J Plant Growth Regul (2003) 22:259–271
DOI: 10.1007/s00344-003-0119-3

J P G R
Journal of Plant Growth Regulation

Physiological Roles of Brassinosteroids in Early Growth of *Arabidopsis:* Brassinosteroids Have a Synergistic Relationship with Gibberellin as well as Auxin in Light-Grown Hypocotyl Elongation

Kiwamu Tanaka,[1] Yasushi Nakamura,[2] Tadao Asami,[3] Shigeo Yoshida,[4] Tomoaki Matsuo,[4] and Shigehisa Okamoto[1,*]

[1]Department of Agricultural Science, Kagoshima University, Kagoshima 890-0065, Japan; [2]Department of Food Science and Nutritional Health, Kyoto Prefectural University, Sakyo-ku, Kyoto 606-8522, Japan; [3]The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-0198, Japan; [4]Department of Biochemical Science and Technology, Kagoshima University, Kagoshima 890-0065, Japan

Abstract

We examined the physiological effects of brassinosteroids (BRs) on early growth of *Arabidopsis*. Brassinazole (Brz), a BR biosynthesis inhibitor, was used to elucidate the significance of endogenous BRs. It inhibited growth of roots, hypocotyls, and cotyledonous leaf blades dose-dependently and independent of light conditions. This fact suggests that endogenous BRs are necessary for normal growth of individual organs of *Arabidopsis* in both photomorphogenetic and skotomorphogenetic programs. Exogenous brassinolide (BL) promoted hypocotyl elongation remarkably in light-grown seedlings. Cytological observation disclosed that BL-induced hypocotyl elongation was achieved through cell enlargement rather than cell division. Furthermore, a serial experiment with hormone inhibitors showed that BL induced hypocotyl elongation not through gibberellin and auxin actions. However, a synergistic relationship of BL with gibberellin A_3 (GA_3) and indole-3-acetic acid (IAA) was observed on elongation growth in light-grown hypocotyls, even though gibberellins have been reported to be additive to BR action in other plants. Taken together, our results show that BRs play an important role in the juvenile growth of *Arabidopsis*; moreover, BRs act on light-grown hypocotyl elongation independent of, but cooperatively with, gibberellins and auxin.

Key words: Brassinosteroids; *Arabidopsis*; Hormonal interaction; Auxin; Gibberellins; Brassinazole

Received: 6 March 2003; accepted: 28 August 2003; Online publication: 3 November 2003

**Corresponding author; e-mail:* okamoto@farm.agri.kagoshima-u.ac.jp

Introduction

Brassinosteroids (BRs) are unique steroidal phytohormones widely distributed throughout the plant kingdom. They exhibit a broad spectrum of physiological functions of plant growth, differentiation, and development such as stem elongation, leaf development, xylem differentiation, pollen tube growth, and senescence (Close and Sasse 1998). These phenomena are thought to be accomplished through BRs' regulation of target gene expression, ATPase activity, and cell wall construction (Bishop and Yokota 2001).

Recently, pathways of BR biosynthesis and signal transduction have been unveiled at the molecular level. Most intermediate molecules in the course of BR biosynthesis have been identified by biochemical studies using *Arabidopsis* and *Catharanthus roseus* (Noguchi and others 2000; Sakurai 1999). Furthermore, numerous genes such as *Arabidopsis DET2*, *CPD*, tomato *DWARF*, and pea *LKB*, which encode enzymes catalyzing each step in the BR biosynthetic pathway, were cloned and characterized molecularly (Li and others 1996; Szekeres and others 1996; Bishop and others 1996; Schultz and others 2001). On the other hand, disclosure of components in BR signaling has commenced from the time that a *BRI1* gene encoding a putative BR receptor was cloned in *Arabidopsis* (Li and Chory 1997). Genetic screening has so far identified BR mutants such as *brs1*, *bin2*, *bes1*, and *bzr1*, all of which affect BR signaling in *Arabidopsis* (Li and others 2001a, 2001b; Yin and others 2002; Wang and others 2002). These precedent studies indicate the necessity of BRs for plant growth and development.

Arabidopsis is an appropriate plant for experiments addressing molecular genetics because it has many features suited for these analyses (Meyerowitz and Pruitt 1985). In fact, molecular genetic studies using *Arabidopsis* have greatly contributed to the resolution of numerous biological questions including those for BR biosynthesis and signal transduction. However, the small size of *Arabidopsis* plants has sometimes prevented researchers from tackling these problems with physiological techniques. Thus, other larger plants with an acute sensitivity to BRs, such as mung bean, cucumber, and pea, have generally been used to elucidate BR physiology (Gregory and Mandava 1982; Katsumi 1985; Sasse 1990). Thereupon, information obtained from these studies has been utilized to explain the relationship of physiological roles of BRs with mechanisms of BR action that were obtained by molecular genetic studies using *Arabidopsis*. However, interpretation must be done with great caution because response to BRs is often species-dependent. For instance, exogenously applied BRs promote stem elongation in various plants (Mandava 1988), with some exceptional cases of inhibitory effects, as in alfalfa (Hata and others 1986), and ineffectiveness in rice (Yokota and Takahashi 1986). *Arabidopsis* has been used to reveal physiological roles of BRs in several reports (Clouse and others 1993; Li and others 1996; Arteca and Arteca 2001; Steber and McCourt 2001). These studies clarify that BRs promote seed germination, hypocotyl elongation, petiole elongation, upward bending of rosette leaves, and peduncle elongation, but they inhibit root growth in *Arabidopsis*. Nevertheless, our knowledge of physiological roles of BRs on early growth of *Arabidopsis* seedlings is rather limited, probably because of their small body size.

Therefore, we decided to do a comprehensive physiological study of early growth of *Arabidopsis* to better understand BR functions. In this study, we used a BR biosynthesis inhibitor, brassinazole (Brz), to elucidate the significance of endogenous BRs; we also used a natural active BR, brassinolide (BL). A triazole derivative, Brz inhibits two steps in BR biosynthesis that are catalyzed by two P450 monooxygenases encoded by *CPD* and *DWF4* (Asami and Yoshida 1999; Asami and others 2001). In fact, Brz-treated *Arabidopsis* has been demonstrated to mimic phenotypes displayed by BR-deficient mutants (Asami and others 2000; Nagata and others 2000). Recently, Brz was applied for microarray analyses to comprehensively identify BR-regulated genes and examine their expression in *Arabidopsis* (Goda and others 2002). The advantages of Brz treatment are that it can control endogenous BR levels more freely than BR-deficient mutations and it can be applied to different growth stages and to different organs, tissues, and cells (Asami and Yoshida 1999). Thus, it is important to do a further physiological analysis using Brz in identical plants to corroborate recent knowledge of BR functions. Furthermore, we employed a new application procedure for BRs to *Arabidopsis* seedlings instead of conventional methods. Previous studies have supplied BRs exogenously to intact plants by absorption from either roots or leaf surfaces (Sasse 1999). Root application of BRs is, in fact, more prevalent than spraying on leaf surfaces (Arteca and Arteca 2001). However, root growth of plants is reported to be severely impaired by exogenously applied BRs (Clouse and others 1993). As a result, impaired roots might become unable to absorb BRs efficiently. Therefore, we considered and adopted another application method

in which BRs and other chemicals were given to whole plants by covering their seeds with a solidified soft medium containing these substances. By this method, we were able to supply them to individual organs of seedlings directly and over a long period. We also evaluated the effect of two phytohormones, gibberellins (GAs) and auxin, on BL-induced hypocotyl elongation of *Arabidopsis* to clarify how these hormones are related with BRs. We did so because both hormones are well known to have stem-growth-promoting effects on many plants (Goodwin 1978).

Herein, we present that endogenous BRs are necessary for normal growth of juvenile organs of *Arabidopsis* in both light and dark, and that BRs can elongate light-grown hypocotyls separately from auxin and GA action. Moreover, we demonstrate that BR has a synergistic relationship with GAs on hypocotyl growth of *Arabidopsis*, which is the first evidence to our knowledge.

Materials and Methods

Chemicals

Phytohormones used in this study were brassinolide (BL; Fuji Chemical Ind. Co., Ltd., Japan), indole-3-acetic acid (IAA; Nacalai Tesque, Inc., Japan) and gibberellin A_3 (GA_3; Kyowa Hakko Kogyo Co. Ltd., Japan). Uniconazole-P (Uni; Wako Pure Chemical Ind. Ltd., Japan), 2-(p-chlorophenoxy)-2-methylpropionic acid (PCIB; Sigma-Aldrich Co., St. Louis, MO), and 2,3,5-triiodobenzoic acid (TIBA; Sigma) were used to inhibit GA biosynthesis, auxin action, and auxin transport, respectively. Brassinazole (Brz), an inhibitor of BR biosynthesis was synthesized and purified according to methods described elsewhere (Min and others 1999). For preparation of stock solutions, these chemicals were dissolved in dimethylsulfoxide and stored at −20°C until use.

Plant Material and Growth Conditions

Seeds of *Arabidopsis thaliana* (L.) ecotype Wassilewskija were sterilized for 5 min with 5% (w/v) sodium hypochlorite solution and then sterilized with strong alkaline water (>pH 11) and strong acidic water (<pH 2.5) for 15 min each. Sterilized seeds were rinsed twice and resuspended in sterile reverse osmotic (RO) water with neutral pH. Then the seeds were sown along a straight line on MS solid medium (Murashige and Skoog 1962) containing 10 g/L sucrose and 5 g/L gellan gum (Wako Pure Chemical) in 90 × 15-mm petri dishes. After incubation at 4°C for three days to synchronize seed germination, chemical treatments were performed as described below. First, stock solutions of phytohormones and those of other chemicals were diluted in MS softly solidified medium containing 3.5 g/L of gellan gum. Second, MS soft medium containing chemicals was dropped to cover the seeds completely. In a series of experiments using Uni, however, chemicals were given to 2-day-old seedlings because Uni had been reported to inhibit seed germination by depressing endogenous levels of GAs (Nambara and others 1991). After chemical treatments, petri dishes with the seeds were placed vertically in the plant growth chamber (Eyelatron FLI-301N; Tokyo Rikakikai Co. Ltd., Japan) set at 22°C under continuous light (35μmol m^{-2} s^{-1}) with white fluorescent lamps (FLR40S; Toshiba Lighting and Tech. Corp., Japan). When seedlings were grown under continuous darkness, seeds in petri dishes were pre-exposed to white fluorescent light for 24 h to facilitate germination. Then, dishes were transferred to an opaque box. Seedling age was measured from the time of transferral of petri dishes to the plant growth chamber: day 0.

Measurement and Morphological Observation

The fresh weight of *Arabidopsis* seedlings was measured with a chemical balance (AC 100; Mettler, Toledo, OH). Lengths of seedling organs were measured with a digital slide caliper (DIGIMATIC CD-15C; Mitsutoyo-Kiko Co. Ltd., Japan). Because a cotyledonous petiole of 7-day-old seedlings was too small to be measured using a digital slide caliper, we could not estimate the effect of chemicals on the growth of this organ. Thus, we designate cotyledonous leaf blades as cotyledons in figures where not otherwise specified. Morphometric measurements in each experiment were performed at least three times. Statistical significance of the data was examined by Student's *t*-test.

For microscopic observation, *Arabidopsis* seedlings were fixed in a mixture of ethanol:acetic acid (9:1). The seedlings were then soaked sequentially in 90, 70, 50, and 30% (v/v) ethanol for 20 min each. After rinsing with RO water, seedlings were cleared by incubation in a mixed solution of chloral hydrate:glycerol:RO water (8:1:2, w:v:v) for 1 hour more. Cell numbers and lengths of hypocotyls were counted and measured under a differential interference microscope (ECLIPSE E600; Nikon Corp., Japan).

Results

Effects of Brassinazole, a Brassinosteroid Biosynthesis Inhibitor, on Organ Growth of *Arabidopsis* Seedlings

To examine physiological roles of endogenous BRs on early growth of seedlings, *Arabidopsis* seeds were treated with different concentrations of brassinazole (Brz). Then growth of hypocotyls, taproots, and cotyledonous leaf blades was measured. We observed that Brz inhibited growth of every organ in 7-day-old seedlings both dose-dependently and independent of light conditions (Figures 1, 2A, 2B). The total fresh weight of seedlings was also decreased by Brz treatment. This decrease was probably caused by growth reduction of organs described above. Figure 2B shows that de-etiolated growth traits, such as open cotyledons and a short hypocotyl, were clearly observed in a Brz-treated seedling grown in darkness. Such traits are similar to those of BR-deficient mutants (Li and others 1996; Szekeres and others 1996). Also, this observation is consistent with results obtained with Brz in previous reports (Asami and Yoshida 1999; Asami and others 2000; Nagata and others 2000). These results indicate that our feeding method, in which Brz is given to a whole body of seedlings, is as workable as conventional methods. However, we also observed Brz-induced growth reduction of the cotyledonous leaf blades along their longitudinal axes in darkness (Figure 1B). It continued, at least, to day 17 (data not shown). The phenomenon is apparently incompatible with an expanded cotyledon that is a reported feature of de-etiolated growth caused by BR deficiency and loss of BR signaling (Li and others 1996; Nagata and others 2000). Hypocotyls were most severely inhibited with respect to their growth among all organs of dark-grown *Arabidopsis* (Figure 1B). At 10^{-5} M Brz, hypocotyl length became less than one-tenth that of nontreated controls. The actual mean length of 10^{-5} M Brz-treated hypocotyls was 1.07 ± 0.14 mm. However, this length was comparable to that of light-grown hypocotyls (0.97 ± 0.03 mm) treated with 10^{-5} M Brz (Figure 1A). Results imply that *Arabidopsis* hypocotyls could hardly elongate if endogenous BR levels were heavily depressed. Consequently, endogenous BRs largely contribute to elongation of hypocotyls in both photomorphogenetic and skotomorphogenetic programs. Interestingly, Brz arrested growth of taproots as severely as BL did (compare Figure 1 and Figure 3). This issue is discussed later.

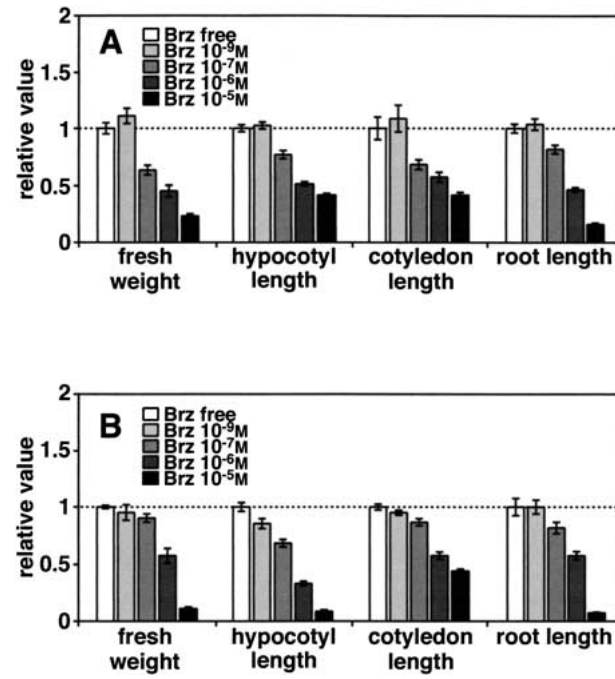

Figure 1. Effects of brassinazole on organ growth of *Arabidopsis* seedlings. *Arabidopsis* seeds were sown on MS solid medium and then covered with different concentrations of Brz. Germinated seedlings were grown for 7 days under either constant light **(A)** or constant darkness **(B)**. The fresh weights of seedlings and lengths of individual organs were measured. Histograms show means with SE (n = 20) as relative values to those of untreated controls (Brz free). ''Cotyledon length'' represents the longitudinal length of cotyledonous leaf blades as described in Materials and Methods. Actual mean values of untreated controls under continuous light were: fresh weight, 1.6 mg; hypocotyl length, 2.3 mm; root length, 15.4 mm; cotyledon length, 2.0 mm. Those under darkness were: fresh weight, 1.3 mg; hypocotyl length, 11.8 mm; root length, 19.5 mm; cotyledon length, 1.4 mm.

Effects of Brassinolide on Organ Growth of *Arabidopsis* Seedlings

Arabidopsis seeds were treated with different concentrations of BL to assess the effects of a naturally active BR, brassinolide (BL), on early growth of seedlings. Then organ growth of seedlings was measured, as in the case of Brz. Exogenous BL remarkably promoted growth of hypocotyls and cotyledonous leaf blades in a dose-dependent manner (Figures 3, 2C, 2D). The BL-induced hypocotyl elongation was shown only in conditions of light. Hypocotyls of seedlings that were treated with 10^{-6} M of BL (4.98 ± 0.18 mm) became 2.4 times longer than nontreated seedlings (2.09 ± 0.07 mm; Figure 3A). In darkness, BL did not promote but rather inhibited hypocotyl elongation at concentrations

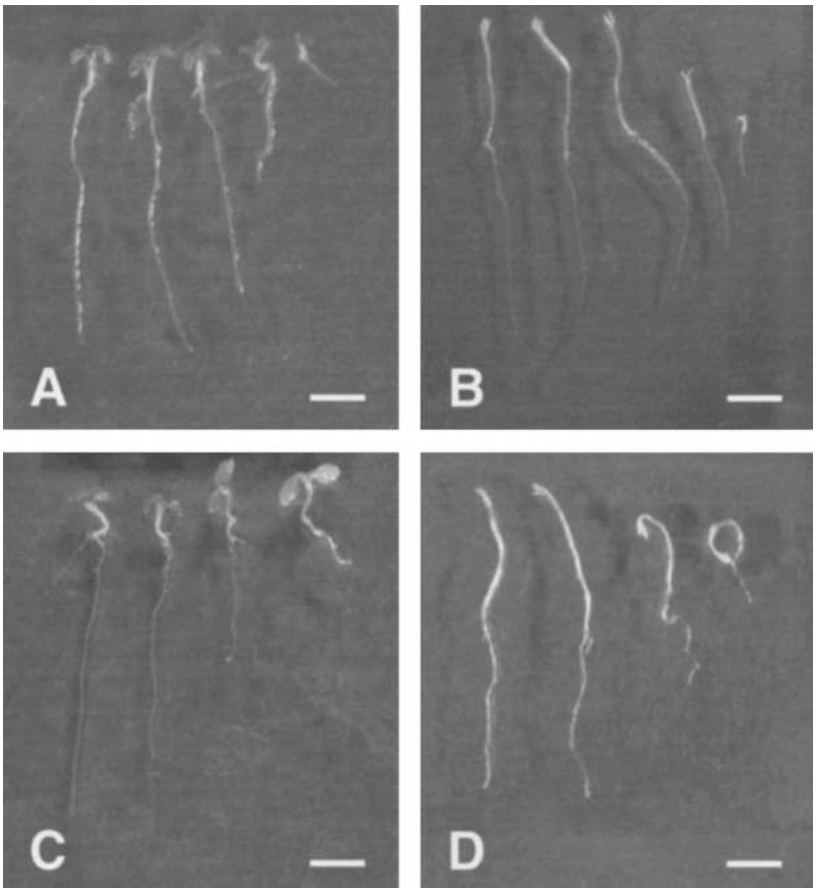

Figure 2. Morphological observation of *Arabidopsis* seedlings treated with either brassinazole or brassinolide. Photographs show *Arabidopsis* seedlings grown in the presence of Brz **(A, B)** or BL **(C, D)** for 7 days under continuous light **(A, C)** or continuous darkness **(B, D)**. In each panel, concentrations of Brz from left to right are 0, 10^{-9}, 10^{-7}, 10^{-6}, and 10^{-5} M; those of BL are 0, 10^{-10}, 10^{-8}, and 10^{-6} M. Scale bars = 5 mm.

higher than 10^{-8} M (Figures 3B,2D). On the other hand, BL-induced elongation of cotyledonous leaf blades was found in both light and dark, although BL was less effective in darkness (Figure 3). Taproot elongation was severely inhibited by exogenously applied BL in a dose-dependent fashion in both light and dark conditions (Figures 3, 2C, 2D). Inhibition of taproot growth in the light was observed when seedlings were treated with BL concentrations greater than 10^{-10} M. Conversely, in darkness, taproot growth was inhibited when treated with 100-fold higher concentration of BL (10^{-8} M) at a minimum. These results indicate that responsiveness of organs to BL differs among organs. They also indicate that such responsiveness is affected by light conditions. Interestingly, fresh weights of seedlings were not much affected by applications of BL in either light or dark (Figure 3). This could be explained in part by compensation through overgrowth of hypocotyls and cotyledonous leaf blades for loss of weight as a result of inhibition of root growth.

Morphological observations of seedlings yielded different information than those obtained by morphometric analyses. Figure 2 shows that growth of a cotyledonous leaf blade in a lateral direction was observed when the light-grown seedlings were treated with concentrations of BL greater than 10^{-8} M (Figure 2C). In addition, BL was shown to cause abnormal growth of organs, such as swelling and twisting of hypocotyls and roots, in both light and dark, whereas Brz did not (Figure 2).

BRs Are Involved in Cell Elongation of Hypocotyls in *Arabidopsis*

Growth promotion of hypocotyls by BL was most obvious in the light. Therefore, we further analyzed effects of BRs on hypocotyl elongation. First, we carried out microscopic observations of hypocotyls to determine whether cell division, cell elongation, or both contribute to promotion or inhibition of hypocotyl elongation caused by either BL or Brz, respectively. As shown in Figure 4, cell lengths of hypocotyls along their longitudinal axes were increased dramatically by treatment of 10^{-6} M BL when compared to that of the nontreated control. Actual length of BL-treated cells was 199.1 ± 5.4 μm on average, and approximately 2.4 times longer than that of the control (83.3 ± 3.9 μm). These re-

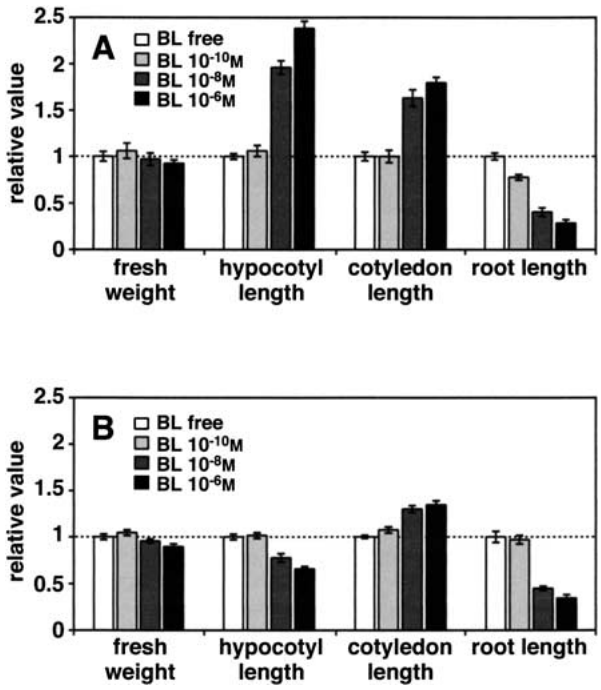

Figure 3. Effects of brassinolide on organ growth of *Arabidopsis* seedlings. Experimental procedures, growth conditions, and histograms are identical to those in Figure 1. The actual values of means of untreated controls (BL free) under continuous light were: fresh weight, 1.5 mg; hypocotyl length, 2.1 mm; root length, 18.4 mm; cotyledon length, 1.5 mm. Those under darkness were: fresh weight, 1.1 mg; hypocotyl length, 11.3 mm; root length, 13.2 mm; cotyledon length, 1.2 mm.

sults are comparable to the difference of hypocotyl length between 10^{-6} M BL-treated and nontreated seedlings (Figure 3). Furthermore, high magnification of the micrograph revealed that cells located at subapical as well as basal regions of the hypocotyl were elongated by exogenous BL, even though upper cells were slightly longer than lower cells. On the other hand, 10^{-6} M Brz reduced cell length of hypocotyls remarkably. The average length of Brz-treated hypocotyl cells (40.3 ± 1.8 μm) was nearly one-half that of the nontreated control (83.3 ± 3.9 μm) and one-fifth that of BL-treated cells (199.1 ± 5.4 μm). This result was also comparable to that of hypocotyl measurement (Figures 1, 3). Furthermore, we counted the number of individual cells in each cell file along with longitudinal axes of hypocotyls. Cell numbers in a single cell file of the BL-, Brz-, and nontreated hypocotyls were 25.4 ± 0.6, 24.6 ± 0.4, and 23.6 ± 0.7 on average, respectively. Taken together, these results strongly suggest that BRs regulate hypocotyl elongation *via* cell enlargement rather than cell division in the case of *Arabidopsis*.

Relationship of BRs with Auxin and Gibberellins on Hypocotyl Elongation in *Arabidopsis*

Auxin and gibberellins (GAs) reportedly have important roles in elongation growth of organs including stems and roots in higher plants (Goodwin 1978; Feldman 1984). Therefore, we investigated the effects of two growth-promoting hormones, IAA and GA_3, on BL-induced hypocotyl elongation in light-grown *Arabidopsis* to clarify a relationship of BL with those two hormones.

As shown in Figure 5, neither an auxin antagonist, PCIB, nor an auxin transport inhibitor, TIBA, reduced hypocotyl elongation of light-grown *Arabidopsis* in the absence of BL, whereas they decreased hypocotyl elongation in darkness (data not shown). The results indicate that these chemicals were incorporated efficiently into the hypocotyls, and that auxin action on hypocotyl elongation is dependent on conditions of light. Furthermore, the effect of exogenous IAA on hypocotyl elongation in the light condition was much weaker than that of exogenous BL or GA_3 (Figure 6). These observations indicate that auxin is less involved in this phenomenon in the light than in the dark. Exogenous BL induced hypocotyl elongation of light-grown *Arabidopsis* to the same extent, regardless of the absence or presence of PCIB (Figure 5). These results suggest that BL can elongate hypocotyls independent of auxin action. In addition, TIBA enhanced BL-induced hypocotyl elongation synergistically (Figure 5B). This finding is similar to that of Yopp and others (1979), who demonstrated that TIBA and brassin-complex enhanced synergistically hook closure of bean in darkness and that they also retarded opening of cotyledons in red light. Furthermore, we observed that IAA synergistically enhanced BL-induced hypocotyl elongation of *Arabidopsis* (Figure 6A), whereas the effect of IAA was not synergistic but additive on GA_3-induced hypocotyl elongation (Figure 6C). Considered together, these results imply that BL-induced hypocotyl elongation in *Arabidopsis* is not mediated through the action of endogenous auxin. Results also indicate that auxin can potentially promote BL-induced hypocotyl elongation of *Arabidopsis* in a synergistic manner.

Previous studies reported that GAs and BRs act additively on growth of organs such as the epicotyl segment of mung bean and hypocotyl segment of cucumber (Gregory and Mandava 1982; Katsumi 1985). For that reason, the relationship of the two hormones is inferred to be ''independent interaction'' (Evans 1984). However, no physiological

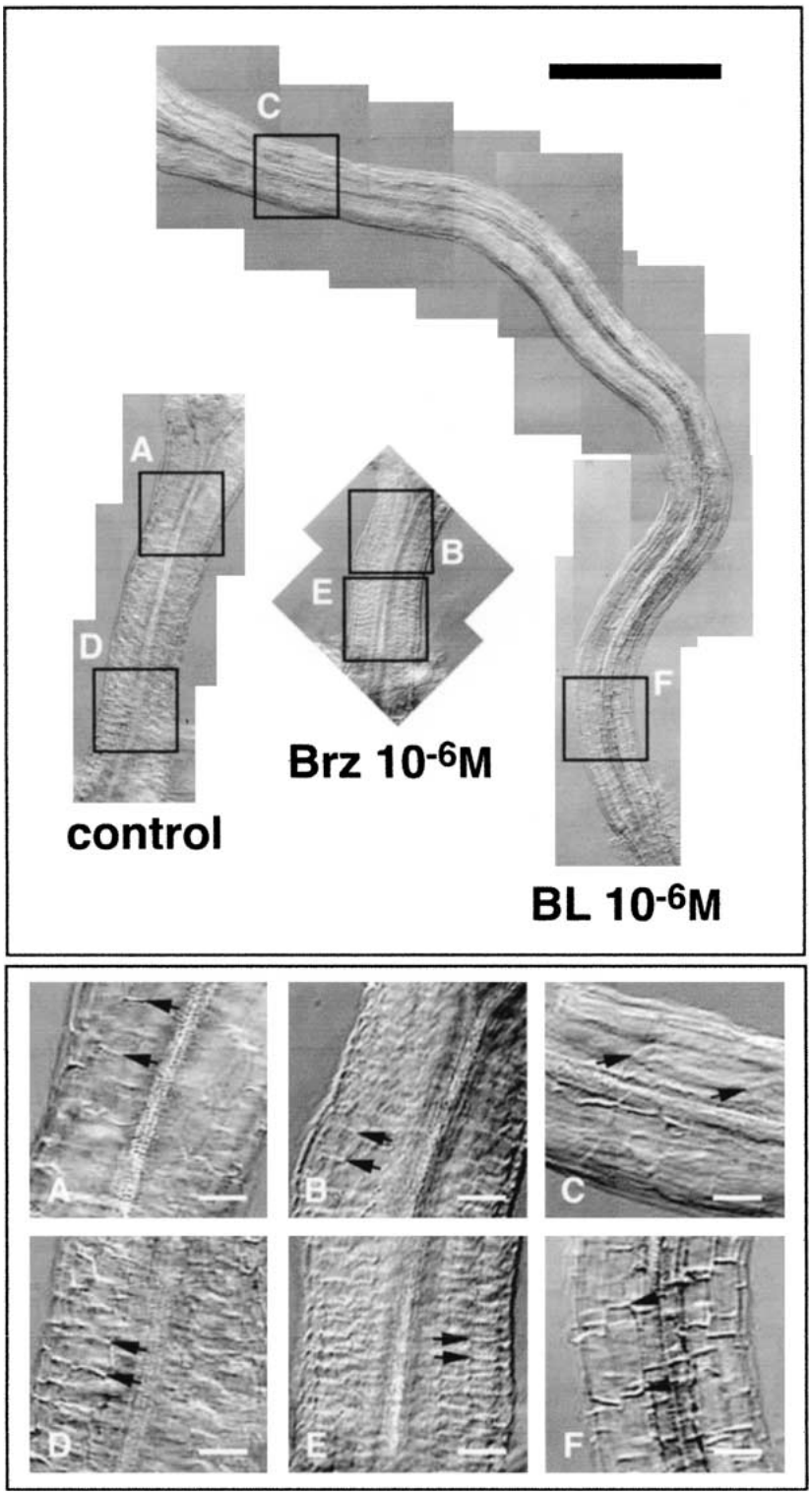

Figure 4. Microscopic observation of *Arabidopsis* hypocotyls treated with brassinazole or brassinolide. *Arabidopsis* seedlings were grown in the presence of either Brz or BL for 7 days under continuous light. Seedlings were fixed and cleared as mentioned in Materials and Methods. Prepared specimens were then observed under a differential interference microscope. Upper panel photographs show total views of seedling hypocotyls. Lower panel photographs show apical and basal regions of hypocotyls (closed area in the upper panel) that are similarly magnified. Note that longitudinal cell length (distance between two arrows) of the hypocotyl was strongly affected by BL and Brz. Black and white bars indicate 1 mm and 100 μm, respectively.

study has described the relationship of BRs with GAs on hypocotyl elongation in *Arabidopsis*. Therefore, we examined the effect of GA_3 on BL-induced hypocotyl elongation in the light. As shown in Figures 6B and 6C, a solely applied GA_3 promoted hypocotyl elongation dose-dependently. In contrast, solely applied Uni dose-dependently arrested hypocotyl elongation in the light (Figure 7A, left panel) as well as in darkness (data not shown). These results imply that GAs play an essential role in hypocotyl elongation of *Arabidopsis*, just as BRs do.

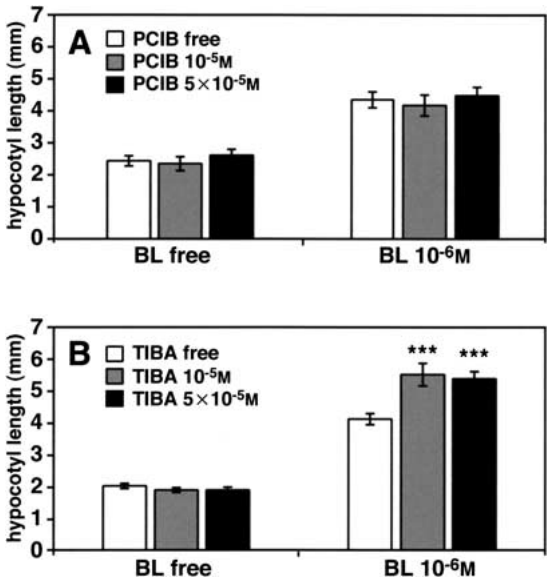

Figure 5. Effect of anti-auxin on brassinolide-induced hypocotyl elongation in light-grown *Arabidopsis*. *Arabidopsis* seedlings were grown for 7 days under continuous light in the presence of BL with either PCIB **(A)** or TIBA **(B)**. Then hypocotyl lengths of seedlings were measured. Values represent a mean of 20 seedlings with SE. Student's *t*-test: $***p < 0.01$ versus TIBA-free control in BL-treated seedlings.

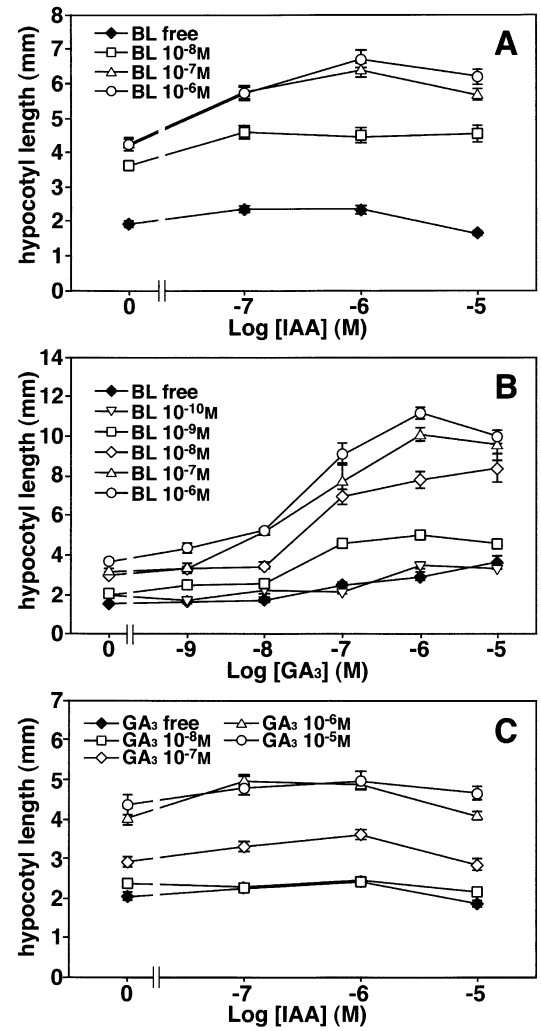

Figure 6. Interactions between brassinolide, indole-3-acetic acid, and gibberellin A_3 on hypocotyl elongation in light-grown *Arabidopsis*. Two hormones among three (BL, IAA, and GA_3) were simultaneously applied to *Arabidopsis* seeds. *Arabidopsis* seedlings were grown for 7 days after treatment and hypocotyl lengths were measured. Values represent a mean of 20 seedlings with SE. Panels show simultaneous application of two chemicals as follows: BL and IAA **(A)**; BL and GA_3 **(B)**; GA_3 and IAA **(C)**.

Growth inhibition of hypocotyls by 10^{-6} M Uni was recovered completely by addition of 10^{-6} M GA_3, whereas that by 10^{-5} M Uni was partially recovered by identical treatment (Figure 7A, right panel). Similar results were obtained in the case of Brz and BL, namely, growth inhibition by 10^{-6} M Brz was recovered fully by 10^{-6} M BL, but that by 10^{-5} M Brz was not recovered completely by 10^{-6} M BL (Figure 7B, center panel). These findings concurrently support the concept that both Uni and Brz are related triazole derivatives and target heme irons of cytochrome P450 monooxygenases, which exist commonly in biosynthesis pathways of BRs and GAs (Yoshida and Aoyama 1991; Asami and others 2001). Furthermore, Uni and Brz were shown to reciprocally affect phenomena induced by either BRs or GAs in some plants (Yokota and others 1991; Iwasaki and Shibaoka 1991; Sekimata and others 2001). Together, these findings caution that a proper concentration of inhibitors, such as 10^{-6} M each of Uni and Brz in our case, must be used to exhibit their own specificity of inhibitory actions on biosynthesis of GAs and BRs, respectively.

A concentration of 10^{-6} M Uni suppressed hypocotyl elongation induced by 10^{-6} M BL (Figure 7A, center panel). This Uni concentration is thought to affect only GA biosynthesis but not BR biosynthesis, as described above. Conversely, 10^{-6} M Brz largely reduced 10^{-6} M GA_3-induced elongation of hypocotyls, which seems not to inhibit GA biosynthesis (Figure 7B, right panel). These observations suggest that hypocotyl elongation, apparently caused by a solely applied BL (Figure 3A), was, in fact, achieved by cooperative action of exogenous BL with endogenous GAs, and *vice versa* in the case of exogenous GA_3 with endogenous BRs (Figure 7B). That is, the results imply that BRs and GAs can individually promote hypocotyl elongation of *Arabidopsis*, at least in light conditions.

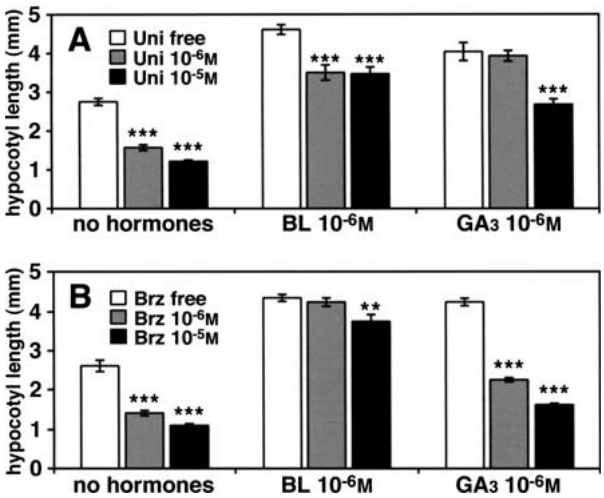

Figure 7. Effects of brassinolide or gibberellin A_3 in the presence of uniconazole-P or brassinazole on hypocotyl elongation in light-grown *Arabidopsis*. Experimental procedures, growth conditions, and histograms are essentially identical to those in Figure 5, except Uni administration (see Materials and Methods for details). Hypocotyl length of 7-day-old seedlings was measured. Values represent a mean of 20 seedlings with SE. **(A)** Simultaneous application of Uni with either BL or GA_3; **(B)** simultaneous application of Brz with either BL or GA_3. Student's *t*-test: $^{**}0.01 < p < 0.05$; $^{***}p < 0.01$ versus Uni- or Brz-free control.

Coapplication experiments suggest a novel relationship between BRs and GAs in *Arabidopsis:* one that is different from the additive relationship proposed in early physiological studies (Gregory and Mandava 1982; Katsumi 1985). As shown in Figure 6B, simultaneously applied GA_3 and BL promoted elongation of light-grown hypocotyls more than one application of either hormone. When both 10^{-6} M of BL and 10^{-6} M of GA_3 were applied, hypocotyl length reached approximately 11.2 ± 0.3 mm. In contrast, lengths by treatment with BL or GA_3 were 3.7 ± 0.2 mm or 2.9 ± 0.2 mm, respectively. This finding indicates that the relationship between BL and GA_3 is synergistic, according to the definition of Evans (1984), who referred to it as a synergism that the effect of two hormones applied simultaneously exceeds the sum of each effect. In conclusion, the results imply that BL and GA_3 act synergistically on elongation of light-grown hypocotyls when more than 10^{-8} M of BL and of GA_3 were given to *Arabidopsis* seedlings, whereas they act additively when less than 10^{-8} M of both hormones each was administered (Figure 6B). This is the first evidence demonstrating a synergistic relationship of BL with GA_3 on plant growth.

Discussion

In this report, we present physiological roles of BRs in the early growth of *Arabidopsis* to combine new information on BR physiology directly with molecular knowledge of BRs obtained using identical plants.

Significant growth reduction of hypocotyls, cotyledons, and roots was observed irrespective of light conditions when Brz was given to *Arabidopsis* seedlings (Figure 1). The results suggest that endogenous BRs are necessary for normal growth of juvenile organs of *Arabidopsis* in both photomorphogenetic and skotomorphogenetic programs.

De-etiolated growth of plants in darkness has been reported to be caused by BR deficiency in the BR mutants or the Brz-treated plants (Asami and Yoshida 1999; Asami and others 2000; Li and others 1996; Szekeres and others 1996). Typical traits of de-etiolation, such as a short hypocotyl and open cotyledons, were also detected in our experiment although the other traits—accumulation of anthocyanins and expanded cotyledons—were not clear (Figures 1, 2). Furthermore, we found that Brz resulted in remarkable growth reduction of cotyledonous leaf blades, at least along their longitudinal axes in darkness (Figure 1), which obviously conflicts with a reported feature of de-etiolation, an expanded cotyledon (Li and others 1996; Nagata and others 2000). At present, we cannot explain why different results were obtained in these studies. The difference might reflect different experimental conditions with respect to media constituents, photoperiod, temperature, procedure for chemical application, or timing of size measurement among distinct studies. Alternatively, the difference might arise from different plant materials, for example, *Arabidopsis* ecotype. In either case, further morphometric analyses at the cellular level using Brz-treated plants, BR-deficient mutants, or both are required to determine whether ''an expanded cotyledon'' is truly caused by BR deficiency.

It is worth further discussion to note that both BL and Brz inhibited root elongation in a dose-dependent fashion (Figures 1, 2, 3). Roots are reported to be more sensitive to auxin than stems for elongation growth (Thimann 1937). In this case, exogenous auxin promotes root elongation at concentrations much less than those for stem elongation. However, excess auxin inhibits root growth, perhaps not through the direct action of auxin but mediated by ethylene produced in the presence of excessive auxin (Chadwick and Burg 1967). Similar to the case of auxin, ultralow concentrations, such as 10^{-14} M of BL, have recently

been shown to stimulate root elongation in *Arabidopsis* (Li and others 2001b). Thus, concentration ranges of exogenous BL used in our study (10^{-10}–10^{-6} M) would be too high to elongate *Arabidopsis* roots. The BL-induced growth inhibition of roots might also be explained by ethylene action because ethylene production is elevated in *Arabidopsis* by application of 24-epiBL (Woeste and others 1999). Furthermore, we observed swelling and twisting of roots in BL-treated seedling of *Arabidopsis* (Figure 2). A similar phenomenon was reported to occur in alfalfa seedlings treated with micromolar levels of BL (Hata and others 1986). These observations also suggest ethylene involvement in this process. Although ethylene is inferred to inhibit growth of roots treated with auxin or BRs, interaction between auxin and BRs is unclear regarding this phenomenon. Eun and others (1989) reported that BRs increase endogenous levels of auxin and ethylene in segments of etiolated hypocotyls of squash, but that the increase of ethylene is less than that of auxin. This result suggests that BRs inhibit root growth *via* the action of ethylene, the production of which depends on enhanced levels of auxin in the presence of BRs. On the other hand, Clouse and others (1993) demonstrated that BRs inhibit root elongation in an auxin-insensitive mutant, *axr1* of *Arabidopsis*. This fact implies that BR-induced growth inhibition of roots is not mediated through auxin action, at least in *Arabidopsis*, and that there is another pathway of ethylene production regulated by BRs but not through auxin. Further analysis is necessary to resolve this issue.

On the other hand, how should root elongation be inhibited in Brz-treated seedlings? Concentrations of 10^{-7} M and 10^{-6} M Brz clearly and dose-dependently inhibited root growth (Figure 1). As described before, those concentrations are considered to affect only BR biosynthesis but not GA biosynthesis (Figure 7B). Furthermore, we observed that Brz did not cause swelling and twisting of roots in *Arabidopsis* as BL did (Figure 2). This implies that ethylene is not associated with Brz-induced growth inhibition of roots. Thus, growth retardation of roots might be caused by Brz-induced depletion of endogenous BRs. Those reduced levels must be less than the threshold for stimulating root elongation (Li and others 2001b). However, the more severe arrest of root elongation observed in 10^{-5} M Brz-treated seedlings (Figure 1) appears to be brought about in different and complicated ways. Tanimoto (1987), (1988) reported that inhibitors of GA biosynthesis, such as CCC, ancymidol, and AMO-1718, cause root-growth retardation in lettuce and Alaska pea when these chemicals are used at higher concentration ranges than 10^{-5} M to inhibit hypocotyl elongation. Furthermore, these studies also indicated that such retardation is recovered by GA_3 at lower concentration ranges than those for stimulating hypocotyl elongation. These results imply that GAs are also involved in root elongation and that roots are more sensitive to GAs than stems with respect to elongation growth, as is the case for auxin and BRs. Arrested growth of roots under 10^{-5} M Brz is probably caused by a deficiency of GAs in addition to BRs because 10^{-5} M Brz affects not only BR biosynthesis but also GA synthesis (Sekimata and others 2001).

Involvement of BRs in hypocotyl elongation has been studied intensively in many plants including *Arabidopsis*, cucumber, and pea (Sasse 1999). For example, reduction of cell size, but not cell number of hypocotyls, has been observed in BR-deficient mutants of *Arabidopsis* such as *cbb1–3*, *cpd*, *dwf7*, and *sax1* (Kauschmann and others 1996; Azpiroz and others 1998; Catterou and others 2001; Ephritikhine and others 1999). Exogenous BRs are also reported to promote cell elongation of stem explants in some plants (Sasse 1999). However, BRs have recently been demonstrated to contribute to leaf expansion of *Arabidopsis* via regulation of both cell division and cell expansion (Nakaya and others 2002). Moreover, *Arabidopsis CycD3*, which encodes a part of the machinery for cell division control, has also been reported to be upregulated by application of 24-epiBL (Hu and others 2000). These two facts suggest that BRs are likely to be involved in cell division and cell expansion. However, our microscopic observation indicates that BL-induced growth promotion of light-grown hypocotyls is caused by enhancement of cell elongation but not that of cell division (Figure 4). Correspondingly, Brz-induced growth inhibition is likely to occur by failure of cell enlargement (Figure 4). Thus, our observations, together with previous studies, strongly suggest that BRs mainly affect hypocotyl growth via cell enlargement, at least in *Arabidopsis*.

Auxin has been reported to exert major effects on growth of organs such as stems, leaves, and roots (Goodwin 1978; Feldman 1984). Hypocotyl elongation in *Arabidopsis* has been demonstrated to be affected by auxin (Smalle and others 1997; Collett and others 2000; Saibo and others 2003). Interactions of BRs with auxin have been discussed extensively hitherto. Effects of BRs on plant growth, for instance, have been claimed to be mediated through enhancement of auxin action, that is, increased sensitivity of BR-treated tissues to endogenous auxin and an elevated level of endogenous auxin in tissues (Mandava 1988). Those claims arose on the

basis of data obtained from physiological studies using bean, rice, cucumber, and squash (Cohen and Meudt 1983; Takeno and Pharis 1982; Katsumi 1985; Eun and others 1989). However, there exists contradictory evidence such as BR-induced cell enlargement in an auxin-starved cultured cell line of carrot (Sala and Sala 1985) and BR-promoted elongation of the auxin-depleted soybean epicotyls (Clouse and others 1992) and *Arabidopsis* peduncles (Clouse and others 1993). We observed that PCIB as well as TIBA did not inhibit hypocotyl elongation of *Arabidopsis* grown in continuous light (Figure 5), although Brz and Uni did (Figures 1, 7). This suggests that endogenous auxin is less involved in hypocotyl elongation than the endogenous BRs and GAs under our conditions. Our conclusion contradicts results of Collett and others (2000), who demonstrated that endogenous levels of auxin in the wild-type hypocotyls grown in a 16-h-light/8-h-dark photoperiod are optimal for elongation. However, exogenous IAA weakly enhanced hypocotyl growth of the wild-type *Arabidopsis* in our study (Figure 6), indicating the suboptimal levels of auxin for elongation. Similarly, auxin levels seem to be suboptimal in the hypocotyls of the wild-type *Arabidopsis* grown on a low nutrient medium in 16-h-light/8-h-dark photoperiod because exogenous auxin can elongate hypocotyls in such conditions (Smalle and others 1997; Saibo and others 2003). Furthermore, the *Arabidopsis* ecotype we used was Wassilewskija, whereas Columbia was used in their studies. Thus, auxin levels could fluctuate by experimental conditions with respect to nutrients, photoperiods, and genetic backgrounds. The different results concerning auxin action among these studies could reflect a difference in endogenous auxin levels or levels of auxin response resulting from the different experimental conditions. Our observation, that BL was able to induce hypocotyl elongation of *Arabidopsis* in the presence of either PCIB or TIBA, concurs with that of Sasse (1990), who demonstrated that PCIB did not affect BR-promoted elongation of stem sections in pea. Thus, both observations indicate that BRs can act separately from auxin action on stem elongation in pea and *Arabidopsis*. Furthermore, BRs negatively regulate root elongation in *Arabidopsis* not via auxin action as described previously (Clouse and others 1993). Together, these results suggest that auxin-mediated action caused by BRs, at least in certain conditions, may be less prevalent than direct action of BRs on elongation growth of hypocotyls, peduncles, and roots in *Arabidopsis*.

In addition, a synergistic relationship between BRs and auxin has been discussed (Cohen and Meudt 1983; Katsumi 1985; Takeno and Pharis 1982). We observed significant synergism between BL and TIBA on hypocotyl elongation in light-grown seedlings (Figure 5B). Because TIBA is a specific inhibitor of efflux control of the auxin transport system (Karabaghli and others 1998), this chemical is thought to lead to an increased endogenous level of auxin because it prevents normal polar transport. Thus, elevated levels of auxin in local cells appear to act synergistically with exogenously applied BL on hypocotyl elongation. Alternatively, TIBA itself might function as a synergist because it has been shown to have a weak auxin-like activity (Fujita and Syono 1996). A synergistic relationship of BRs with auxin for hypocotyl elongation was confirmed by simultaneous application of BL and IAA (Figure 6A). Results described so far suggest that BRs can induce, separately from auxin action, hypocotyl elongation in light-grown seedlings, but, at the same time, auxin can potentiate BR action on the same event.

Early physiological studies demonstrated additive effects of GAs and BRs on elongation growth of organs in pea, mung bean, and cucumber (Mandava and others 1981; Gregory and Mandava 1982; Katsumi 1985), suggesting that a relationship of the two hormones is ''independent interaction'' defined by Evans (1984). We found, however, that BL and GA_3 acted synergistically on elongation of light-grown hypocotyls when more than 10^{-8} M each of BL and GA_3 were given simultaneously to *Arabidopsis* seedlings, whereas they acted additively when less than 10^{-8} M of both hormones each was given (Figure 6B). Additivities observed at lower levels of BL concur well with those of Gregory and Mandava (1982) with mung bean epicotyls, whereas synergies found at relatively higher levels of BL obviously contradict those of previous studies (Mandava and others 1981; Gregory and Mandava 1982; Katsumi 1985). What causes this discrepancy of results? In the former studies, tissue segments of hypocotyls, epicotyls, or apical hooks of plants were used as targets of BR administration, in contrast with the intact plants in our present study. This difference in methods may cause differential results. Alternatively, result discrepancy may merely reflect different plant species used, indicating the existence of species-dependent differences of actions of individual hormones and interactions among them. However, other possibilities cannot be excluded. Recently, molecular studies have disclosed cross-talk between GAs and BRs such as antagonistic (*γ-TIP*, *GASA1*, *GA5*) and cooperative (*MERI5*) interactions at the level of gene expression (Kauschmann and others 1996; Bouquin and others 2001).

The synergism between BRs and GAs on hypocotyl elongation may be elucidated by a positive interaction of two hormones on gene expression because *MERI5* encodes a xyloglucan-endotransglycosylase that is probably involved in cell wall loosening, thereby controlling cell expansion growth. Further studies are required to disclose the nature of synergism between BRs and GAs in *Arabidopsis*.

In conclusion, we shed some light on the physiological roles of BRs at a juvenile stage of *Arabidopsis* growth and demonstrated that endogenous BRs are essential to attain normal growth of all organs. Among them, hypocotyls and roots were shown to be most sensitive to BRs with respect to their growth. Moreover, we found that BRs have synergistic relationships with GAs as well as auxin for hypocotyl elongation. Based on our results, we are currently analyzing and comparing expression profiles of BR-regulated genes between wild-type *Arabidopsis* and morphological mutants that impair the growth of hypocotyls and roots.

ACKNOWLEDGEMENTS

We thank Dr. Sumio Iwai at Kagoshima University for technical advice and support on differential interference microscopic observations.

REFERENCES

Arteca JM, Arteca RN. 2001. Brassinosteroid-induced exaggerated growth in hydroponically grown Arabidopsis plants. Physiol Plant 112:104–112.

Asami T, Yoshida S. 1999. Brassinosteroid biosynthesis inhibitors. Trends Plant Sci 4:348–353.

Asami T, Min YK, Nagata N, Yamagishi K, Takatsuto S, Fujioka S, Murofushi N, Yamaguchi I, Yoshida S. 2000. Characterization of brassinazole, a triazole-type brassinosteroid biosynthesis inhibitor. Plant Physiol 123:93–99.

Asami T, Mizutani M, Fujioka S, Goda H, Min YK, Shimada Y, Nakano T, Takatsuto S, Matsuyama T, Nagata N, Sakata K, Yoshida S. 2001. Selective interaction of triazole derivatives with DWF4, a cytochrome P450 monooxygenase of the brassinosteroid biosynthetic pathway, correlates with brassinosteroid deficiency in planta. J Biol Chem 276:25687–25691.

Azpiroz R, Wu Y, LoCascio JC, Feldmann KA. 1998. An Arabidopsis brassinosteroid-dependent mutant is blocked in cell elongation. Plant Cell 10:219–230.

Bishop GJ, Harrison K, Jones JDG. 1996. The tomato *Dwarf* gene isolated by heterologous transposon tagging encodes the first member of a new cytochrome P450 family. Plant Cell 8:959–969.

Bishop GJ, Yokota T. 2001. Plants steroid hormones, brassinosteroids: current highlights of molecular aspects on their synthesis/metabolism, transport, perception and response. Plant Cell Physiol 42:114–120.

Bouquin T, Meier C, Foster R, Nielsen ME, Mundy J. 2001. Control of specific gene expression by gibberellin and brassinosteroid. Plant Physiol 127:450–458.

Catterou M, Dubois F, Schaller H, Aubanelle L, Vilcot B, Sangwan NBS, Sangwan RS. 2001. Brassinosteroids, microtubules and cell elongation in *Arabidopsis thaliana*. I. Molecular, cellular and physiological characterization of the *Arabidopsis bul1* mutant, defective in the delta 7-sterol-C5-desaturation step leading to brassinosteroid biosynthesis. Planta 212:659–672.

Chadwick AV, Burg SP. 1967. An explanation of the inhibition of root growth caused by indole-3-acetic acid. Plant Physiol 42:415–420.

Clouse SD, Sasse JM. 1998. Brassinosteroids: essential regulators of plant growth and development. Annu Rev Plant Physiol Plant Mol Biol 49:427–451.

Clouse SD, Hall AF, Langford M, McMorris TC, Baker ME. 1993. Physiological and molecular effects of brassinosteroids on *Arabidopsis thaliana*. J Plant Growth Regul 12:61–66.

Clouse SD, Zurek DM, McMorris TC, Baker ME. 1992. Effect of brassinolide on gene expression in elongating soybean epicotyls. Plant Physiol 100:1377–1383.

Cohen JD, Meudt WJ. 1983. Investigations on the mechanism of the brassinosteroid response. Plant Physiol 72:691–694.

Collet CE, Harberd NP, Leyser O. 2000. Hormonal interactions in the control of Arabidopsis hypocotyl elongation. Plant Physiol 124:553–561.

Ephritikhine G, Fellner M, Vannini C, Lapous D, Barbier BH. 1999. The *sax1* dwarf mutant of *Arabidopsis thaliana* shows altered sensitivity of growth responses to abscisic acid, auxin, gibberellins and ethylene and is partially rescued by exogenous brassinosteroid. Plant J 18:303–314.

Eun JS, Kuraishi S, Sakurai N. 1989. Changes in levels of auxin and abscisic acid and the evolution of ethylene in squash hypocotyls after treatment with brassinolide. Plant Cell Physiol 30:807–810.

Evans M. 1984. Functions of hormones at the cellular level of organization. In: Scott TK Ed. Encyclopedia of plant physiology, New Series vol. 10. Berlin: Springer-Verlag. pp 23–79.

Feldman LJ. 1984. Regulation of root development. Annu Rev Plant Physiol 35:223–242.

Fujita H, Syono K. 1996. Genetic analysis of the effects of polar auxin transport inhibitors on root growth in *Arabidopsis thaliana*. Plant Cell Physiol 37:1094–1101.

Goda H, Shimada Y, Asami T, Fujioka S, Yoshida S. 2002. Microarray analysis of brassinosteroid-regulated genes in Arabidopsis. Plant Physiol 130:1319–1334.

Goodwin PB. 1978. Phytohormones and growth and development of organs of the vegetative plant. In: Letham DS, Goodwin PB, Higgins TJV Eds. Phytohormones and Related Compounds: A Comprehensive Treatise. Vol. II. Amsterdam: Elsevier/North-Holland Biomedical Press. pp 31–173.

Gregory LE, Mandava NB. 1982. The activity and interaction of brassinolide and gibberellic acid in mung bean epicotyls. Physiol Plant 54:239–243.

Hata S, Takagishi H, Egawa Y, Ota Y. 1986. Effects of compactin, a 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitor, on the growth of alfalfa (*Medicago sativa*) seedlings and the rhizogenesis of pepper (*Capsicum annuum*) explants. Plant Growth Regul 4:335–346.

Hu Y, Bao F, Li J. 2000. Promotive effect of brassinosteroids on cell division involves a distinct *CycD3*-induction pathway in Arabidopsis. Plant J 24:693–701.

Iwasaki T, Shibaoka H. 1991. Brassinosteroids act as regulators of tracheary-element differentiation in isolated Zinnia mesophyll cells. Plant Cell Physiol 32:1007–1014.

Karabaghli C, Sotta B, Bonnet M, Gay G, Le Tacon F. 1998. The auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) inhibits the stimulation of *in vitro* lateral root formation and the

colonization of the tap-root cortex of Norway spruce (*Picea abies*) seedlings by the ectomycorrhizal fungus *Laccaria bicolor*. New Phytol 140:723–733.

Katsumi M. 1985. Interaction of a brassinosteroid with IAA and GA_3 in the elongation of cucumber hypocotyl sections. Plant Cell Physiol 26:615–625.

Kauschmann A, Jessop A, Koncz C, Szekeres M, Willmitzer L, Altmann T. 1996. Genetic evidence for an essential role of brassinosteroids in plant development. Plant J 9:701–713.

Li J, Nagpal P, Vitart V, McMorris TC, Chory J. 1996. A role for brassinosteroids in light-dependent development of Arabidopsis. Science 272:398–401.

Li J, Chory J. 1997. A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell 90:929–938.

Li J, Lease KA, Tax FE, Walker JC. 2001a. BRS1, a serine carboxypeptidase, regulates BRI1 signaling in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 98:5916–5921.

Li J, Nam KH, Vafeados D, Chory J. 2001b. BIN2, a new brassinosteroid-insensitive locus in Arabidopsis. Plant Physiol 127:14–22.

Mandava NB. 1988. Plant growth-promoting brassinosteroid. Ann Rev Plant Physiol Plant Mol Biol 39:23–52.

Mandava NB, Sasse JM, Yopp JH. 1981. Brassinolide, a growth-promoting steroidal lactone. Physiol Plant 53:453–461.

Meyerowitz EM, Pruitt RE. 1985. *Arabidopsis thaliana* and plant molecular genetics. Science 229:1214–1218.

Min YK, Asami T, Fujioka S, Murofushi N, Yamaguchi I, Yoshida S. 1999. New lead compounds for brassinosteroid biosynthesis inhibitors. Bioorg Med Chem Lett 9:425–430.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497.

Nagata N, Min YK, Nakano T, Asami T, Yoshida S. 2000. Treatment of dark-grown *Arabidopsis thaliana* with a brassinosteroid-biosynthesis inhibitor, brassinazole, induces some characteristics of light-grown plants. Planta 211:781–790.

Nakaya M, Tsukaya H, Murakami N, Kato M. 2002. Brassinosteroids control the proliferation of leaf cells of *Arabidopsis thaliana*. Plant Cell Physiol 43:239–244.

Nambara E, Akazawa T, McCourt P. 1991. Effects of the gibberellin biosynthetic inhibitor uniconazole on mutants of Arabidopsis. Plant Physiol 97:736–738.

Noguchi T, Fujioka S, Choe S, Takatsuto S, Tax FE, Yoshida S, Feldmann KA. 2000. Biosynthetic pathways of brassinolide in Arabidopsis. Plant Physiol 124:201–209.

Saibo NJM, Vriezen WH, Beemster GTS, Van Der Straeten D. 2003. Growth and stomata development of Arabidopsis hypocotyls are controlled by gibberellins and modulated by ethylene and auxins. Plant J 33:989–1000.

Sakurai A. 1999. Brassinosteroid biosynthesis. Plant Physiol Biochem 37:351–361.

Sala C, Sala F. 1985. Effect of brassinosteroid on cell division and enlargement in cultured carrot (*Daucus carota* L.) cells. Plant Cell Rep 4:144–147.

Sasse JM. 1990. Brassinolide-induced elongation and auxin. Physiol Plant 80:401–408.

Sasse JM. 1999. Physiological actions of brassinosteroids. In: Sakurai A, Yokota T, Clouse SD Eds. Brassinosteroids: Steroidal Plant Hormones. Tokyo: Springer-Verlag. pp 137–161.

Schultz L, Kerckhoffs LHJ, Klahre U, Yokota T, Reid JB. 2001. Molecular characterization of the brassinosteroid-deficient *Ikb* mutant in pea. Plant Mol Biol 47:491–498.

Sekimata K, Kimura T, Kaneko I, Nakano T, Yoneyama K, Takeuchi Y, Yoshida S, Asami T. 2001. A specific brassinosteroid biosynthesis inhibitor, Brz2001: evaluation of its effects on Arabidopsis, cress, tobacco, and rice. Planta 213:716–721.

Smalle J, Haegman M, Kurepa J, Van Montagu M, Van Der Straeten D. 1997. Ethylene can stimulate Arabidopsis hypocotyl elongation in the light. Proc Natl Acad Sci USA 94:2756–2761.

Steber CM, McCourt P. 2001. A role for brassinosteroids in germination in Arabidopsis. Plant Physiol 125:763–769.

Szekeres M, Nemeth K, Koncz–Kalman Z, Mathur J, Kauschmann A, Altmann T, Redei GP, Nagy F, Schell J, Koncz C. 1996. Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in Arabidopsis. Cell 85:171–182.

Takeno K, Pharis RP. 1982. Brassinosteroid-induced bending of the leaf lamina of dwarf rice seedlings: an auxin-mediated phenomenon. Plant Cell Physiol 23:1275–1281.

Tanimoto E. 1987. Gibberellin-dependent root elongation in *Lactuca sativa*: recovery from growth retardant-suppressed elongation with thickening by low concentration of GA_3. Plant Cell Physiol 28:963–973.

Tanimoto E. 1988. Gibberellin regulation of root growth with change in galactose content of cell walls in *Pisum sativum*. Plant Cell Physiol 29:269–280.

Thimann KV. 1937. On the nature of inhibitions caused by auxin. Am J Bot 24:407–412.

Wang ZY, Nakano T, Gendron J, He J, Chen M, Vafeados D, Yang Y, Fujioka S, Yoshida S, Asami T, Chory J. 2002. Nuclear-localized BZR1 mediates brassinosteroid-induced growth and feedback suppression of brassinosteroid biosynthesis. Dev Cell 2:505–513.

Woeste KE, Vogel JP, Kieber JJ. 1999. Factors regulating ethylene biosynthesis in etiolated *Arabidopsis thaliana* seedlings. Physiol Plant 105:478–484.

Yin Y, Wang ZY, Mora–Gracia S, Li J, Yoshida S, Asami T, Chory J. 2002. BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. Cell 109:181–191.

Yokota T, Takahashi N. 1986. Chemistry, physiology and agricultural application of brassinolide and related steroids. In: Bopp M Ed. Plant growth substances 1985. Berlin: Springer-Verlag. pp 129–138.

Yokota T, Nakamura Y, Takahashi N, Nonaka M, Sekimoto H, Oshio H, Takatsuto S. 1991. Inconsistency between growth and endogenous levels of gibberellins, brassinosteroids and sterols in *Pisum sativum* treated with uniconazole antipodes. In: Takahashi N, Phinney BO, McMillan J Eds. Gibberellins. New York: Springer-Verlag. pp 339–349.

Yopp JH, Colclasure GC, Mandava NB. 1979. Effects of brassin-complex on auxin and gibberellin mediated events in the morphogenesis of the etiolated bean hypocotyl. Physiol Plant 46:247–254.

Yoshida Y, Aoyama Y. 1991. Sterol 14 alpha-demethylase and its inhibition: structural considerations on interaction of azole antifungal agents with lanosterol 14 alpha-demethylase ($P\text{-}450_{14DM}$) of yeast. Biochem Soc Trans 19:778–782.