded to be essential substances for growth and development of plants.

The occurrence of BRs has been demonstrated in almost every aerial part of plants such as pollen, flower, shoot, vascular cambium, leaf, fruit, and seed (Kim, 1991; Fujioka and Sakurai, 1997; Fujioka, 1999). It is possible that because of very low concentrations, however, the presence of BRs in roots has not yet been demonstrated. Nevertheless, exogenously applied BRs inhibited primary root extension and lateral root formation, and they occasionally promote elongation and adventitious rooting at less than 1 pM of BRs (Roddick and Guan, 1991; Clouse et al., 1993, 1996; Sasse, 1994; Fujioka and Sakurai, 1997). In *Lotus japonicus*, treatment with uniconazole induced stunted lateral roots, but simultaneous treatment of BR reduced the number to the control value, suggesting that endogenous BRs may regulate initiation of lateral roots (Kawaguchi et al., 1996). Recently, a BR-insensitive mutant, *bri1*, has been isolated from mutagenized Arabidopsis plants in which root elongation was not inhibited by BRs, but by other plant hormones, auxin, gibberellin, cytokinin, abscisic acid, and ethylene, similar to the wild type (Clouse et al., 1996). These results indicate that BRs also have important regulatory functions in growth and development of plant roots.

Gravitropism is the directional movement of a plant in response to the stimulus of gravity. When a seedling is placed in the horizontal position, the primary root exhibits a downward curvature, known as positive gravitropism, whereas the primary shoot shows upward curvature, known as negative gravitropism. The gravitropic curvature of both gravitropisms is a consequence of differential cell elongation on opposite sides of the organ (root or shoot), which

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is believed to be mediated by an auxin gradient caused by a redistribution of auxin across the organs (Chen et al., 1999). Some responses induced by BRs seem to be similar to those induced by auxin. Some auxin-induced responses are synergistically enhanced by BRs, indicating that both hormones are interactive in some aspects of plant growth and development (Yopp et al., 1981; Takeno and Pharis, 1982; Cohen and Meudt, 1983; Katsumi, 1985; Eun et al., 1989; Kim et al., 1990; Fujii et al., 1991; Fujioka et al., 1998; Sasse, 1999). Meudt (1987) has reported that the gravitropic curvature of bean hypocotyls (negative gravitropism) is enhanced by the application of BR. The enhancement of the gravitropic response by BR was also demonstrated in tomato hypocotyls (Park, 1998), indicating that BRs might participate in the regulation of shoot gravitropism. These findings suggested that BRs may also be involved in the regulation of root gravitropism and led us to examine the effects of BRs on gravitropic response in maize (*Zea mays*) primary roots, a system whose gravitropic response has been well characterized. In this work, we found that BRs increased indole-3-acetic acid (IAA)-induced gravitropic response. We also demonstrated the occurrence of BR in the maize primary roots, which provides the first evidence for the presence of BR in plant roots.

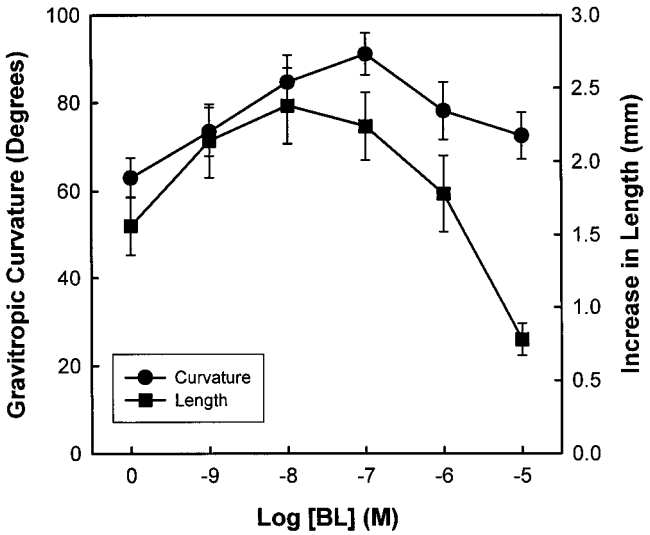

Figure 1. Dose response for the effect of BL on gravitropic response and elongation in maize primary roots. For gravitropic response experiments, the roots were pre-incubated in 5 mM MES-Tris (pH 6.8) containing various concentrations of BL for 2 h and placed horizontally for 4 h at 25°C ± 1°C for gravistimulation treatment. The pre-incubation and gravistimulation treatment was performed in a water-saturated chamber. In growth experiments, the roots were pre-incubated and treated as in gravitropic response experiments, except that roots were positioned vertically during treatment. All experiments were undertaken at 25°C ± 1°C with aeration in the dark. Values in the figure represent the means ± SE of five replicates.

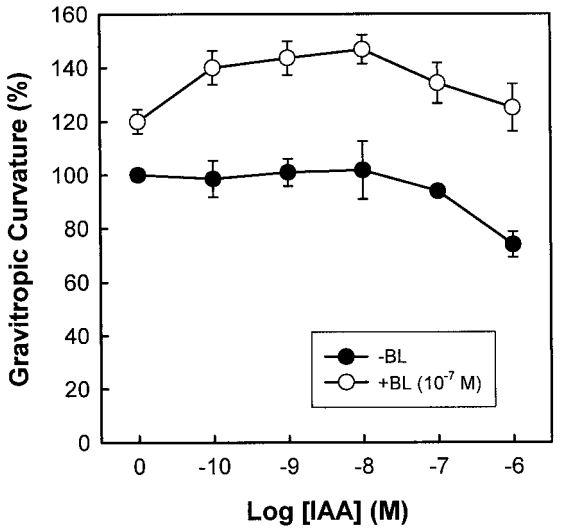

Figure 2. Dose response for the effect of IAA on gravitropic curvature in maize primary roots in the presence or absence of 10^{-7} M BL. The roots were pre-incubated in a solution of 5 mM MES-Tris (pH 6.8) containing various concentrations of IAA with or without 10^{-7} M BL for 2 h, and placed horizontally for 5 h at 25°C ± 1°C for gravistimulation treatment. Values in the figure represent the means ± SE of five replicates.

RESULTS

Effects of BL on Elongation and Gravitropic Curvature

Dose response of BL for elongation and gravitropic curvature of maize primary roots was investigated at concentrations between 10^{-9} and 10^{-5} M. The gravitropic curvature was increased by BL at all concentrations tested and was greatest at 10^{-7} M (Fig. 1). In contrast, elongation of the roots was increased by BL from 10^{-9} to 10^{-7} M, with the maximum increase at 10^{-8} M BL. Concentrations of BL over 10^{-6} M inhibited elongation. The fact that BL shows differential dose response between elongation and gravitropic response in maize roots indicates that BL may control and/or affect both events separately.

Interaction of BL and IAA in Gravitropic Curvature

It is well known that IAA is involved in root gravitropic response (Chen et al., 1999) and that BR interacts with IAA. The results illustrated in Figure 1 prompted us to investigate whether BR interacts with IAA during the gravitropic response. IAA alone showed no effect at concentrations less than 10^{-8} M, but showed inhibitory effects at 10^{-7} and 10^{-6} M IAA. In the presence of 10^{-7} M BL, which exhibits the strongest stimulation of gravitropic response, significant promotion of gravitropic curvature was found at 10^{-10} to 10^{-8} M IAA (Fig. 2). Moreover, the reduced gravitropic curvature at 10^{-7} IAA was clearly reversed to levels that exceeded control value in the presence of BL. Taken together, it is suggested that

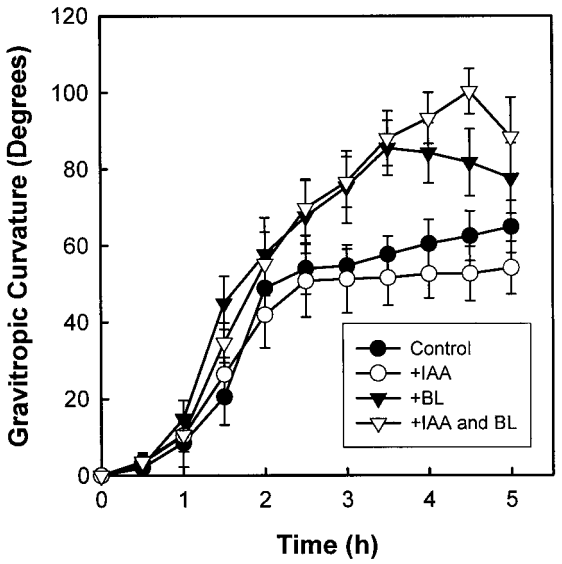

Figure 3. Time-course analysis of gravitropic curvature in the presence and absence of IAA and BL. The primary roots were pre-incubated in solutions of 5 mM MES-Tris (pH 6.8) containing no hormone (control), 10^{-8} M IAA, 10^{-7} M BL, or 10^{-8} M IAA plus 10^{-7} M BL for 2 h and then placed horizontally at 25°C ± 1°C. Values in the figure represent the means ± SE of five replicates.

BL and IAA might have an interactive effect on the gravitropic response.

The time-course analysis of gravitropic response in the presence of BL and/or IAA shows that IAA (10^{-8} M) alone slightly reduced gravitropic curvature compared with untreated controls from 2 h after gravistimulation (Fig. 3). BL (10^{-7} M)-increased gravitropic response is evident from 2 h after the horizontal orientation. Combined application of IAA and BL shows increased gravitropic curvature similar to BL alone for the first 3 h of graviresponse. Thereafter, the curvature continues over the level of BL alone. Therefore, BL increases the gravitropic curvature of maize roots in the presence of low levels of exogenously applied IAA, as well as normal IAA levels.

The order of treatment of IAA and BL was important for showing synergistic activity in several bioassays (Takeno and Pharis, 1982; Cohen and Meudt, 1983; Katsumi, 1985; Mandava, 1988; Kim et al., 1990). Thus, the order of both hormone treatments on the gravitropic response was investigated. However, significant difference at 5 h after the start of gravistimulation treatment was observed, irrespective of the order of treatments (data not shown).

The stimulatory effect of BL on the gravitropic response in the presence of IAA indicates that BRs might be involved in IAA-mediated gravitropic processes in the maize roots. To confirm that notion, the influence of antiauxins on the gravitropic response promoted by BL was investigated. *p*-Chlorophenoxy isobutric acid (*PCIB*), an auxin action antagonist, decreased the root gravitropic curvature and diminished the interactive effect between IAA and BL on the gravitropic response (Fig. 4A). 2,3,5-triiodobenzoic acid (TIBA), an auxin transport inhibitor, greatly delayed the gravitropic curvature regardless of BL and/or IAA treatments (Fig. 4B). These results strongly indicate that BL exerts its role through auxin effects and auxin transport is essential for the regulation of the root gravitropic response by BL.

Identification of Castasterone from Maize Primary Roots

The results described above prompt the question of whether BRs exist in maize roots. Thus, we examined the presence of BRs in maize primary roots. BRs in 3-d-old whole seedlings (150 g) were preliminarily analyzed by gas chromatography-mass spectrometry (GC-MS) after purifying as described in "Methods and Materials." As summarized in Table I, bismethaneboronate (BMB) of an endogenous BR showed prominent ions at *m/z* 512, 497, 399, 358, 327, 287, and 155, which are identical to those of castasterone (CS) BMB. On GC, the retention time (R_t) of BMB of the BR (16.45 min) was exactly the same as that of authentic CS BMB. Thus, the endogenous BR in maize seedlings was determined to be CS. Coexistence of teasterone, typhasterol, dolichosterone, and 28-norCS with CS has been demonstrated in different cultivars of maize (Suzuki et al., 1986; Gamoh et al., 1990;

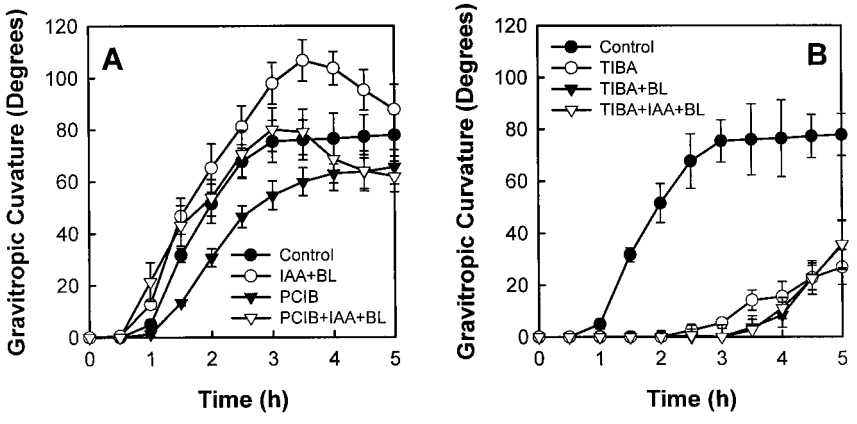

Figure 4. Effects of antiauxins, PCIB (A) or TIBA (B) on gravitropic curvature induced by BL in the presence or absence of IAA in maize primary roots. The roots were pre-incubated in 5 mM MES-Tris (pH 6.8) containing BL (10^{-7} M) or BL plus IAA (10^{-8} M) in the presence or absence of PCIB ($10^{\text{minus}]6}$ M) and TIBA (10^{-5} M) for 2 h, and placed horizontally for 5 h at 25°C ± 1°C. Values in the figure represent the means ± SE of five replicates.

Table I. *GC-MS data for an endogenous BR in 3-d-old seedlings of maize and authentic CS*

Compound[a]	R_t on GC	Prominent Ions
	min	*m/z, relative intensity*
Endogenous BR	16.45	155 (100), 287 (48), 327 (9), 358 (40), 399 (23), 497 (5), 512 (82)
Authentic CS	16.45	155 (100), 287 (32), 327 (6), 358 (31), 399 (13), 497 (5), 512 (74)

[a] The sample was analyzed by a derivative of bismethaneboronate.

Sekimoto et al., 1997). However, no trace of these BRs was detected in the seedlings.

Thus, CS in primary roots of the seedlings was quantified by GC-selected ion monitoring (SIM) using deuterium-labeled ([26,28-$^2H_6$]) CS as an internal standard. As shown in Figure 5, ions at *m/z* 512 and 155, due to BMB of CS, were detected at the same R_t (16.45 min) as those of the authentic specimen. Thus, the presence of CS in primary roots of maize was unequivocally demonstrated. The endogenous level of CS in the roots was determined on the basis of the deuterated standard being approximately 0.3 ng g^{-1} fresh weight roots.

Effect of Other BRs on Gravitropic Response in Maize Primary Roots

The identification of CS as an endogenous BR prompted us to investigate its gravitropic activity in the maize primary roots. As shown in Figure 6, CS also increased the gravitropic curvature, and the CS-induced gravitropic response was also stimulated by IAA. However, the CS-induced gravitropic response was lower than that induced by BL. This suggested that there is a structure-activity relationship of BRs in the gravitropic response of maize primary roots. Tyhasterol and 6-deoxo-CS, direct precursors of CS (Yokota, 1997; Clouse and Sasse, 1998), are hard to detect in the rice lamina inclination assay that is known to the most sensitive assay for BRs. Thus, the possibility for detection of these BRs in gravitropic response of maize roots was investigated. Unfortunately, no significant increments of gravitropic response by either BR was observed, regardless of additional application of IAA or not.

DISCUSSION

The role of BRs in gravitropic response has been demonstrated in shoots such as hypocotyls of bean and tomato (Meudt, 1987; Park, 1998). In both plants, however, BR-enhanced gravitropic response in the presence of added auxin has not been clearly detected. In contrast, current study shows that the gravitropic response induced by BRs was clearly enhanced in the presence of auxin in maize primary roots. Moreover, the enhanced gravitropic curvature in the presence of auxin was diminished by application of antiauxins, PCIB and TIBA. These results indicate that there are differential action mechanisms of BRs between shoot and root gravitropic response, and that root gravitropism is a complex process in terms of mode of action of BRs.

BL displayed its stimulatory effect in the presence of IAA (Fig. 2), indicating that BL increases the sensitivity of maize roots to IAA. BL enlarged the IAA dose response. Therefore, it can be argued that BL increases overall capacity of the root to the number of occupied IAA receptors according to Firn's terminology (1986). However, it is not certain how the sensitivity is increased by BL. To characterize the BL-induced changes in root sensitivity to IAA, more studies are needed. One possibility is a study of BL effects on auxin binding capacity.

Prior to this study, BRs have not been identified in the roots, which has limited our understanding of the physiological roles of BRs in roots. To date, the possible functions of BRs in the roots include inhibition of growth and formation of adventitious and lateral roots with occasional promotions by very low concentrations (Roddick and Guan, 1991; Clouse et al., 1993, 1996; Sasse, 1994; Fujioka and Sakurai, 1997). This concept is still controversial because of the uncertain occurrence of BRs in roots. This study is the first to confirm the presence of BRs in roots, which suggests that BRs are important regulatory substances for growth and development of roots. The endogenous level of BR in maize roots (approximate-

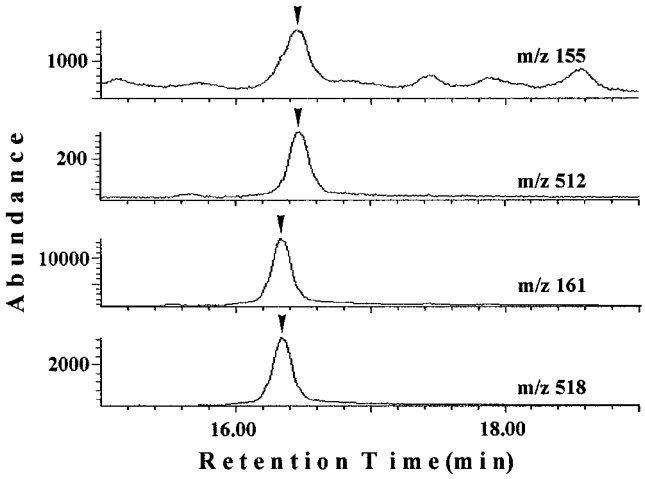

Figure 5. Identification and quantification of CS in maize primary roots by GC-SIM. The ions at *m/z* 512 and 155 for BMB of endogenous CS were detected at 16.45 min, which is identical with that of authentic CS BMB (Table I). The relative ratio of endogenous CS against [$^2H_6$]CS added as an internal standard (1 μg) measured by 512/518 or 155/161 was approximately 1:13.

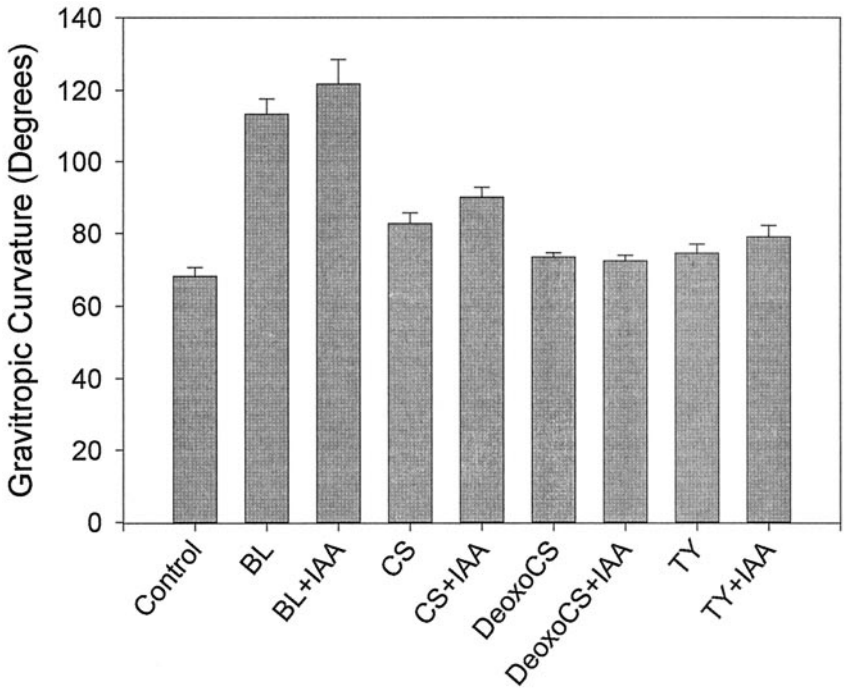

Figure 6. Effect of several BRs on the gravitropic response of maize primary roots in the presence or absence of IAA. The roots were pre-incubated in solutions of 5 mM MES-Tris (pH 6.8) containing no hormone (control) or 10^{-7} M BRs with or without 10^{-8} M IAA for 2 h, and placed horizontally for 3 h at 25°C ± 1°C. Values in the figure represent the means ± SE of five replicates.

ly 0.3 ng g^{-1} fresh weight) is comparable to leaves and shoots in other higher plants (Roddick and Guan, 1991; Fujioka, 1999) that show abnormal growth and differentiation by defects of BRs. Therefore, more data on activities of BRs in plant roots other than growth inhibition and the gravitropic response can be expected in the near future.

CS, a 6-keto derivative of BL, shows less biological activity than that of BL in BRs bioassays. In *Catharanthus roseus* cells, exogenously applied [^{3}H]CS was successfully converted into [^{3}H]BL, demonstrating that CS is a direct biosynthetic precursor of BL (Yokota et al., 1990). Recently, the conversion of CS to BL was confirmed by cell-free systems prepared from cultured cells of *Marchantia polymorpha* and *Phaseolus vulgaris* (S.-K. Kim, K.-S. Han, T.-W. Kim, S. Takatsuto, and T. Yokota, unpublished data). Nevertheless, the role of CS as only a biosynthetic precursor of BL is debated. CS itself shows strong activity in many bioassays for BRs. In some plant materials, exogenously applied CS or 24-epiCS is not converted into BL or 24-epiBL, respectively, but into other metabolites such as conjugates (Yokota et al., 1991; Suzuki et al., 1993; Adam and Schneider, 1999) or 3-epiCS (Suzuki et al., 1995). Moreover, CS alone has been identified from some plants that did not contain BL (Kim, 1991; Fujioka, 1999). Therefore, it is possible that CS itself has biological activities. In this study we identified CS, but not BL, from maize seedlings and primary roots, which is similar to other reports that indicate CS occurs together with other BRs but not with BL in several different maize cultivars (Suzuki et al., 1986; Gamoh et al., 1990; Sekimoto et al., 1997). The identification of only CS from the primary roots of maize may suggest that CS is the active BR for gravitropic response in the roots. However, our study cannot rule out the possibility that BL is the active principle for the gravitropic response in the maize roots, because exogenously applied BL shows stronger activity than that of CS and endogenous level of BL in plants is sometimes too low to be detected by GC-MS analysis.

The activation of gravitropic curvature of primary roots of maize by application of BL is a fast response. The accelerated gravitropic curvature between BL-treated and untreated primary roots was detected within 3 h after gravitropic stimulation and was amplified by IAA. The activity of BL in the gravitropic curvature of maize roots, preferably in the presence of IAA, might be useful as a BR bioassay. The minimum detectable amount of BR in the curvature assay is approximately 10^{-9} M BL equivalents (Fig. 1), which is comparable with that of the rice lamina inclination assay, where the minimum detectable amount of BL in the rice lamina inclination assay is 0.0001 μL L^{-1}, or approximately 2×10^{-10} M. Furthermore, the curvature assay needs only 3 to 4 d for the entire assay (measurements were made 3–4 h after BL application), which is much shorter than the rice lamina inclination assay, which needs 12 d for the entire assay and the measurements are typically made 2 d after BR application). To develop a time saving bioassay for BRs, the usefulness of the gravitropic activity to detect BRs in maize primary roots is now under investigation.

MATERIALS AND METHODS

Plant Materials and Chemicals

Maize (*Zea mays* L. cv Golden Cross Bantam) seeds were washed several times with tap water and soaked in distilled water for 24 h. After soaking, the seeds were placed on trays (27 × 20 × 2.5 cm) covered by water-saturated paper towels. To keep the seeds moisturized, they were covered with one more layer of water-saturated paper towel. The trays were positioned vertically at 28°C ± 1°C in the dark with 70% relative humidity. After germination in the dark for 2 d, seedlings with 1.5- to 2 cm-long straight-grown primary roots were selected and used in experiments. Seedlings grown in the same condition for 3 d were used to identify BRs in primary roots.

All chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis). The BRs used as authentic standards in this study were provided by Prof. Takao Yokota (Teikyo University, Utsunomiya, Japan).

Measurement of Elongation of Maize Primary Roots

Root caps of seedlings were immersed for 2 h in a MES [2-(*N*-morpholino)-ethanesulfonic acid]-Tris [tris(hydroxymethyl)-aminomethane] buffer (5 mM, pH 6.8), which contained various concentrations of IAA and/or BL, for 2 h with aeration at 25°C ± 1°C. To measure the length of primary roots, seedlings were positioned vertically in a wall of a lucent chamber and exposed to a closed circuit digital camera. The image of the root was then magnified 70 times on a computer monitor with the SECANT computer program (Yongma, Seoul, Korea) and recorded every 30 min up to 4 h.

Measurement of Gravitropic Curvature of Maize Primary Roots

The root caps of seedlings were immersed in the buffer solution (5 mM MES-Tris, pH 6.8) containing various concentrations of IAA and/or BRs for 2 h with aeration at 25°C ± 1°C. The hormone-treated roots were then placed horizontally in a lucent Plexiglas container (13 × 9 × 6 cm) for gravistimulation. Using the closed circuit digital camera and computer program Image-Pro Plus (Yongma), the image of the root was magnified 10 times on a monitor and recorded every 30 min for 5 h. The angles of curvature were measured using the program after the gravistimulation treatment.

Bioassay for BRs

The rice lamina inclination assay was used to detect BRs activity (Arima et al., 1984).

Identification of BRs in Whole Seedlings and Primary Roots of Maize

The 3-d-old whole seedlings (150 g) were homogenized and extracted with 90% (v/v) methanol (three times in 500 mL). The extracts were concentrated to aqueous phase in vacuo and re-extracted with chloroform (three times in 500 mL). The chloroform-soluble extracts were reduced to dryness in vacuo and partitioned between *n*-hexane and 80% (v/v) methanol (three times in 500 mL). After drying, the 80% (v/v) methanol soluble fraction was partitioned again between phosphate buffer (pH 7.4) and ethyl acetate (500 mL × 3). The ethyl acetate-soluble fraction (400 mg) was purified by silica gel (40 g, Merck, Rahway, NJ) column chromatography. The elution was performed stepwise with chloroform containing 0%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 20%, 50%, and 100% (v/v) methanol (400 mL each). The 4% and 5% methanol-in-chloroform fractions that showed biological activity in the rice lamina inclination assay were combined and chromatographed on Sephadex LH-20 column (bed volume 340 mL, 22 × 900 mm, Pharmacia LKB Biotechnology, Uppsala) using a 4:1 mixture of methanol:chloroform at a flow rate of 0.5 mL min^{-1}. The bioactive fractions with 0.65 to 0.75 of elution volume/total volume were combined and subjected to octadecylsilane column (10 g, LiChroprep RP-18, Merck) eluted with aqueous methanol with an increase in methanol content every 10 mL (50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, and 100%). The biologically active fraction eluted with 75% (v/v) methanol was analyzed by a capillary GC-MS after methaneboronation.

Segments of maize primary roots (240 g) obtained from 3-d-old seedlings were homogenized and extracted with 90% (v/v) methanol (three times in 500 mL). After concentrating to aqueous phase in vacuo, [26,28-$^2H_6$]CS (1 μg) was added to the extracts as an internal standard for quantitative analysis. The extracts were re-extracted and solvent-partitioned by the same methods described above. The obtained ethyl acetate soluble fraction (300 mg) was subjected to silica gel (20 g) eluted stepwise with chloroform containing 0%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 20%, 50%, and 100% (v/v) methanol (100 mL of each). The fractions eluted with 4% and 5% methanol-in-chloroform were combined and purified by the same Sephadex LH-20 column chromatography mentioned above. The active fractions (0.65–0.75 of elution volume/total volume) was combined, dissolved in small volume of methanol, and subjected to a reversed phase HPLC (8 × 100 mm, 4-μm Novapak C18 column, Waters, Milford, MA) at a flow rate 1 mL/min with 45% (w/v) acetonitrile. The fractions were collected every minute and fractions 13 to 15, which correspond to R_t of authentic CS (13.7 min), showed biological activity. The fractions were combined and analyzed by GC-SIM (*m/z* 155 and 512) after methaboronation.

GC-MS and GC-SIM Analysis

GC-MS and GC-SIM analyses were carried out by a 5973 mass spectrometer (electron impact ionization, 70 electron volt, Hewlett-Packard, Palo Alto, CA) connected to 6890 gas chromatograph fitted with a fused silica capillary column (HP-5, 0.25 × 30 m, 0.25-μm film thickness, Hewlett Packard). GC conditions were as follows: on-column injec-

tion mode, He 1 mL min^{-1}; oven temperature, 175°C for 2 min; and thermal gradient, 40°C to 280°C min^{-1} and then 285°C min^{-1}. Prior to injection the samples were treated with pyridine containing methaneboronic acid (2 mg mL^{-1}) for 30 min at 70°C.

Replication of Experiments and Statistical Analysis of Data

All experiments for gravitropic curvature and elongation of maize primary roots were performed at least three times. In every experiment, 20 primary roots were used. To test for significance of the data, mean values were calculated with Student's *t* tests.

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