J Plant Growth Regul (1993) 12:61–66

Journal of
Plant
Growth
Regulation

Physiological and Molecular Effects of Brassinosteroids on *Arabidopsis thaliana*

Steven D. Clouse,[1] Allen F. Hall,[1] Mark Langford,[1] Trevor C. McMorris,[2] and Michael E. Baker[3]

[1]Department of Biology, San Diego State University, San Diego, California 92182, Departments of [2]Chemistry and [3]Medicine, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093, USA

Received January 25, 1993; accepted March 25, 1993

Abstract. We examined the effects of brassinosteroids on *Arabidopsis thaliana* (L.) Henyh. ecotype Columbia in order to develop a model system for studying gene regulation by plant steroids. Submicromolar concentrations of two brassinosteroids, brassinolide and 24-epibrassinolide, stimulated elongation of *Arabidopsis* peduncles and inhibited root elongation, respectively. Furthermore, brassinolide altered the abundance of specific in vitro translatable mRNAs from peduncles and whole plants of *Arabidopsis*. Root elongation in the auxin-insensitive *Arabidopsis* mutant axr1 was inhibited by 24-epibrassinolide but not by 2,4-D, indicating an independent mode of action for these growth regulators in this physiological response.

Brassinosteroids are plant growth-promoting natural products with structural similarities to insect and animal steroid hormones (Mandava 1988). Brassinolide (BR), the first brassinosteroid to be structurally characterized (Grove et al. 1979), was originally isolated from *Brassica napus* pollen and was shown to induce cell elongation and proliferation at nanomolar concentrations in bean, rice, and many other plants. Subsequent work identified other brassinosteroids with similar structures from a broad range of plants including rice, maize, mustard, and wheat (Mandava 1988). The physiological effects and agricultural applications of brassinosteroids have been widely studied, and there is growing evidence that these compounds have properties characteristic of endogenous plant growth regulators (Sasse 1991a). The molecular mechanism of brassinosteroid action, however, remains unclear. One might argue from structural considerations that they may act by a mechanism similar to that of animal steroid hormones. In general, such hormones act via a soluble receptor/ligand complex that binds to nuclear sites to regulate the expression of specific genes (Evans 1988).

Although the effects of brassinosteroids on cell elongation and proliferation qualitatively resemble those of auxins, we have recently presented evidence that brassinolide can affect elongation and gene expression independently of auxin in soybean epicotyls (Clouse et al. 1992, Clouse and Zurek 1991). While the soybean system has been valuable for studying auxin-BR interactions, the complex genome and incomplete genetic map of soybean make it less than ideal for molecular genetic studies aimed at cloning a BR receptor. The advantages of *Arabidopsis thaliana* for a combined mutational and molecular genetic analysis of a physiological problem have been clearly demonstrated (Klee and Estelle 1991). In this paper, we show that brassinosteroids have a physiological effect on stem and root elongation in *A. thaliana* and that they also alter the pattern of gene expression in *Arabidopsis* stem tissue and whole plants. These results indicate that *Arabidopsis* will be a suitable system for a mutational/molecular genetic study of the mechanism of brassinosteroid action in plant growth.

Abbreviations: BR, brassinolide; EBR, 24-epibrassinolide; 2,4-D,2,4-dichlorophenoxyacetic acid; KPSC, 10 mM potassium phosphate, pH 6.0, 2% sucrose, 50 μg/ml chloramphenicol; PAGE, polyacrylamide gel electrophoresis.

Materials and Methods

Plant Growth and Peduncle Elongation Assay

Seeds of *Arabidopsis thaliana* (L.) Henyh. ecotype Columbia were sown in a 1:1:1 mixture of Perlite/vermiculite/sphagnum moss and covered with a thin layer of fine vermiculite. Plants

were grown in a growth chamber at a constant temperature of 22°C with 100 μE m^{-2} s^{-1} light intensity on a 14 h light/10 h dark cycle. Plants were watered regularly with a standard mineral solution (Estelle and Somerville 1987) until peduncles emerged (approximately 25 days). Peduncles of ≤4 cm were harvested and the apical 1 cm directly below the terminal infloresence was excised and floated on ice-cold KPSC buffer until required for the assay. Assay protocols were as previously described for the soybean epicotyl elongation assay (Clouse et al. 1992). Brassinolide (2α,3α22(R),23(R)-tetrahydroxy-24(S)-methyl-B-homo-7-oxa-5α-cholestan-6-one) was synthesized and characterized by NMR as previously described (McMorris et al. 1991). The naturally occurring isomer of BR, 24-epibrassinolide (EBR), was a gift of Dr. Ikekawa (Iwaki Meisei University, Iwaki, Japan) (Ikekawa and Zhao 1991). We have found EBR to have approximately 70% of the activity of BR at 10^{-7} M and 100% of the activity at 10^{-6} M in bioassays in our laboratory. The synthesis of EBR is much more efficient and economical than that of BR. Because of the relatively large amounts of hormone required, we used EBR in place of BR in the root inhibition assay and mutant screen.

Liquid Culture

Seeds of *A. thaliana* were sterilized in 100% ethanol (1 min) followed by a 30% commercial bleach solution containing 20% Triton X-100 (5 min). Seeds were then rinsed five times in a large volume of sterile distilled water and ca. 2500 seeds were aliquoted into a 1 L flask containing 100 ml of Gamborg's B-5 salts (Gamborg et al. 1968), pH 5.8, supplemented with 2% sucrose. Flasks were rotated at 100 rpm in a 22°C chamber with 100 μE m^{-2} s^{-1} constant light intensity. After 5 days, a mat of plant tissue with roots and shoots developed and the flasks were drained and fresh medium (control) or fresh medium plus 10^{-8} M BR was added. After a further 4 h of incubation, the plant material was blotted dry and frozen in liquid N_2 prior to RNA isolation.

RNA Isolation and Two-Dimensional PAGE

Total RNA was isolated from frozen peduncle sections or liquid culture plantlets by the guanidinium isothiocyanate/phenol method (Chomczynski and Sacchi 1987). Polyadenylated RNA was isolated from total RNA using the PolyAT Tract kit from Promega (Madison, WI, USA) or the Poly(A) Quick kit from Stratagene (La Jolla, CA, USA). To ensure reproducibility, control and BR-treated tissues were processed in exactly the same way throughout the experiment and all RNA extractions were done simultaneously on equivalent amounts of tissue with the same lot of reagents. Optimized in vitro translation reactions were performed on equal amounts of polyadenylated RNA using 12.5 μl wheat germ extract (Promega), 0.4 U of RNase inhibitor (Inhibit ACE, 5 prime 3 prime), 50 mM potassium acetate (final concentration), and 1.3 μl of ^{35}S-methionine (1200 Ci/mmol, NEN) in a final volume of 25 μl. A total of 300,000 cpm of acid precipitable counts from each translation was run simultaneously on first dimension tube gels in the same apparatus. Pairs of second dimension gels (control and +BR) were run together in a dual slab gel unit and fluorographed together for equal exposure. Conditions of electrophoresis and fluorography were those described previously (Clouse et al. 1992). Both the peduncle and liquid culture experiments were repeated at least twice.

Root Inhibition Assay

Seeds of wild-type *A. thaliana* (Columbia) or the axr-1 mutant (Lincoln et al. 1990) were surface sterilized as described above and suspended in 0.2% agarose. Approximately 15–20 seeds of each genotype were deposited along a straight line on 15 × 100 mm Petri plates containing 20 ml of 1% agar, 2% sucrose, and standard mineral solution (Estelle and Somerville 1987). EBR or 2,4-D was added to each medium after autoclaving. The plates were placed vertically in a growth chamber at 23°C with a 16 h light/8 h dark cycle at 50 μE m^{-2} s^{-1} intensity. After 6 days of incubation, root length was measured under a dissecting microscope equipped with an eyepiece micrometer. The experiment was replicated so that each data point represents the mean of 30 individual measurements ±SE.

Screening of EMS Mutagenized Plants

M_2 seeds of *Arabidopsis thaliana* (Columbia background), mutagenized with 0.25% ethyl methane sulfonate (EMS), were obtained from Lehle Seeds (Tucson, AZ, USA). The M_2 seeds were supplied in bulked parental groups of 8000 seeds representing 1000 M_1 parents. M_2 seeds (about 23,000 total) were placed along lines on agar plates containing 10^{-7} M EBR as described above and incubated vertically for 6 days. M_2 plants whose roots elongated substantially (>9 mm) were picked and grown to maturity to produce M_3 seeds. A large number of M_3 progeny from individual M_2 picks were then compared to wild type for their ability to elongate roots in the presence of 10^{-7} M EBR.

Results

BR Stimulates Elongation of Arabidopsis *Peduncles*

BR has pronounced effects on the elongation of stem tissue from a variety of plants including pea, Azuki bean, soybean, mung bean, cucumber, and wheat (Mandava 1988). We have found that BR, in the absence of other exogenous growth regulators, also stimulates elongation of *Arabidopsis* peduncle sections. First, we investigated the dependence of *Arabidopsis* peduncle elongation on BR concentration during a 17 h incubation. As shown in Fig. 1A, measurable elongation occurs at 10^{-9} M, and at 10^{-6} M BR there is a 35% increase in peduncle length when compared to the control. We next investigated the time course of elongation at 10^{-7} M BR. Figure 1B shows that significant elongation occurred after 4 h of incubation and a maximum was reached by 24 h. BR at 10^{-7} M was as effective as 10^{-5} M 2,4-D (an optimal concentration for elongation) in promoting peduncle elongation (data not shown).

Brassinolide Alters Gene Expression in Arabidopsis

Both plant and animal hormones are known to affect gene expression in target cells. To determine if BR affects gene expression in *Arabidopsis*, we iso-

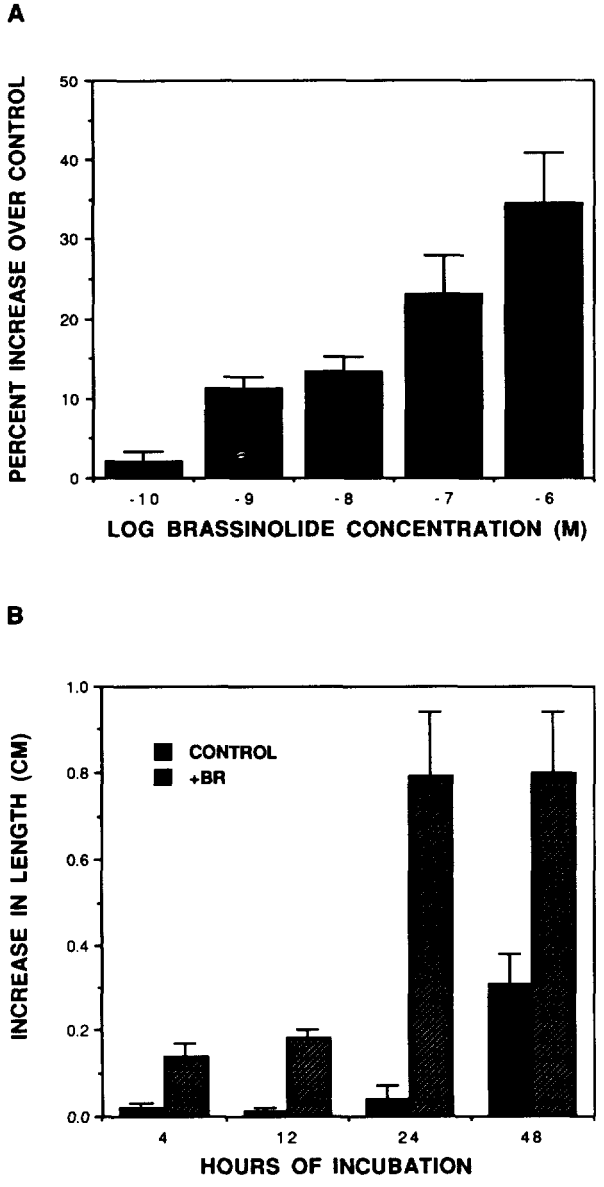

Fig. 1. Effect of brassinolide on *A. thaliana* peduncle elongation. (**A**) A minimum of 13 peduncle sections (1.0 cm) were auxin-depleted for 2 h followed by incubation with the indicated concentrations of BR for 17 h. (**B**) The standard peduncle assay was performed on a minimum of nine 1.0 cm sections (auxin-depleted for 2 h) for the times indicated. CONTROL, KPSC buffer; + BR, 10^{-7} M brassinolide. Other assay conditions are described in Materials and Methods. Error bars are ±SE.

lated mRNA from control and BR-treated peduncle sections and performed two-dimensional PAGE after in vitro translation of the mRNA. Figures 2C and D show that a 17 h incubation with 10^{-7} M BR altered the abundance of several specific in vitro translatable mRNAs. BR caused both increases and decreases in the abundance of different mRNAs, an effect we previously found with BR-treated soybean stem sections (Clouse et al. 1992, Clouse and Zurek 1991). To further investigate BR effects on gene expression, whole *Arabidopsis* plants were grown in shaking liquid cultures for 5 days followed by incubation in 10^{-8} M BR for 4 h. Figures 2A and B show that BR also had a marked effect on the expression of several in vitro translatable mRNAs at this earlier time.

24-Epibrassinolide Inhibits Root Elongation in Arabidopsis

Inhibition of root elongation by exogenous hormones is a common response in wild-type *Arabidopsis* seedlings. Auxin, cytokinin, ethylene, and methyl jasmonate all elicit this effect, and the ability of specific mutant plants from a mutagenized population to elongate their roots in the presence of these compounds has been used as a screening technique to isolate mutants insensitive to each of these growth regulators (Klee and Estelle 1991). Figure 3 shows that EBR effectively inhibits root elongation in wild-type *Arabidopsis*. EBR, at 10^{-7} M, caused a 58% inhibition of root elongation, while the same concentration of 2,4-D led to an 88% inhibition. Figure 3 also shows the effect of EBR on the axr1 mutant of *A. thaliana*, which is insensitive to exogenously applied auxin (Lincoln et al. 1990). The insensitivity of axr1 to 2,4-D is clearly shown in Fig. 3. However, EBR, unlike 2,4-D, inhibits root elongation in axr1 by 52%.

Our finding that brassinosteroids inhibit root elongation in *Arabidopsis* has allowed us to screen EMS mutagenized M_2 plants for brassinosteroid-insensitive genotypes based on their ability to elongate roots in the presence of 10^{-7} M EBR. We are currently characterizing several likely brassinosteroid-insensitive mutants (Langford and Clouse in preparation). Figure 4 shows a preliminary result on one such putative BR-insensitive mutant. Comparison of Fig. 4B and C clearly shows again the potent inhibitory effect of 10^{-7} M EBR on wild-type *Arabidopsis* root elongation. However, comparison of Fig. 4D and E shows that the selected M_3 genotype has lost sensitivity to the EBR inhibition. Furthermore, comparison of Fig. 4A and F shows that both wild-type and the putative BR-insensitive mutant are inhibited by 10^{-7} M 2,4-D. Figure 5 confirms this effect in a more quantitative manner. In the presence of 10^{-7} M EBR, the roots of M_3 seedlings of the putative mutant were approximately three times the length of the wild-type roots, while in the presence of 1.25×10^{-7} M 2,4-D the roots of both genotypes were inhibited equally.

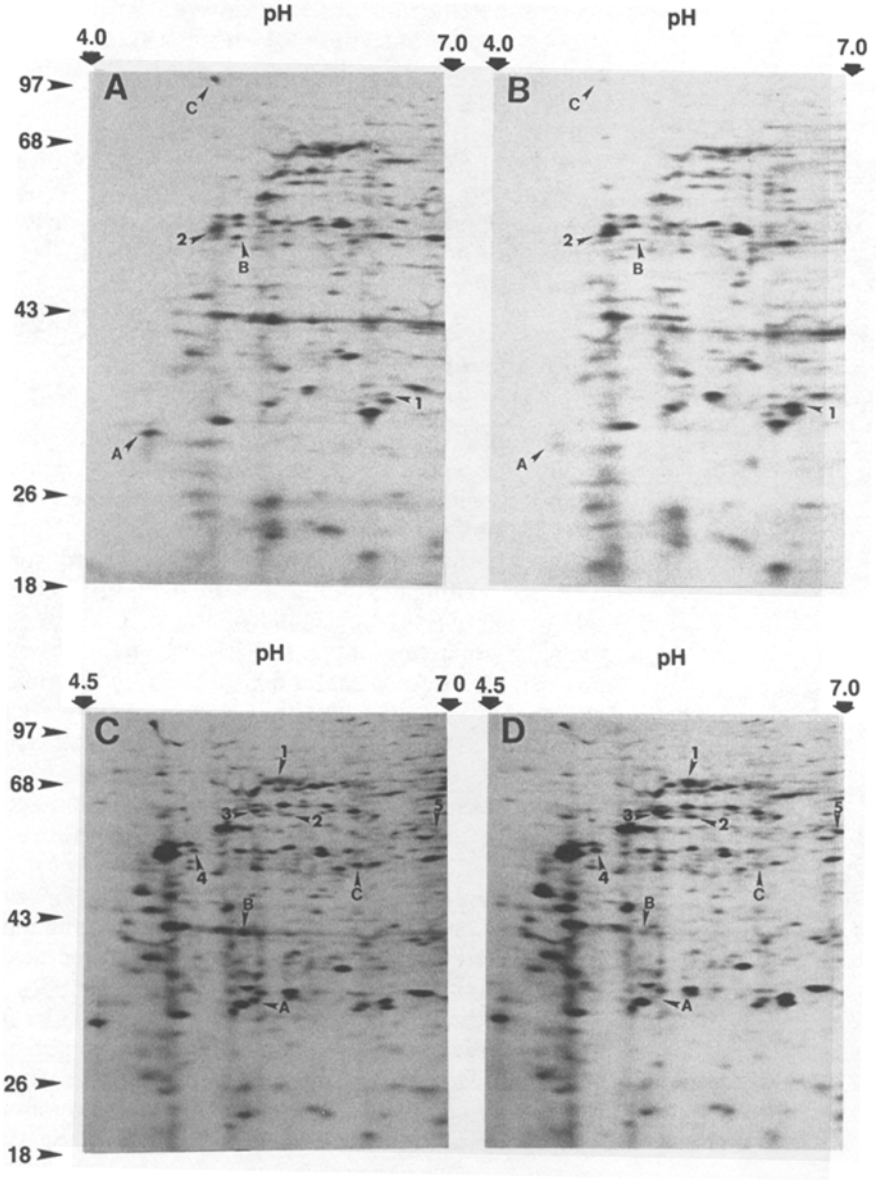

Fig. 2. Two-dimensional gel electrophoresis of in vitro translation products encoded by *Arabidopsis thaliana* mRNA. Polyadenylated RNA was isolated from 5-day-old liquid cultures treated for 4 h with buffer (**A**) or 10^{-8} M BR (**B**); or from auxin-depleted peduncle sections 17 h after treatment with buffer (**C**) or 10^{-7} M BR (**D**). RNA was translated in vitro with wheat germ extract and ^{35}S-methionine and equal counts were loaded on first dimension IEF gels. Numbers to the left of the photograph indicate molecular mass in kD. Numbered arrows point to in vitro translation products that are increased in the presence of BR, while lettered arrows point to those that are decreased in the presence of BR. Only those spots were marked with arrows which showed reproducible differences in replicate experiments.

Discussion

The marked elongation of bean second and third internodes caused by extracts of *Brassica napus* pollen was the first reported example of brassinosteroid-like activity (Mitchell 1970). Since then, purified or synthesized brassinosteroids have been shown to promote stem elongation at low concentrations in maize mesocotyls, pea, Azuki bean, mung bean, and soybean epicotyls (Yopp et al. 1981, Gregory and Mandava 1982, Mandava 1988, Clouse et al. 1992); bean and cucumber hypocotyls (Mandava et al. 1981, Katsumi 1985) and wheat coleoptiles (Sasse 1985). We show here that *Arabidopsis* peduncle sections also exhibit BR-promoted stem elongation. In fact, Sasse (1991b) has suggested that the effect of brassinosteroids on the elongation of young vegetative tissue is probably a general phenomenon. The sensitivity of *Arabidopsis* stem tissue to BR (10^{-9} M) and the kinetics of elongation are similar to the reported effects of BR on stem tissue from other species, cited above.

Our objective in studying BR-induced stem elongation in *Arabidopsis* was to establish a model system in which the molecular mechanisms underlying elongation could be examined. In particular, we are interested in the regulation of gene expression by BR. We have confirmed here that BR affects the abundance of several specific in vitro translatable mRNAs in elongating peduncle sections—

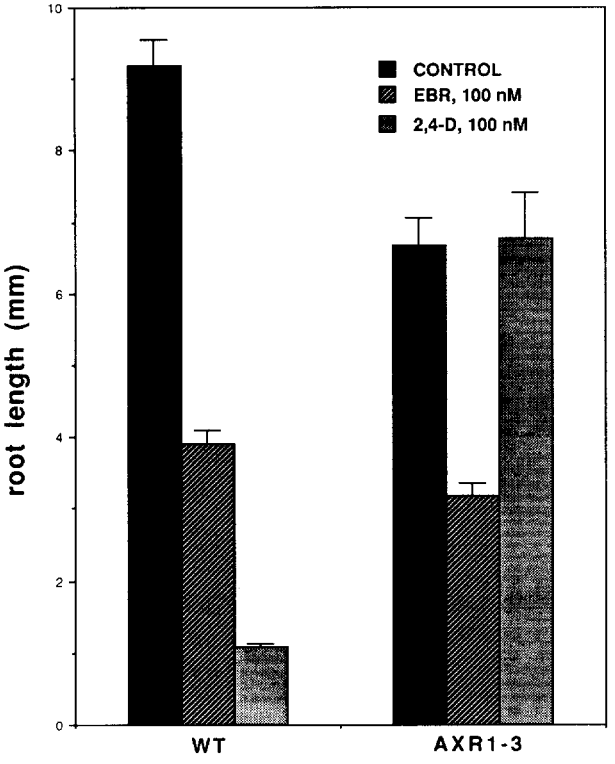

Fig. 3. Comparison of brassinosteroid and auxin effects on root elongation in *Arabidopsis thaliana*. Thirty seeds for each treatment of wild-type Columbia (WT) and an auxin-resistant mutant (axr1-3) were plated on three different media as indicated. Plates were incubated vertically for 6 days and root length was determined to the nearest 0.1 mm with a dissecting microscope. Error bars are ±SE.

increasing some and decreasing others. The results are consistent with our previous findings that BR regulates gene expression in elongating soybean hypocotyls (Clouse and Zurek 1991) and epicotyls (Clouse et al. 1992). Because of the small size of *Arabidopsis* stems, it is very tedious to collect enough peduncle sections for RNA isolation. To overcome this problem, we also examined the effect of BR on whole plants of *Arabidopsis* grown in shaking liquid culture; again, finding that BR increased some in vitro translatable mRNAs and decreased others. In order to determine whether transcriptional or posttranscriptional mechanisms are operative in this control of specific mRNA levels by BR, it will be necessary to clone the corresponding genes. We have used differential screening techniques to isolate several clones for potential BR-induced genes which we are currently characterizing (Hall and Clouse in preparation). The availability of such molecular probes will enlarge the scope of studies on BR-regulated gene expression.

A major advantage of using *Arabidopsis* as a model system is the comparative ease with which

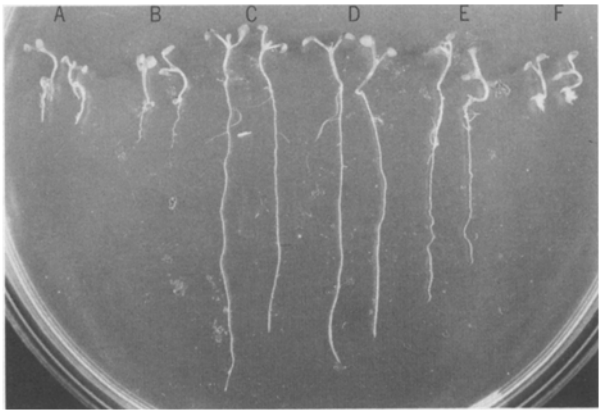

Fig. 4. EMS mutagenized *A. thaliana* plants with the ability to elongate roots in the presence of EBR. Representative duplicate 6-day-old seedlings were picked from treatment plates and placed on a single agar plate for photography. **(A)** Wild type (Columbia) treated with 10^{-7} M 2,4-D; **(B)** wild type treated with 10^{-7} M EBR; **(C)** wild type control **(D–F)** M_3 progeny of a single M_2 plant selected for root elongation in the presence of 10^{-7} M EBR. **D**, control; **E**, 10^{-7} M EBR; **F**, 10^{-7} M 2,4-D.

mutants can be isolated. During the past decade, several groups have isolated mutant *Arabidopsis* plants with defective perception or response to plant hormones. The axr1 (Lincoln et al. 1990), AXR2 (Wilson et al. 1990), and aux1 (Pickett et al. 1990) auxin-resistant mutants and the ckr1 (Su and Howell 1992) cytokinin-resistant mutant were selected by the ability of the mutant plants to elongate roots in the presence of hormone concentrations that inhibited root growth in wild-type *Arabidopsis*. We have shown here that brassinosteroids exhibit a similar inhibitory effect on *Arabidopsis* roots and that EMS mutagenized plants can be selected which are insensitive to this response. To confirm that our putative BR-resistant mutant is controlled by a single genetic locus, we are currently analyzing F_1 and F_2 progeny of M_3 plants backcrossed to the wild-type parent, in addition to characterizing further the response of the M_3 plants to brassinosteroids and auxins. The preliminary data presented here, however, clearly show the promise of this approach as well as clarifying the interaction of brassinosteroids and auxin.

Based on physiological studies, it has been suggested that the effects of BR are mediated through auxin or that BR increases tissue sensitivity to endogenous auxins (Mandava 1988). Our observation that EBR inhibits root elongation in the axr1 auxin-insensitive mutant suggests that brassinosteroids can function independently of auxins. The inhibition of root elongation by auxin in the putative BR-insensitive mutant further supports this independence. This novel genetic data gives independent corroboration of our previous finding using RNA blot analysis that BR can act independently of auxin

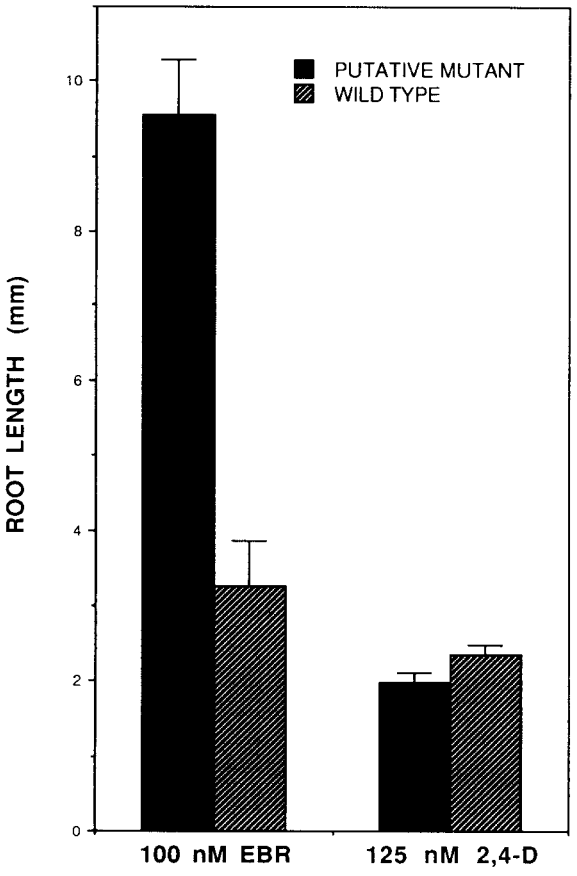

Fig. 5. Root elongation of wild-type *A. thaliana* and a putative BR-resistant mutant in the presence of EBR and 2,4-D. A minimum of 10 M_3 progeny from a single M_2 parent (putative mutant) or from wild type (Columbia) were assayed for root elongation as described in Materials and Methods. Error bars are ±SE.

in elongating soybean epicotyls (Clouse et al. 1992) and those of Sasse (1990), who showed that the anti-auxin, 2-(p-chlorophenoxy) isobutryic acid, did not affect BR-induced elongation in *Pisum sativum*.

The availability of BR-insensitive mutants may facilitate identification and cloning of genes for a BR receptor, as well as genes regulating other aspects of the diverse effects of brassinosteroids on plants. Our findings of both physiological and molecular effects of brassinosteroids on *Arabidopsis thaliana* will allow the development of a model system useful for studying steroid gene regulation in plants.

Acknowledgments. This research was supported in part by USDA/CRGO grant no. 90-37261-5700 and NSF grant no. DCB-9013409. We thank Dr. Ikekawa for the 24-epibrassinolide sample and Drs. David Rayle and Ken Johnson for critically reading the manuscript.

References

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium extraction. Anal Biochem 162:156–159

Clouse SD, Zurek DM (1991) Molecular analysis of brassinolide action in plant growth and development. In: Cutler HG, Yokota T, Adam G (eds) Brassinosteroids: Chemistry, bioactivity and applications, ACS Symposium Series; 474. American Chemical Society, Washington, DC, pp 122–140

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

Estelle MA, Somerville CR (1987) Auxin-resistant mutants of Arabidopsis with an altered morphology. Mol Gen Genet 206:200–206

Evans RM (1988) The steroid and thyroid hormone receptor superfamily. Science 240:889–895

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:148–151

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

Grove MD, et al. (1979) A unique plant growth promoting steroid from *Brassica napus* pollen. Nature 281:216–217

Ikekawa N, Zhao YJ (1991) Application of 24-Epibrassinolide in Agriculture. In: Cutler HG, Yokota T, Adam G (eds) Brassinosteroids: Chemistry, bioactivity and applications, ACS Symposium Series; 474. American Chemical Society, Washington, DC, pp 280–291

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

Klee H, Estelle M (1991) Molecular genetic approaches to plant hormone biology. Ann Rev Plant Physiol Plant Mol Biol 42:529–551

Lincoln C, Britton JH, Estelle M (1990) Growth and development of the axr1 mutants of *Arabidopsis*. Plant Cell 2:1071–1080

Mandava NB, Sasse JM, Yopp JH (1981) Brassinolide, a growth-promoting steroidal lactone. II. Activity in selected gibberellin and cytokinin bioassays. Physiol Plant 53:453–461

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

McMorris TC, Donaubauer JR, Silveira MH, Molinski TF (1991) Synthesis of brassinolide. In: Cutler HG, Yokota T, Adam G (eds) Brassinosteroids: Chemistry, bioactivity and applications, ACS Symposium Series, 474. American Chemical Society, Washington, DC, pp 36–42

Mitchell JW, Mandava NB, Worley JP, Plimmer JR (1970) Brassins: A new family of plant hormones from rape pollen. Nature 225:1065–1066

Sasse JM (1985) The place of brassinolide in the sequential response to plant growth regulators in elongating tissue. Physiol Plant 63:303–308

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

Sasse JM (1991a) The case for brassinosteroids as endogenous plant hormones. In: Cutler HG, Yokota T, Adam G (eds) Brassinosteroids: Chemistry, bioactivity and applications, ACS Symposium Series; 474. American Chemistry Society, Washington, DC, pp 158–166

Sasse JM (1991b) Brassinolide-induced elongation. In: Cutler HG, Yokota T, Adam G (eds) Brassinosteroids: Chemistry, bioactivity and applications, ACS Symposium Series, 474. American Chemical Society, Washington, DC, pp 255–264

Yopp JH, Mandava NB, Sasse JM (1981) Brassinolide, a growth-promoting steroidal lactone. I. Activity in selected auxin bioassays. Physiol Plant 53:445–452