Molecular Cloning and Characterization of a Brassinosteroid-Regulated Gene from Elongating Soybean (*Glycine max* L.) Epicotyls[1]

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Brassinosteroids promote elongation and regulate gene expression in soybean (*Glycine max* L.) stems. We constructed a cDNA library from brassinosteroid-treated soybean epicotyls and used differential hybridization to isolate a cDNA (pBRU1) corresponding to a transcript whose abundance is increased by brassinosteroid treatment. Sequence analysis of pBRU1 revealed an open reading frame of 283 amino acids with a putative signal peptide of 29 amino acids. The sequence had extensive homology (77% identity, 89% similarity) over 114 contiguous amino acids to the *meri-5* gene of *Arabidopsis thaliana* (J.I. Medford, J.S. Elmer, H.J. Klee [1991] Plant Cell 3: 359–370), and significant homology (48% identity, 62% similarity) to a xyloglucan endotransglycosylase localized in the cell walls of nasturtium (J. de Silva, C.D. Jarman, D.A. Arrowsmith, M.S. Stronach, S. Chengappa, C. Sidebottom, J.S. Reid [1993] Plant J 3: 701–711). RNase protection studies showed that *BRU1* transcript levels are not increased by 1.0 μM auxins, cytokinins, abscisic acid, or gibberellic acid and that *BRU1* expression is highest in stem tissue. Findings from studies with run-on transcripts from isolated soybean nuclei most likely indicate that the regulation of *BRU1* by brassinosteroids is largely posttranscriptional. The elevated levels of *BRU1* transcripts in elongating tissue and the homology with a xyloglucan endotransglycosylase suggest a possible role for the BRU1 protein in brassinosteroid-stimulated elongation.

Brassinosteroids are widely distributed natural products that promote growth at nanomolar concentrations and appear to possess all of the properties necessary for classification as plant hormones (Sasse, 1991a, 1992). Brassinosteroids are unique among plant growth regulators because of their close structural similarity to animal and insect steroid hormones (Mandava, 1988). Although a great deal is known about the molecular mechanisms by which steroid hormones regulate gene expression in vertebrates and insects (Evans, 1988), the gene-regulating properties of steroids found in higher plants are largely unknown. We recently used two-dimensional gel analysis to show that BR, a highly active brassinosteroid, altered the abundance of specific in vitro translatable mRNAs in elongating stem sections of soybean (*Glycine max* L.) and *Arabidopsis thaliana* (Clouse et al., 1992, 1993). Brassinoster-

oids were also shown to alter the levels of specific polypeptides in wheat leaves (Kulaeva et al., 1991) and pea stems (Sasse, 1991b). Moreover, Arteca et al. (1988) found that BR and IAA increased levels of ACC synthase in mung bean hypocotyls. In no case, however, has the cloning of a BR-responsive cDNA been reported. The objective of the present study was to clone and characterize a gene regulated by BR.

To clone a BR-regulated gene, we chose soybean epicotyls as a model system in which the molecular mechanisms underlying elongation could be examined. Brassinosteroids have a range of physiological effects, but promotion of stem elongation is perhaps the best characterized. BR-promoted elongation of young vegetative tissue has been observed in at least 15 different species and appears to be a general effect (Sasse, 1991a). We previously showed that BR is a potent enhancer of epicotyl elongation in soybeans (Clouse and Zurek, 1991), and we examined the effect of BR on cell wall mechanical properties and gene expression in this system (Clouse et al., 1992; Zurek et al., 1994). It is well known that auxin also promotes stem elongation (Taiz, 1984), and comparisons of auxin versus BR-stimulated growth showed that the kinetics of elongation and effects on gene expression were quite different for BR than for auxin (Clouse et al., 1992).

Soybean stems have also been used to clone auxin-regulated genes, and several gene families have been identified that are rapidly induced by auxin in elongating soybean hypocotyls (Walker and Key, 1982; Hagen et al., 1984; McClure and Guilfoyle, 1987). However, the functions of the corresponding gene products are unknown, and their causal roles in elongation have not been established. Because load-bearing bonds in the cell wall must be disrupted and re-formed during elongation (Cosgrove, 1993), it is important to identify the genes responsible for altering wall properties. Recently, de Silva et al. (1993) reported the cloning of a cDNA for an XET from nasturtium that has been proposed to be a wall-loosening enzyme. Whether it is truly a wall-loosening enzyme is currently being debated (McQueen-Mason et al., 1993), but the localization of the enzyme in the cell wall and its substrate specificity for wall xyloglucans are known (de Silva et al., 1993; Fanutti et al., 1993).

In this paper we describe the cloning and characterization

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Abbreviations: BR, brassinolide; KPSC, 10 mM potassium phosphate (pH 6.0), 2% sucrose, 25 μg mL⁻¹ chloramphenicol; XET, xyloglucan endotransglycosylase.

of a cDNA (pBRU1) corresponding to a soybean epicotyl mRNA whose abundance is increased by BR treatment. Sequence analysis shows that pBRU1 has significant homology with the nasturtium XET and even greater homology with the *meri-5* gene of *A. thaliana* (Medford et al., 1991), which is expressed in meristematic and stem tissues of crucifer species. Results concerning the kinetics of induction, effects of other growth regulators on BRU1 expression, and the mechanism of regulation are presented.

MATERIALS AND METHODS

Plant Growth

Soybean seeds (*Glycine max* L., cv Williams 82, purchased from Wilkens Seed Grains, Pontiac, IL) imbibed water overnight and were sown in flats containing 50% vermiculite/50% perlite. For experiments requiring elongating epicotyl sections or for BRU1 expression studies of young plants, seedlings were grown for 10 to 14 d in a greenhouse under natural lighting conditions before harvesting. For *BRU1* expression studies of older plant organs, 14-d-old seedlings were transplanted to individual pots containing University of California soil mix and grown for an additional 14 to 60 d in the same greenhouse.

Epicotyl Elongation Assays

BR [2α,3α,22(*R*),23(*R*)-tetrahydroxy-24(*S*)-methyl-B-homo-7-oxa-5α-cholestan-6-one] was synthesized by Dr. Trevor McMorris (University of California, San Diego) as previously described (McMorris et al., 1991) and stored as a 1 mM stock solution in absolute ethanol at −20°C. Epicotyl sections were obtained from the first 1.5 cm immediately below the plumule of 10- to 14-d-old soybean seedlings and floated on ice-cold KPSC buffer until required for the assay. Sections (20–25) were placed in a 50-mL Erlenmeyer flask containing 10 mL of KPSC and rotated at 125 rpm in a 27°C shaking incubator under continuous illumination (25 μE m^{-2} s^{-1}). Sections were preincubated for 2 h as previously described (Clouse et al., 1992) before the addition of fresh KPSC containing BR. Controls were incubated in KPSC with appropriate concentrations of ethanol. Epicotyl length was measured to the nearest 1 mm, and sections were homogenized for isolation of nuclei or immediately frozen in liquid N$_2$ for RNA isolation.

RNA Isolation and Analysis

Total RNA was isolated from frozen epicotyl sections with 4.0 M guanidinium isothiocyanate and acidified phenol by the method of Chomczynski and Sacchi (1987). Northern blot analysis was performed as previously described (Clouse et al., 1992) using as a probe 1 × 10^6 cpm mL^{-1} of the pBRU1 cDNA insert labeled with [^{32}P]dCTP by random priming (Prime-It II kit, Stratagene, La Jolla, CA). To confirm equal loading of RNA in each lane, blots were stripped and reprobed with a soybean actin cDNA (Clouse et al., 1992). Each blot was repeated a minimum of three times.

The probe for RNase protection assays was generated by digesting pBRU1 with *Pst*I and rendering the resulting 3' overhang blunt ended with Klenow fragment of DNA polymerase I. After proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation, the purified DNA fragment was used as a template for in vitro RNA transcription. The final reaction contained 1 μg of DNA; 1× transcription buffer (40 mM Tris [pH 8.0], 8 mM MgCl$_2$, 2 mM spermidine, 50 mM NaCl); 30 mM DTT; 40 units of RNase Block I; 50 μCi of [α-^{32}P]UTP (800 Ci mmol^{-1}); 400 μM each ATP, GTP, and CTP; and 10 units of T7 RNA polymerase. After 30 min at 37°C, 10 units of RNase-free DNase was added, and the reaction was incubated for an additional 15 min. Labeled probe was separated from unincorporated nucleotides by passage through a NucTrap column (Stratagene). The resulting probe represented a 281-bp antisense RNA complementary to the 3' end of the BRU1 mRNA. RNase protection was performed by hybridizing 8 × 10^4 cpm of this probe with 5 μg of total RNA from various treatments using the Ribonuclease Protection Assay II kit (Ambion, Austin, TX) according to manufacturer's instructions. Gels were exposed to preflashed film with intensifying screens (Laskey and Mills, 1975) and quantitated by scanning densitometry in the linear range of A. The RNase protection experiment was performed in triplicate, and the densitometry results were averaged.

cDNA Library Construction and Screening

Apical epicotyl sections (1.5 cm) were excised from 11-d-old soybean seedlings and treated with KPSC buffer, buffer containing 0.1 μM BR, or buffer containing 50 μM 2,4-D for 17 h as described above. After total RNA isolation, polyadenylated RNA was purified from each treatment using biotinylated oligo(dT) and streptavidin-coated magnetic beads (PolyATtract system; Promega, Madison, WI). A cDNA library was constructed in λZap II from 7.2 μg of polyadenylated RNA (0.1 μM BR treatment), following the manufacturer's instructions (Stratagene). The unamplified library contained 1.11 × 10^6 primary plaques with a 2.3% non-recombinant background. After a single amplification, the library was stored in 7% DMSO at −80°C.

For differential screening of the cDNA library, duplicate lifts were made from 10 plates (150 mm, 1000 plaque-forming units/plate). Each duplicate set was hybridized with ^{32}P-labeled first-strand cDNA synthesized from polyadenylated RNA isolated from the buffer or BR-treated epicotyls. Details of probe synthesis, hybridization, and washing conditions are described elsewhere (Cochran et al., 1987). Examination of the resulting autoradiographs revealed 12 plaques that hybridized more strongly to the plus-BR probe than to the control probe. The 12 plaques were picked and rescued into plasmid form (Bluescript SK−) by in vivo excision with R408 m13 helper phage as described by the manufacturer (Stratagene).

The 12 recombinant plasmids were subjected to dot blot analysis in duplicate using Zeta-Probe nylon membranes (Bio-Rad, Richmond, CA). One set of dot blots was hybridized with ^{32}P-labeled first-strand cDNA synthesized from polyadenylated RNA isolated from BR-treated epicotyls, and the duplicate set was hybridized with cDNA derived from polyadenylated RNA from 2,4-D-treated epicotyl tissue. A

single cDNA clone, pBRU1, that hybridized to the plus-BR but not the plus-auxin probe was identified.

DNA Sequence Analysis

Nested deletions of pBRU1 were made with exonuclease III by the procedure of Henikoff (1984). Both strands of the original clone and the deletions were sequenced by two independent methods: manually, using dideoxy thermal cycle sequencing (Vent Polymerase; New England Biolabs, Beverly, MA), and with Taq polymerase on an Applied Biosystems 373A automated sequencer. Areas of ambiguity were resolved manually with Sequenase 2.0 (United States Biochemical, Cleveland, OH).

The DNA sequence was analyzed using the University of Wisconsin Genetics Computer Group package (Devereux et al., 1984) on a VAX computer. After the open reading frame was identified, the program PEPTIDESTRUCTURE was used to determine molecular mass, isoelectric point, and hydrophobicity (using the algorithm of Kyte and Doolittle, 1982). Initial sequence comparisons were performed using the National Library of Medicine NIH BLAST program (Altschul et al., 1990). Positives were retrieved from SwissProt, Protein Information Resource, and GenBank libraries, and percentages of identity and similarity were determined with the University of Wisconsin Genetics Computer Group program BESTFIT.

Genomic DNA Isolation and Southern Blot Analysis

Soybean seedlings were grown in a greenhouse for 12 d and then transferred to a dark growth chamber for 48 h to reduce starch content. Leaf tissue (5 g) was ground to a fine powder in liquid N_2 and transferred to a 50-mL conical tube. After the liquid N_2 was sublimated, the powder was suspended in 25 mL of extraction buffer (100 mM Tris [pH 8.0], 100 mM EDTA [pH 8.0], 250 mM NaCl). Sarkosyl was added (1% [v/v] final concentration), followed by proteinase K (1 mg mL^{-1} final concentration), and the mixture was incubated at 55°C for 2 h. After the mixture was centrifuged for 10 min at 2000g, the supernatant was extracted twice with phenol:chloroform:isoamyl alcohol (24:24:1, equilibrated with extraction buffer). Finally, the aqueous phase was ethanol precipitated with high salt as described by Fang et al. (1992) to remove polysaccharides.

Aliquots (20 µg) of genomic DNA were digested with various restriction enzymes overnight in the presence of 3 mM spermidine before separation on 0.8% agarose gels. DNA was transferred under pressure to a Duralon-UV nylon membrane and UV cross-linked as directed by the manufacturer (Stratagene). The blot was prehybridized and hybridized as previously described (Clouse et al., 1992). The pBRU1 cDNA insert, labeled with [^{32}P]dCTP by random priming (Prime-It II kit), was used for the probe at 4 × 10^6 cpm mL^{-1}. Washes were as previously described (Clouse et al., 1992) with the addition of a high stringency wash in 0.1× SSC, 0.1% SDS at 68°C. The blot was exposed with two intensifying screens at −80°C for 7 d.

Nuclear Isolation and Run-On Transcription

Epicotyl sections (apical 1.5 cm) were excised from 9- to 11-d-old soybean seedlings and treated as described above with or without 0.1 µM BR. Nuclei were isolated using the procedure of Luthe and Quatrano (1980) as modified by Lawton and Lamb (1987) with the exceptions that (a) tissue was used immediately without freezing; (b) homogenized tissue was filtered successively through 60-, 52-, and 20-µm meshes; and (c) the Percoll step gradient was centrifuged for 45 min rather than for 30 min. Isolated nuclei were examined microscopically after staining with 4',6'-diamidine-2-phenylindole dihydrochloride and quantitated in a hemocytometer. Nuclei were assayed for transcriptional activity by determining the incorporation of [α-^{32}P]UTP (600 Ci mmol^{-1}) in response to different times of incubation and number of nuclei. Typical reaction conditions were a 30-min incubation with 5 × 10^5 nuclei at 26°C. Reaction buffers and other details of preparing labeled run-on transcripts were as described by Lawton and Lamb (1987).

Slot blots were generated by blotting (in triplicate) 10 µg of test or control cDNAs on Zeta-Probe nylon membranes (Bio-Rad) following manufacturer's instructions. The pBRU1 and soybean actin cDNAs were obtained from the 17-h BR-treated soybean epicotyl library. A partial cDNA for tomato hydroxymethylglutaryl CoA reductase cDNA was obtained from Dr. Carole Cramer (Virginia Polytechnic Institute and State University, Blacksburg, VA). As an additional control, we included 100 ng of a cDNA for 25S rRNA obtained from a *Helianthus tuberosus* cDNA library (M.B. Buchanan and S.D. Clouse, unpublished data).

Duplicate blots were hybridized for 18 h at 42°C with 10^7 cpm mL^{-1} of nuclear run-on transcripts from control or BR-treated tissue in 40% formamide, 1.0% SDS, 5× Denhardt's solution, and 100 µg mL^{-1} of sheared, denatured salmon sperm DNA. Blots were washed in 2× SSC, 0.1% SDS once at room temperature and once at 42°C and in 0.2× SSC, 0.1% SDS twice at 42°C and twice at 50°C. Blots were exposed to preflashed film with one intensifying screen (Laskey and Mills, 1975) and developed in the linear range of intensity for scanning densitometry.

RESULTS

Cloning of a BR-Regulated cDNA

We previously found by in vitro translation and two-dimensional PAGE that BR altered the abundance of specific transcripts in elongating soybean stems (Clouse and Zurek, 1991; Clouse et al., 1992). To clone a representative gene whose expression is regulated by BR, polyadenylated RNA was isolated from BR-treated soybean epicotyl sections and used to construct a cDNA library in λZAPII. Using differential hybridization, we isolated a clone (subsequently named pBRU1) corresponding to an mRNA of approximately 1050 nucleotides whose abundance was increased by BR treatment. Figure 1 shows the kinetics of *BRU1* expression in response to BR and auxin during a typical elongation assay in excised soybean epicotyls. The growth data for the epicotyl sections used in Figure 1 can be found in figure 4 of Clouse et al.
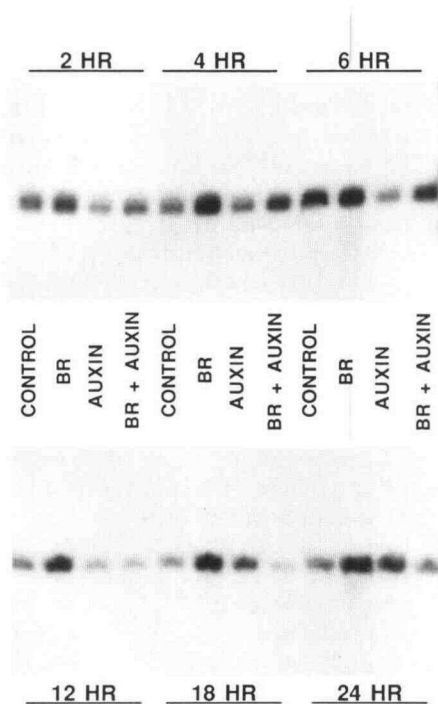
Figure 1. Kinetics of *BRU1* expression. Soybean epicotyl sections were obtained from the first 1.5 cm immediately below the plumule of 10- to 14-d-old soybean seedlings and treated for the times indicated as described in "Materials and Methods." CONTROL, KPSC buffer; BR, 10^{-7} M BR; AUXIN, 5×10^{-5} M 2,4-D; BR + AUX, 10^{-7} M BR plus 5×10^{-5} M 2,4-D. Total RNA was isolated, and northern blot analysis was performed on 40 μg of RNA per lane as previously described (Clouse et al., 1992), using as probe 1×10^6 cpm mL^{-1} of the pBRU1 cDNA insert labeled with [^{32}P]dCTP. Molecular size markers indicated that the band was approximately 1050 nucleotides.

(1992). At early times auxin failed to increase, and, in fact, effectively decreased, *BRU1* transcript levels. At 18 and 24 h auxin slightly increased *BRU1* message abundance but not to the same extent as BR treatment. Scanning densitometry of duplicate blots showed that 0.1 μM BR increased *BRU1* transcript levels 1.5-fold after 2 h, 2.8-fold after 4 h, and 4.9-fold after 18 h. The elevated level of *BRU1* expression in the 6-h control appears to be an artifact of hybridization (not of loading, because probing the blot with an actin cDNA showed equal amounts of RNA loaded). Three repetitions of the northern experiment and duplicate RNase protection experiments (using the same 6-h RNA set) showed a pattern of expression similar to that seen for the 4-h time in Figure 1.

We attempted to determine the earliest time of *BRU1* induction by RNase protection experiments 15, 30, and 60 min after addition of 0.1 μM BR or 50 μM 2,4-D to soybean epicotyl sections. However, scanning densitometry showed no detectable difference in *BRU1* transcript levels between control and hormone-treated segments at any of these early times (data not shown). Thus, 2 h appears to represent the earliest increase in *BRU1* transcript abundance stimulated by BR. A dose-response experiment (data not shown) indicated

that 10^{-9} M BR caused a slight increase in *BRU1* transcript levels with maximum expression reached at 10^{-7} M, followed by a slight decease or plateau at 10^{-6} M BR. This parallels closely the dose-response curve of the effect of different BR concentrations on overall epicotyl elongation found previously (Clouse et al., 1992).

Sequence Analysis of pBRU1

The initial differential screening of 10,000 plaques from the BR-induced soybean epicotyl cDNA library resulted in a positive clone with an insert of 820 bp, which is too short to represent the transcript of 1050 nucleotides estimated by RNA gel blot analysis. We sequenced this clone and synthesized an oligonucleotide complementary to the 5' end that was used as a probe to rescreen the cDNA library. A positive clone with an insert of 1075 bp was isolated and completely sequenced as shown in Figure 2. The cDNA contained an open reading frame of 283 amino acids beginning with an ATG at +27 and terminating with TGA at +875. The initial AUG codon was in the context of the plant consensus sequence for the start site of translation, with 9 of 12 bases conserved, including the highly conserved A residue 3 bases upstream of the AUG (Joshi, 1987). An AAUAAA polyadenylation signal (Darnell, 1982) was located 22 bases upstream of the polyadenylation start site.

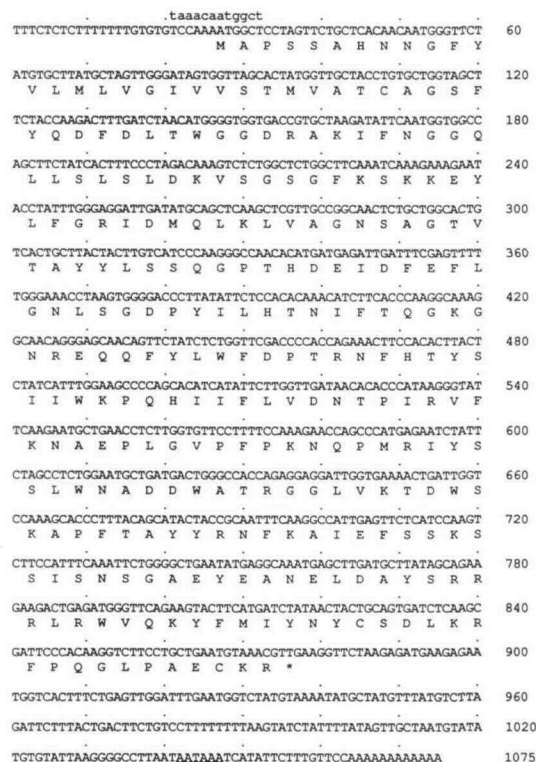Computer analysis of the putative polypeptide revealed a

Figure 2. Nucleotide sequence of pBRU1 with derived amino acid sequence. The lowercase sequence above the first line represents the consensus sequence for plant translation initiation (Joshi, 1987). A putative polyadenylation sequence (AATAAA) is underlined.

basic isoelectric point of 9.71 and a predicted molecular mass of 32,255 D for the unprocessed protein. Figure 3 shows a hydropathy analysis using the algorithim of Kyte and Doolittle (1982). A hydrophobic core of 21 contiguous amino acids near the N terminus suggests the presence of a signal peptide. A number of possible cleavage sites for the putative signal peptide were examined using the statistical weighting method of von Heijne (1983), based on an analysis of 78 eukaryotic signal sequences. The Ala/Gly at positions 29/30 scored highest as the most likely cleavage site. If this is the correct cleavage site, the processed protein would have a predicted molecular mass of 29,288 D and an isoelectric point of 9.88.

Comparison with Other Sequences

The NIH BLAST program was used to search updated versions of protein sequence data banks for homologies to the open reading frame of pBRU1. The most striking homology was found between pBRU1 and the meri-5 gene of A. thaliana (Medford et al., 1991). Significant homology was also observed with the recently cloned xyloglucan-specific endo-(1,4)-β-D-glucanase (XET) from nasturtium seeds (de Silva et al., 1993) and with a group of bacterial endo-β-(1,3)-(1,4)-glucanases (lichenases), primarily from Bacillus sp. (Borriss et al., 1990). Both the nasturtium XET and the bacterial lichenases contain signal peptides and are secreted from the cell, and both have plant cell walls as their substrates. The XET has been localized in the cell wall and has an absolute specificity for xyloglucan (de Silva et al., 1993; Fanutti et al., 1993). It acts in vitro primarily as a transglycosylase except at low substrate concentrations, when glucanase activity predominates. The lichenases hydrolyze β-1,4-linkages adjacent to β-1,3-linkages in mixed linkage β-glucans such as those found in cell walls of barley endosperm. The function of the meri-5 protein is unknown, but based on sequence homology, it is likely to have cell wall activity. No sequence homology was found between pBRU1 and other plant β-(1,4)-glucanases (cellulases). Recently, a partial cDNA corresponding to a maize mRNA induced by anaerobic treatment of seedlings was shown to have 59% identity (78% similarity) with pBRU1 over 167 contiguous amino acids (Peschke and Sachs, 1993).

Figure 4 shows the region of highest sequence conservation among BRU1, meri-5, and the nasturtium XET (NXG1 gene).

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BRU1    48  FNGGQLLSLSLDKVSGSGFKSKKEYLFGRIDMQLKLVAGNSAGTVTAYYLSS 99
            :I.IIII.IIIII  IIIII.II.IIIII:IIII:III:IIIIIIII.:II.I
MERI5   40  LNNGQLLTLSLDKSSGSGFQSKTEYLFGKIDMQIKLVPGNSAGTVTTFYLKS 91
            :...II:.II..IIIII.I . I  I :: .III .I .II.:I.III..
NXG1    59  .DQGSLTIWLDSTSGSGFKSINRYRSGYFGANIKLQSGYTAGVITSFYLSN 108

                **** ****
BRU1    100 Q...GPTHDEIDFEFLGNLSGDPYILHTNIFTQGKGN.....REQQFYLWFD 143
            :  I.I IIIIIIIIII:IIIII.IIII::IIIII:     :IIII.IIII
MERI5   92  E...GSTWDEIDFEFLGNMSGDPYTLHTNVYTQGKGD.....KEQQFHLWFD 135
            :  .:.  IIII:IIII.:.I.IIII:III:.:I.II     :I .:IIIII
NXG1    109 NQDYPGKGHDEIDIEFLGTIPGKPYTLQTNVFIEGSGDYNIIGREMRIHLWFD 160
                ********

BRU1    144 PTRNFHTYSIIWKPQHII 161
            II IIIIIII:I.II:II
MERI5   136 PTANFHTYSILWNPQRII 153
            II.::I.I.I.I.I  II
NXG1    161 PTQDYHNYAIYWTPSEII 178
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Figure 4. Comparison of the translated BRU1 amino acid sequence with the A. thaliana meri-5 (Medford et al., 1991) and the nasturtium XET (NXG1) sequences (de Silva et al., 1993). The region of highest homology over the longest contiguous region was determined with the University of Wisconsin Genetics Computer Group program BESTFIT. The starred amino acid sequence corresponds to the proposed active site of Bacillus lichenases (Borriss et al., 1990).

BRU1 and meri-5 have 77% identity (89% similarity) over the contiguous 114 amino acid region, and BRU1 and NXG1 exhibit 48% identity (62% similarity) over the same region. meri-5 and NXG1 show 50% identity (64% similarity) over this region, indicating that BRU1 and meri-5 are more closely related to each other than either is to NXG1. It is interesting that this conserved region is centered around a core sequence of DEIDFEFLG, which is nearly identical with the sequence DEIDIEFLG conserved in all Bacillus lichenases and proposed to be in the catalytic site (Borriss et al., 1990; Lloberas et al., 1991).

When BRU1 and meri-5 are aligned over their entire lengths the amino acid identity decreases to 49% (68% similarity). However, when the nucleotide sequences are compared, BRU1 and meri-5 share 71% identity over 768 bp. The lower homology of the full-length peptide sequences is due mostly to small insertions and deletions in the nucleotide sequence of the 3' end of BRU1, which changes the reading frame of BRU1 relative to meri-5 in the carboxy terminus. There is also very little homology at the amino terminus because of differences in the putative signal peptides. The homology data are summarized in Table I, where the full-length BRU1 is aligned with meri-5, NXG1, and the bacterial lichenases.

Figure 3. Hydropathy plot of the translated BRU1 polypeptide based on the algorithm of Kyte and Doolittle (1982).

Southern Blot Analysis

Soybean genomic DNA was digested with several restriction enzymes and analyzed by the Southern procedure using an 820-bp fragment from the 3' end of pBRU1 as the probe. As shown in Figure 5, two major bands appeared for each enzyme used. Neither *EcoRI*, *HindIII*, nor *XbaI* cuts within the cDNA sequence, but without knowledge of these sites in potential introns it is not possible to determine whether the *BRU1* gene occurs in one or two copies in the soybean genome. Although several faint bands remained after high-stringency washes, indicating related sequences, it is clear that *BRU1* does not occur as a member of a large, multigene family. This is consistent with the copy number of the homologous *meri-5* and *NXG1* genes, both of which are single-copy genes.

Effect of Different Growth Regulators on *BRU1* Transcript Levels

The effect of various plant growth regulators on *BRU1* expression was determined by RNase protection studies using the standard soybean epicotyl elongation assay. Figure 6 clearly shows that BR was the most effective of the compounds tested in promoting soybean epicotyl elongation and that only BR resulted in an increase in *BRU1* transcript levels under these conditions. Although GA₃, IAA, and 2,4-D also promoted elongation, they resulted in a reproducible decrease in *BRU1* transcript levels. This indicates that increased *BRU1* expression is not simply the result of elongation per se but rather that *BRU1* message increases in response to BR treatment. The results presented in Figure 1 show that auxin may slightly enhance *BRU1* expression at 18 and 24 h, whereas Figure 6 shows a decrease in *BRU1* expression in auxin-treated tissue at 19 h. This apparent discrepancy may be due to differences in auxin concentration between the two experiments (1.0 µM in the RNase protection experiment versus 50 µM in the northern blot). We are investigating further the effect of auxin on *BRU1* expression using competitive polymerase chain reaction (Siebert and Larrick, 1992) to accurately quantitate mRNA levels.
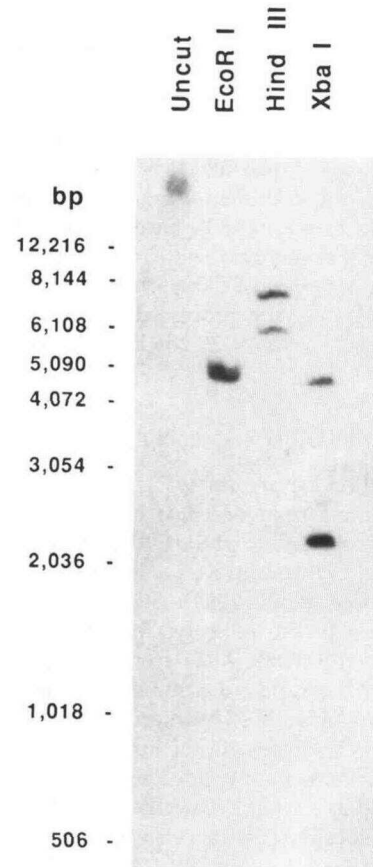
Figure 5. Southern blot of soybean genomic DNA. The probe was 4 × 10⁶ cpm mL⁻¹ of the pBRU1 cDNA insert labeled with [³²P]dCTP.

BRU1 Expression in Untreated Plants

RNase protection assays were used to monitor *BRU1* transcript levels in different tissues and developmental stages of soybean. Figure 7 shows that highest expression was observed in 14-d-old stem tissue, with epicotyl expression (both apical and basal) slightly exceeding that of hypocotyl. Apical

Table I. *Sequences showing highest homology to full-length BRU1 open reading frame*

| Sequence	Percentage Similarity	Percentage Identity	Match Length	Reference
A. thaliana meri-5	68	49	267 amino acids	Medford et al., 1991
meri-5 (nucleotide)	71	71	768 bp	Medford et al., 1991
Nasturtium XET (*NXG1*)	56	37	300 amino acids	de Silva et al., 1993
NXG1 (nucleotide)	57	57	352 bp	de Silva et al., 1993
Bacterial lichenases				
Bacillus polymyxa	54	30	254 amino acids	Gosalbes et al., 1991
Bacillus subtilis	52	29	259 amino acids	Tezuka et al., 1989
Bacillus licheniformis	52	29	260 amino acids	Lloberas et al., 1991
Bacillus amyloliquefaciens	53	28	246 amino acids	Hofemeister et al., 1986
Bacillus macerans	49	28	247 amino acids	Borriss et al., 1990
Bacillus subtilis	51	27	259 amino acids	Murphy et al., 1984
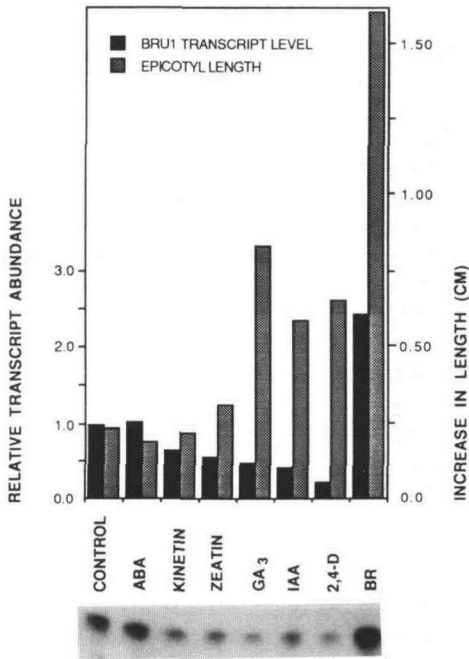
Figure 6. Comparison of the effect of various growth regulators on elongation and *BRU1* expression in soybean epicotyls. Soybean epicotyls (25 replicates) were treated for 19 h as described in "Materials and Methods" with 1.0 μM of the indicated compound. After elongation was measured, total RNA was isolated, and 5-μg total RNA samples were subjected to RNase protection studies using a 281-bp antisense RNA complementary to the 3' end of the *BRU1* mRNA as the probe. Gels were exposed to preflashed film and quantitated by scanning densitometry. The RNase protection experiment was performed in duplicate, and the densitometry results were averaged.
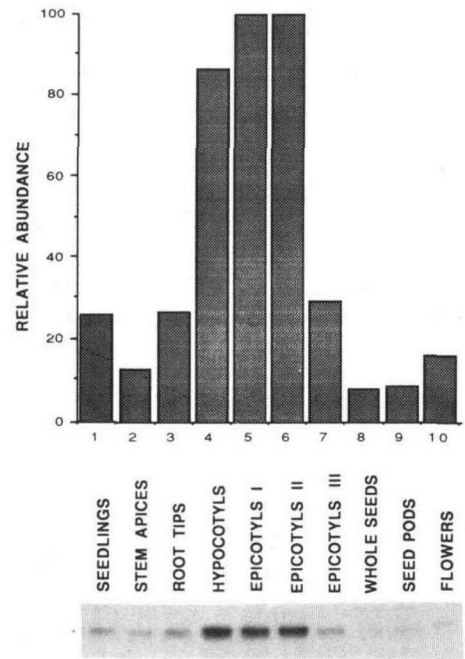
Figure 7. *BRU1* expression in untreated soybean plants. Total RNA was isolated from various soybean organs and analyzed by RNase protection as described in Figure 6. Seedlings were from 2-d etiolated sprouts; stem apices and root tips were from 14-d plants; hypocotyls were 14-d apical sections; epicotyls I were 14-d apical sections; epicotyls II were 14-d basal sections; epicotyls III were 28-d apical sections; seeds, seed pods, and flowers were from 75-d plants. The RNase protection experiment was performed in triplicate, and the densitometry results were averaged.

epicotyl expression decreased 4-fold by 28 d. Stem apices showed low levels of *BRU1* expression. However, we did not dissect out meristems to determine whether *BRU1*, like *meri-5*, had high levels of expression in this localized tissue. Because meristematic cells make up only a small portion of the shoot apex, genes whose expression is limited to the dome of the meristem would not be detected in an RNA sample isolated from the entire stem apex (Medford et al., 1991). *BRU1* transcript levels in 14- and 28-d primary leaves were less than 7% of the level in 14-d epicotyls (data not shown).

Regulation of *BRU1* Expression by BR Appears to Be Posttranscriptional

Northern blots and RNase protection experiments do not definitively show whether an increased abundance in a specific transcript is due to transcriptional or posttranscriptional (such as mRNA stability) events. The most common method used to distinguish between these levels of eukaryotic gene control is run-on transcription in isolated nuclei (Darnell, 1982). To investigate the mechanism of increased *BRU1* expression, we isolated nuclei from soybean epicotyls treated with or without 0.1 μM BR for various times and performed run-on transcription assays. Figure 8 shows that BR does not
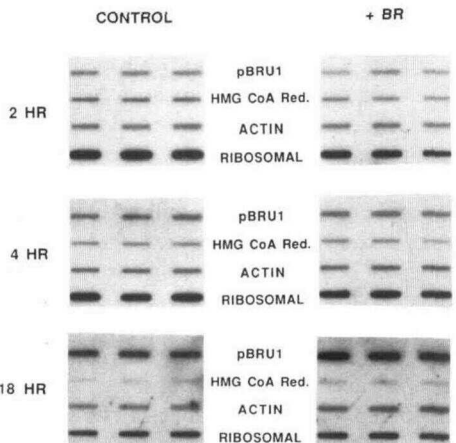

Figure 8. Run-on transcription in isolated nuclei. Epicotyl sections (apical 1.5 cm) were excised from 9- to 11-d-old soybean seedlings and treated with 0.1 μM BR for 2, 4, or 18 h or were untreated. Nuclei were isolated, and labeled run-on transcripts were prepared and hybridized to duplicate slot blots as described in "Materials and Methods." HMG CoA Red, Hydroxymethylglutaryl CoA reductase.

cause induction of *BRU1* at the transcriptional level in soybean epicotyls after 2 or 4 h, even though northern blot analysis shows that *BRU1* transcript levels are higher in BR-treated tissue than in control tissue at these times (see Fig. 1). Although Figure 8 appears to show a slight induction by BR at 18 h, scanning densitometry of duplicate experiments (six data points for each treatment) showed no significant difference between control and BR-treated tissue. Recall that 18 h (Fig. 1) was the time of maximum difference in *BRU1* transcript levels between control and BR-treated tissue in northern blot analysis. Therefore, at all times examined, BR appears to be regulating *BRU1* expression at a posttranscriptional level.

DISCUSSION

In this paper we have presented the cloning and partial molecular characterization of a cDNA corresponding to a BR-responsive gene. Several lines of evidence suggest that the *BRU1* gene encodes an enzyme that has some type of cell wall activity, possibly associated with BR-stimulated elongation. The sequence analysis gives clear evidence of a signal peptide, which would be required for export to the cell wall, and the sequence homology with the XET (de Silva et al., 1993), which has a documented catalytic activity on cell wall polymers, is further evidence that the *BRU1* gene product may act on cell walls. Moreover, *BRU1* shows an eight of nine amino acid identity with the region proposed to be the catalytic site of *Bacillus* lichenases (Borriss et al., 1990), which have plant cell wall-degrading activity. Also, expression of *BRU1* is quite high in apical regions of hypocotyls and epicotyls, which are very responsive to BR-promoted elongation. It should be noted that *BRU1* transcript levels are also high in basal epicotyls, which do not elongate in response to BR (Clouse and Zurek, 1991), but one could argue that changes in wall structure in the more mature tissue result in insensitivity to BR-promoted elongation.

Although the evidence cited above suggests that the *BRU1* gene product may act on cell walls, we do not have sufficient data to claim that *BRU1* encodes a wall-loosening enzyme. First, the homology with the nasturtium XET is significant but does not prove that the BRU1 protein is a xyloglucan-specific endotransglycosylase; it may belong to a family of related enzymes with a variety of cell wall activities of which XET, BRU1, and meri-5 are all members. Furthermore, although it has recently been proposed that XET is a wall-loosening enzyme (Fry et al., 1992; Nishitani and Tominaga, 1992; de Silva et al., 1993), no direct biophysical evidence has been provided. In fact, McQueen-Mason et al. (1993) recently showed that a crude extract containing high XET activity did not cause extension in isolated cucumber walls. Nevertheless, even if XET is ultimately shown not to be a wall-loosening enzyme, it still may have an important role in elongation, such as anchoring newly deposited xyloglucan into the wall or controlling xyloglucan chain length (Cosgrove, 1993). To further define *BRU1* function, we have cloned the full-length cDNA into a baculovirus vector and are currently overexpressing the product in insect cells in an attempt to recover a functional *BRU1* gene product. Functional characterization of *BRU1* should also be revealing with

respect to the *meri-5* gene because the extensive homology between the two genes makes it highly likely that they both have the same function. We have previously shown that BR also promotes elongation in *Arabidopsis* stem sections (Clouse et al., 1993), and we are currently examining the effects of BR on *meri-5* expression in *Arabidopsis* stems.

The cloning of a gene regulated by BR allows for the first time direct examination of the mechanisms of gene regulation by a plant steroid. One might argue from structural considerations that brassinosteroids may act by a mechanism similar to that of animal steroid hormones. In general, such hormones function via a soluble receptor/ligand complex that binds to nuclear sites to regulate the expression of specific genes at the transcriptional level (Evans, 1988). Our results from run-on transcription experiments in isolated epicotyl nuclei indicate that BR probably regulates *BRU1* expression at a posttranscriptional level and, thus, does not fit the classical animal model. However, although posttranscriptional regulation by hormones is not as common as transcriptional regulation, it is by no means rare. Brock and Shapiro (1983) showed that estrogen stabilizes vitellogenin mRNA against cytoplasmic degradation, and several other examples of posttranscriptional regulation by animal steroid hormones have been cited (Darnell, 1982). Numerous examples of posttranscriptional gene regulation by plant growth regulators have also been reported (reviewed by Gallie, 1993; Green, 1993). ABA (Williamson and Quatrano, 1988), cytokinin (Flores and Tobin, 1986), and ethylene (Lincoln and Fisher, 1988) all have been shown to stabilize specific mRNAs. Furthermore, the auxin-inducible *SAUR* genes (McClure et al., 1989) have recently been shown to possess *cis* elements in the 3' untranslated tail that lead to destabilization of the *SAUR* mRNA (Franco et al., 1990) and also to destabilization of β-glucuronidase mRNA when placed in the 3' tail of a β-glucuronidase reporter construct (Newman et al., 1993). We are currently using protein and RNA synthesis inhibitors in conjunction with BR to further understand the posttranscriptional regulation of *BRU1*.

Whereas *BRU1* appears to be regulated at the posttranscriptional level, we have preliminary evidence that BR can also control gene expression at the level of transcription. Using the same cDNA library from which pBRU1 was cloned, we have isolated a second BR-responsive clone that is unrelated to pBRU1. Run-on transcription assays have shown that this newly cloned gene may be transcriptionally regulated by BR (D.M. Zurek and S.D. Clouse, unpublished data). The cloning of BRU1 and other BR-regulated genes will allow the application of a variety of molecular techniques to help clarify the mechanism of BR action. For example, construction of transgenic plants overexpressing *BRU1* or expressing *BRU1* antisense RNA will allow critical testing of the importance of BRU1 in stem elongation. Furthermore, the availability of BR-specific gene probes will allow detailed studies of the mechanism of gene regulation by endogenous plant steroids.

ACKNOWLEDGMENTS

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