Plant Physiol. (1993) 101: 965–968

Brassinosteroid Stimulation of Hypocotyl Elongation and Wall Relaxation in Pakchoi (*Brassica chinensis* cv Lei-Choi)[1]

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Hypocotyl elongation of pakchoi (*Brassica chinensis* cv Lei-Choi) was stimulated by applying 300 ng of brassinosteroid (2α,3α,22β,23β-tetrahydroxy-24β-methyl-B-homo-7-oxa-5α-cholestan-6-one, BR) in 1 μL of 50% ethanol to the apex of hypocotyls. BR had its greatest effect on elongation of the apical 3-mm region below the cotyledonary node (75% stimulation) between 6 and 18 h after treatment. Stress/strain (Instron) analysis of this 3-mm region revealed that plastic and elastic components of extension were not significantly different between BR-treated and control seedlings. In pressure-block experiments, the initial rate of relaxation was 2-fold faster in BR-treated plants as compared with controls, whereas after 125 min the total amount of relaxation and the relaxation rate were the same for the two treatments. Osmotic pressure of cell sap expressed from this 3-mm region showed a large decrease (28%) in BR-treated seedlings compared to the controls. We conclude that BR stimulates growth in pakchoi by accelerating the biochemical processes that cause wall relaxation, without inducing a large change in wall mechanical properties.

In 1970, brassins were isolated from rape (*Brassica napus* L.) pollen by Mitchell and coworkers (1970). Brassinolide (2α,3α,22α,23α-tetrahydroxy-24α-methyl-B-homo-7-oxa-5α-cholestan-6-one) was identified as a major biologically active component of brassins (Grove et al., 1979). Brassinolide and its active analogs have been synthesized by Thompson and coworkers (1979).

BRs induce elongation of normal and dwarf pea epicotyls, dwarf bean apical segments, mung bean epicotyls, cucumber hypocotyls, Azuki bean epicotyls, and sunflower hypocotyls (Mandava, 1988). In cucumber hypocotyl sections, BR-induced elongation was inhibited in the presence of *p*-chlorophenoxyisobutyric acid and kinetin (Katsumi, 1985). BR acted synergistically with auxin in elongation (Yopp et al., 1981; Katsumi, 1985) but showed an additive effect with GA_3 in cucumber hypocotyl sections (Katsumi, 1985). BR also acted synergistically on auxin-induced ethylene production in etiolated mung bean segments (Arteca et al., 1983). A membrane-bound ATPase inhibitor, dicyclohexylacarbodiimide, inhibited the BR- or IAA-induced elongation but did not affect GA-induced elongation (Katsumi, 1985). These responses have suggested that BR acts through processes involving auxin.

However, northern blot analysis using cDNAs corresponding to auxin-regulated genes as probes has shown that the molecular mechanism of BR-induced elongation is likely to differ from that of auxin-induced elongation in this system (Clouse and Zurek, 1991). In two-dimensional gel analysis of in vitro translated mRNA, BR altered the pattern of gene expression in elongating soybean stem sections in both the presence and absence of added auxin (Clouse and Zurek, 1991).

Auxins stimulate growth by increasing stress relaxation of the wall (Cosgrove, 1985), and this effect is usually accompanied by a mechanical weakening of the wall (Cleland, 1984) and enhanced xyloglucan turnover (Labavitch and Ray, 1974; Bret-Harte et al., 1991). In contrast, almost nothing is known about the biophysical and biochemical mechanisms for BR stimulation of stem elongation.

In this study, we examined BR stimulation of elongation in pakchoi (*Brassica chinensis* cv Lei-Choi) and analyzed its biophysical mechanism of action. For this approach, we separated stem elongation into processes that control wall expansion and water uptake to determine which physical growth parameters are affected by BR. Osmotic pressure was measured directly, and in vivo wall relaxation properties were assessed in living tissue using the pressure-block technique. We also assessed wall mechanical properties in frozen-thawed tissue by Instron analysis. The results show that BR stimulates growth by increasing wall relaxation without a concomitant change in wall mechanical properties.

MATERIALS AND METHODS

Plant Preparation

Pakchoi (*Brassica chinensis* cv Lei-Choi) hypocotyls were grown in a slant board sandwich, as described previously (Wang and Arteca, 1992), for 5 d under continuous cool-white fluorescent light (3 μmol m^{-2} s^{-1}) at 25 ± 2°C. For pressure-block experiments, each plant was grown in a vial filled with paper matrix (KimPak, Seedburo Equipment Co., Chicago, IL) under the same conditions as above.

Effects on Elongation

BR(2α,3α,22β,23β-tetrahydroxy-24β-methyl-B-homo-7-oxa-5α-cholestan-6-one; provided by Dr. N.B. Mandava) was

[1] Contribution No. 220, Department of Horticulture, The Pennsylvania State University. This research was partially supported by a U.S. Department of Energy grant to D.J.C.

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Abbreviations: BR, brassinosteroid; ε, volumetric elastic modulus; *P*, turgor pressure; φ, wall yield coefficient; *r*, initial relaxation rate of wall; *Y*, yield threshold.

dissolved in 50% ethanol, and 1 μL of the solution was applied to the apex of the hypocotyls. To the control plants, 1 μL of 50% ethanol was applied.

The elongation of hypocotyls was measured from the node below the cotyledons to the edge of the slant board with a ruler. Five-day-old seedlings (approximately 10–15 mm tall) were used.

Marking Experiments (Growth Kinematics)

From the time course of BR action, the time of the greatest effect of BR on elongation was found to be between 6 and 18 h. Therefore, at 6 h we applied fine horizontal marks to the hypocotyl with an eyebrow hair coated with black water-based paint (Liquitex "Ivory Black" acrylic; Binney and Smith, Inc., Easton, PA). The zones were numbered sequentially from the node to the base, and photographs were taken at 6 and 18 h. Displacement of the marks was analyzed with a digitizing tablet, microcomputer, and custom software.

Instron Analysis

This method to assess wall mechanical properties was used according to that described by Taylor and Cosgrove (1989) with slight modifications. The region with maximal BR effect, as determined from the marking experiments, was the 3-mm zone below the cotyledonary node. For Instron testing, seedlings were frozen 12 h after treatment (the time of maximal growth response), pressed to remove excess cell sap, and clamped so that the apical 3-mm zone was held between the two clamps. The section was extended in two cycles at 3 mm min^{-1} until a limiting load value of 10 g was reached. The slopes at the end of each cycle were used to calculate total, elastic, and plastic extensibilities, normalized to a 100-g load.

Pressure-Block Experiments and Osmotic Pressure

Twelve hours after treatment with BR or ethanol, seedlings were sealed into the pressure-block chamber as previously described (Cosgrove, 1988; Taylor and Cosgrove, 1989) except that the relaxation measurement was restricted to the 3-mm apical zone showing maximal BR response. For osmotic pressure measurement, 20 of the 3-mm apical hypocotyl segments were cut, and their expressed cell sap was assayed with a vapor pressure osmometer (model 5500; Wescor, Logan, UT).

The Instron analysis pressure-block and osmotic pressure data were subjected to analysis of variance (StatView 512+, version 1.1; BrainPower, Inc., Calabasas, CA) at the 95% confidence level.

RESULTS

Effect of BR on Pakchoi Hypocotyl Elongation

In preliminary experiments, a series of concentrations (0–1000 ng μL^{-1}) of BR were applied to the apex of pakchoi hypocotyl (data not shown). We obtained the same growth rate when BR concentrations above 300 ng μL^{-1} were applied, and hypocotyls treated with 1000 ng μL^{-1} of BR showed twisting. This twisting response might be caused by BR-stimulated ethylene production (Arteca et al., 1983). Therefore, we decided to use 300 ng μL^{-1} in the remaining studies.

BR had the greatest effect on elongation between 6 and 18 h after treatment; the ratio of elongation rate of the BR-treated seedlings to the control was approximately 1.75 (Fig. 1). To identify the stem region with maximal response, marking experiments were performed at 6 and 18 h. BR had the greatest growth effect on the apical 3-mm region below the cotyledonary node (Fig. 2).

Instron Analysis

Instron analysis of this 3-mm region revealed that plastic and elastic components of wall extension were not significantly affected by BR treatment (at 12 h) when a 10-g load limit was used (Table I). We also tried a 15- or 20-g load limit, but the hypocotyl segments broke too frequently for reliable results.

Pressure-Block Experiments and Osmotic Pressure

The pressure-block experiments proved challenging because not only were the pakchoi seedlings very small but their cell wall relaxation rates were unusually slow (Fig. 3). The initial pressure required to stop stem elongation was very small (<0.1 bar), which means that the internal water potential gradient that sustained cell growth was small and that tissue hydraulic conductance did not limit growth (Cosgrove, 1988). BR increased the initial rate of relaxation approximately 2-fold (Table I) but had no significant effect on the total amount of relaxation or the rate of relaxation at 125 min. In theory (Cosgrove, 1985) the initial relaxation rate (r) is equal to $\phi\epsilon$ ($P - Y$). The fact that the relaxation value at 125 min was statistically the same for control and BR-treated seedlings indicated that BR had little or no effect on $P - Y$, which was the maximal relaxation attainable. Given that ϵ is unchanged, as evidenced by the Instron data, the results argue that BR increased ϕ by 2-fold. However, we should

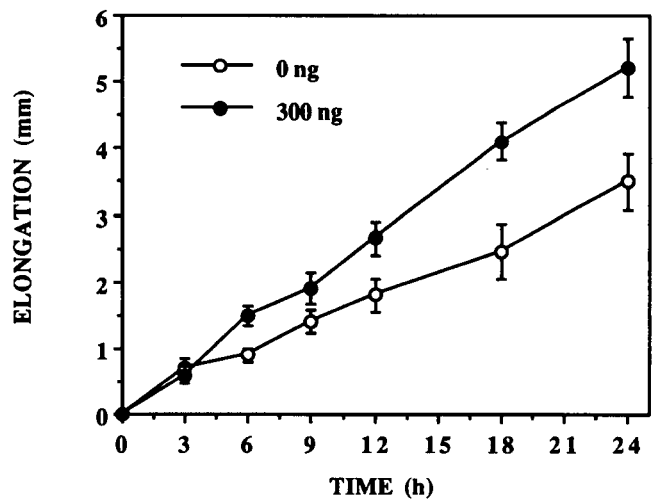

Figure 1. Effect of BR on hypocotyl length of pakchoi seedlings. Each point is the mean of 15 replications; bar, ±SE.

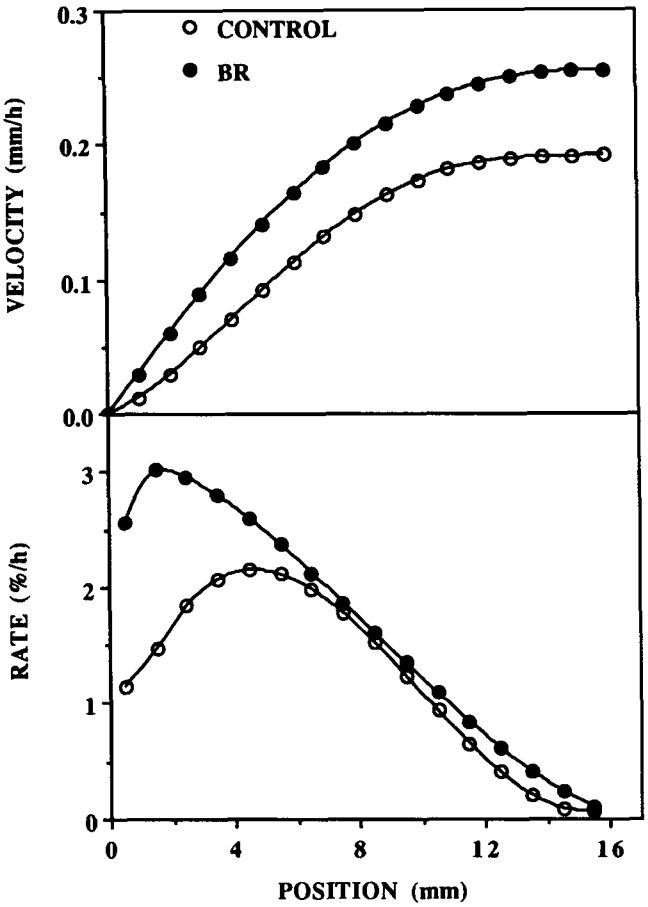

Figure 2. Effect of BR on the growth kinematics of pakchoi hypocotyls, determined by marking experiments. Plants were marked and photographed 6 h after BR application and photographed again 12 h later. The velocity graph was calculated from these data (top), and its derivative gives the growth rate graph (bottom). Ten plants were marked for each treatment.

note that the cell walls of most seedlings kept relaxing for >150 min, which meant that that total relaxation value at 125 min was an underestimate of $P - Y$.

Direct measurement of the osmotic pressure of cell sap expressed from the apical 3-mm region revealed a large decrease in BR-treated seedlings compared to the control (Table I). This is likely a consequence of dilution of cell sap by the faster growth in the BR-treated seedlings.

DISCUSSION

Cell elongation has been postulated to be controlled by at least two processes, namely the uptake of water by osmosis and the relaxation and subsequent expansion of the cell wall. In this study, BR decreased the cell osmotic pressure when it promoted cell elongation (Table I). This result argues against the possibility that BR stimulates growth by increasing cell osmotic pressure and P. Instron analysis (Table I) shows that BR did not alter the cell wall mechanical properties while stimulating elongation. However, in preliminary assays using a 15- or 20-g load limit, breakages were more frequent in BR-treated seedlings than in controls, suggesting that there might be some mechanical weakening of the wall by BR. The pressure-block experiments (Fig. 3 and Table I) showed that BR increased r but did not change the maximal relaxation.

These results are best understood as an increase in ϕ without a change in $P - Y$. If we assume that growth rate is given by $\phi(P - Y)$, then the 2-fold increase in growth rate by BR is evidently the result of a 2-fold increase in ϕ (evidenced by the 2-fold higher r) with little or no change in $P - Y$ (evidenced by the fact that the same relaxation values were observed at 125 min in the two treatments). This conclusion assumes that ϵ is unchanged by BR, which seems likely from the Instron results. The small pressures needed to block growth at the start of the pressure-block relaxations (Fig. 3) indicated that internal water potential gradients were small, and therefore, tissue hydraulic conductance was large (Cosgrove, 1988). Because there is no net water uptake during pressure-block relaxations (recall that tissue size is held constant during the measurement), hydraulic conductance does

Table I. *Instron analysis, pressure-block experiments, and osmotic pressure measurements on pakchoi hypocotyls 12 h after treatment with or without BR*

Means ± SE. *n*, Sample number.

Treatment	BR	
	0 ng	300 ng
Instron analysis (n = 25)		
Total extension (% per 100-g load)	78.8 ± 5.28	89.5 ± 5.47
Elastic extension (% per 100-g load)	34.9 ± 0.94	37.4 ± 1.49
Plastic extension (% per 100-g load)	44.0 ± 4.64	52.1 ± 4.64
Pressure-block experiments (n = 13)		
Initial relaxation rate (bar h^{-1})	0.75 ± 0.14[a]	1.73 ± 0.30[a]
Total relaxation at 125 min (bar)	1.43 ± 0.14	1.68 ± 0.19
Relaxation rate at 125 min (bar h^{-1})	0.319 ± 0.045	0.362 ± 0.058
Osmotic pressure (n = 13)[b]	6.55 ± 0.149[a]	4.73 ± 0.059[a]

[a] Significantly different at the 95% confidence level (treated by analysis of variance). [b] Twenty 3-mm hypocotyl segments for each sample.

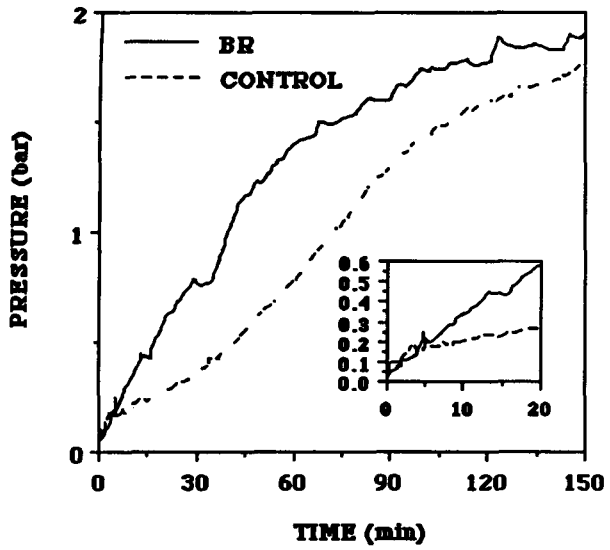

Figure 3. Typical cell wall relaxation time courses for pakchoi hypocotyls after BR or ethanol treatment, using the pressure-block technique. The 3-mm apical zone of the seedling was sealed into the pressure-block chamber 12 h after treatment with BR or ethanol. Chamber pressure was continuously adjusted to hold stem length constant. The inset shows the initial 20-min relaxation of both treatments. Note that the small pressures required to stop expansion indicates that the internal water potential gradients sustaining cell expansion are small.

not complicate the interpretation of these data. This lack of water flow gives in vivo stress-relaxation methods a decided advantage over other biophysical methods of growth analysis, which rely on growth or tissue deformation as part of the measurement and which, therefore, can be complicated by possible hydraulic limitations. Therefore, we conclude that BR stimulates stem elongation by increasing ϕ, and the decrease in the osmotic pressure of the cell sap is an indirect effect of greater dilution by the faster growth.

Like BR, auxin does not stimulate cell elongation by increasing the osmotic pressure of the cell sap in pea stem tissue (Cosgrove and Cleland, 1983) but acts by increasing ϕ (Cosgrove, 1985). However, unlike auxin action (Masuda, 1978; Cleland, 1984), BR stimulation of growth was not associated with enhanced mechanical extensibility, at least as assayed by the Instron technique. This result implies that BR acts by a different mechanism of wall loosening than does auxin. The only caveat to this conclusion is that the action of auxin on *Brassica* hypocotyls has not been well investigated and may differ from responses typically found in oat coleoptiles (Cleland, 1984), pea epicotyls (Kutschera and Briggs, 1987), and many other stem tissues (Cleland, 1986).

To summarize, our experiments have shown that BR increases elongation of *B. chinensis* hypocotyls with little or no change in the mechanical properties of cell walls but with an increase in wall relaxation properties (ϕ) and a passive dilution of the osmotic pressure of the cell sap. The molecular mechanisms of these actions may differ from those of auxin and GA in inducing elongation and, thereby, produce the well-known synergistic and additive effects of BR with these agents.

ACKNOWLEDGMENT

Sincere thanks are extended to Dr. N.B. Mandava for providing brassinosteroid used in this study.

Received September 24, 1992; accepted December 7, 1992.
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