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Isolation and expression of an elongation-dependent gene of mung bean (*Vigna radiata*) hypocotyl

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Abstract

A cDNA clone of an auxin up-regulated gene, *ARG8*, was isolated from hypocotyl sections of etiolated mung bean (*Vigna radiata* (L.) Wilczek) seedlings by differential screening. The deduced amino acid sequence suggested that *ARG8* may encode a cell wall protein. The steady state mRNA level of *ARG8* increased by treatment of hypocotyl sections not only with indole-3 acetic acid (IAA) but also with fusicoccin, and the auxin inducibility was inhibited by the addition of 0.3 *M* mannitol in the incubation medium. This indicated that it was not auxin but elongation that regulated the expression of *ARG8*. The promoter activity of the 5'-flanking region of *ARG8* was determined by assaying the transient expression of a luciferase fusion gene that was introduced into mung bean hypocotyl section by the particle bombardment technique. The basal activity of the *ARG8* upstream region was about a few tenths of that of a modified cauliflower mosaic virus 35S promoter, and it was increased a few fold by treatment with IAA. The auxin inducibility was completely suppressed by the addition of mannitol. A 5'-deletion analysis showed that a 53 bp region in the *ARG8* promoter was important for the basal and elongation-dependent promoter activities.

Abbreviations – CaMV, cauliflower mosaic virus; FC, fusicoccin; GPI, glycosylphosphatidylinositol; KPSC buffer, buffer that consisted of 50 m*M* potassium phosphate (pH 6.8), 2%(w/v) sucrose and 50 μg ml^{-1} chloramphenicol; LUC, luciferase; PCR, polymerase chain reaction; 5'-RACE, 5'-rapid amplification of cDNA ends.

Introduction

Growth is a critical aspect of morphogenesis in plants. Over the years, physiological, biochemical, and molecular biological studies have revealed many of the components that are involved in the process (Ray 1987, Cosgrove 1997). Many genes have been postulated to be involved in growth as their mRNA levels are abundant in growing tissues of plants, and at the same time their gene expressions show good correlations with the growth rates of the tissues when the growth is modulated experimentally by exposing the plants to light, water deficit, and so on. These genes include genes for cytoskeletal proteins such as soybean β-tubulin and actin (Creelman and Mullet 1991), cell wall proteins such as soybean and rice glycine-rich proteins (Creelman and Mullet 1991, D. Xu et al. 1995), soybean hydroxyproline-rich protein (Creelman and Mullet 1991), and chickpea CanST of unknown function (Munoz et al. 1997), and other various proteins including tomato H^+-ATPase (Mito et al. 1996), pea small G-protein (Nagano et al. 1995), and petunia *S*-adenosylmethionine synthetase (Izhaki et al. 1996). A GA-induced gene (*GAST1*; Shi et al. 1992, Ben-Nissan and Weiss 1996) and genes for tonoplast intrinsic protein (Ludevid et al. 1992), expansin (Cho and Kende 1997) and endo β-1,4-D-glucanase (Wu et al. 1996, Brummell et al. 1997, Catalá et al. 1997, Shani et al. 1997), have been reported to be expressed at higher levels in elongating/expanding tissues, but it is not known whether their expression is controlled by a specific hormone or stimulus, or is dependent on growth in general. Promoter analysis has not been attempted for the growth-dependent genes except for the rice glycine-rich protein gene, *Osgrpl.* By a 5'- deletion analysis, the 394-bp region of the *Osgrp1* promoter has been suggested to contain *cis*-acting elements necessary for high-level, specific expression in the elongating/differentiating cells of root (D. Xu et al. 1995).

In an attempt to isolate auxin-inducible genes by differential screening of a cDNA library, we found a cDNA of a mung bean gene, *ARG8,* the mRNA levels of which increased in response to not only auxin but also fusicoccin (FC). FC has been shown to promote elongation of plant tissues in a different molecular mechanism from auxin (Hager et al. 1991). The auxin inducibility was inhibited by addition of mannitol to the incubation medium. These results indicate that the steady state level of *ARG8* mRNA in fact responded to elongation growth rather than auxin treatment. We also cloned an approximately 600-bp 5'-flanking sequence of *ARG8* by inverse polymerase chain reaction (PCR), and measured its promoter activity by a transient assay of the luciferase (LUC)-fusion reporter gene introduced into etiolated mung bean hypocotyls by particle bombardment. The present study showed that a part of the 5'- flanking sequence that was as short as 53 bp was responsible for its promoter activity and growth dependency.

Materials and methods

Plant material, growth condition, and treatment with chemicals

Seeds of mung bean (*Vigna radiata* (L.) Wilczek), obtained from a local market, were sterilized in about l%(w/v) sodium hypochlorite solution for 15 min, rinsed with tap water, soaked in running tap water at about 29°C for one day, and grown on 0.7%(w/v) agar in the dark at 29°C. Fifty 1-cm-long sections of the elongating region of the hypocotyls, excised from the etiolated seedlings on the third day in the dark, were rinsed with water at room temperature and then preincubated at 29°C for 8 h in 2 ml of an incubation solution (KPSC buffer) that consisted of 50 m*M* potassium phosphate (pH 6.8), 2%(w/v) sucrose, and 50 μg ml^{-1} chloramphenicol. After preincubation, the buffer was changed to fresh KPSC buffer that contained various concentrations of the chemicals to be tested. Incubation was continued for 1 h at the same temperature. The sections were immediately frozen in liquid nitrogen and stored at −80°C.

Preparation of RNA and genomic DNA and Northern an Southern blot analysis

Preparation of RNA and Northern blot analysis was carried out essentially as described previously (Yamamoto et al. 1992). Genomic DNA was extracted from frozen plumules of 3-day-old etiolated seedlings of mung bean as described by Murrey and Thompson (1980). Southern blot analysis was carried out according to Sambrook et al. (1989), using a nylon membrane (Hybond-N^+; Amersham, Little Chalfont, UK).

Differential screening of the cDNA library and DNA sequence analysis

A cDNA library for poly(A)$^+$ RNA obtained from sections of hypocotyls treated with 30 μ*M* indole-3-acetic acid (IAA) for 1 h was constructed in plasmid pT7T3 by using a TimeSaver cDNA Synthesis Kit (Pharmacia, Uppsala, Sweden) following the manufacturer's instructions. Differential screening was carried out essentially as described previously (Yamamoto et al. 1992) except that first-strand cDNA probes were synthesized by SuperScript II reverse transcriptase (GIBCO BRL, Gaithersburg, MD, USA).

Nucleotide sequences were determined with a Thermo Sequenase cycle sequencing kit (Amersham) using an automated DNA Sequencer (4000L; Li-cor, Lincoln, NE, USA).

Isolation of 5'-flanking regions by inverse PCR

Genomic DNA (150 ng) was completely digested with *Eco*RI. The digest was ligated with T4 DNA ligase (Takara, Kyoto, Japan) at a concentration of 10 ng μl^{-1} for 18 h at 16°C to generate circular genomic DNA. PCR reactions were performed using 0.05 unit μl^{-1} Ex *Taq* DNA polymerase (Takara), 250 μM dNTPs, 1 × Ex *Taq* buffer, 0.2 μM sense primer (P1 primer in Fig. 1; 5'-AATGATCTGACAAATGAGTGTGCCTCAACC-3'), 0.2 μM antisense primer (AP1 primer; 5'-GAAACGAGGAAGAAACCCAGAAGCG-3'), and the self-ligated genomic DNA as a template. The PCR products were cloned into pT7Blue (Novagen, Madison, WI, USA). After determination of the nucleotide sequence, nine independent PCRs were carried out against genomic DNA to establish the nucleotide sequence of the 5'-flanking region. The used 17-bp primers were located immediately 5' of the putative translation start site (5'-GACTAGTTCAAAACTGA-3') and at the 5' *Eco*RI site corresponding to positions −747 to −731 of the Pro-1 sequence (Fig. 3) (5'-GAATTCATAATATCTAA-3').

5'-Rapid amplification of cDNA ends

5'-Rapid amplif1cation of cDNA ends (5'-RACE) was performed by a modified 5'-RACE method (Frohman et al. 1988). In brief, about 3 μg of total RNA from the IAA-treated sections of hypocotyl was reverse-transcribed with SuperScript II using an antisense extension primer located in the *ARG8* cDNA (AP1 primer in Fig. 1). A poly(A) tail was added by using dATP and terminal deoxynucleotidyltransferase (Boehringer-Mannheim, Mannheim, Germany). Second (+) strand synthesis was carried out at 72°C for 10 min by Ex *Taq* DNA polymerase with a $(dT)_{17}$-adaptor primer (5'-GACTCGAGTCGACATCGAT_{17}-3'). The products were amplified by using the adaptor primer and a gene-specific amplification primer (AP2 primer in Fig. 1) consisting of a sequence located upstream of the extension primer sequence (AP1 primer). The amplified product was cloned into pT7Blue and the nucleotide sequence was determined.

Construction of plasmids for transient transformation

A series of 5'-truncated deletions in the *ARG8* promoter region was fused to the firefly *LUC* gene for a transient assay. The GUS open reading frame/nopaline synthase polyadenylation site was excised from pBI101 (Clontech, Palo Alto, CA, USA) at the *Hin*dIII and *Eco*RI sites and inserted into pUC19 at the same sites (named pUC-GUS-NOS). The firefly *LUC* gene excised from pSP-LUC (Promega, Madison, WI, USA) at the *Hin*dIII and *XbaI* sites was blunted with a Klenow fragment (Takara) and

inserted into pUC-GUS-NOS at the *Sma*I and *Sac*I sites that had been blunted with T4 DNA polymerase (Takara) instead of the GUS open reading frame (named pF-LUC). The 5'-flanking region of *ARG8* excised from pT7Blue at the*Hin*dIII and *Bam*HI sites was inserted into pF-LUC at the same sites, and 5' deletion was carried out by mung bean exonuclease III (Takara) digestion. For an internal control of the quantitative assay, modified cauliflower mosaic virus (CaMV) 35S promoter/*Renilla reniformis LUC* was constructed. An *Nhe*I/blunted *Xba*I fragment excised from pRL-TK (Promega) was inserted into pBI221 (Clontech) at the *Xba*I/blunted *Sac*I site instead of the GUS coding region. The CaMV 35S promoter of the pBI221 had been substituted for the modified promoter (a gift from Dr. J. Obokata, Hokkaido University), in which the −90 to −66 portion that almost covered the *as-1* element (Liu and Lam 1994) was changed to 5'-GTCGACCCCGGTAC-3'.

Transient transformation and quantitative LUC assay

Transient plant transformation was accomplished by particle bombardment (GIE-III IDERA; Tanaka, Sapporo, Japan) according to the manufacturer's recommendations. One μg each of the test plasmid and reference plasmid was precipitated with 1 mg of gold particles of 1.5 − 3.0 μm diameter (Aldrich, Milwaukee, WI, USA) by ethanol. The particles coated with DNAs were suspended in 160 μl of ethanol. The elongation region of hypocotyls of four mung bean seedlings, which had been grown in the dark for 3 days and fixed on a slide glass with Scotch tape, was bombarded with 4 μl of the suspension. The bombarded region of hypocotyls, 1 cm long, was excised, preincubated in 10 m*M* potassium phosphate, pH 6.8, at 29°C for 6 h, and further incubated for 3 h in the buffer containing a chemical, whose effects were to be examined. After the incubation, the hypocotyl sections were frozen in liquid nitrogen and kept at −80°C until used. A quantitative LUC assay was conducted using the Dual-Luciferase Reporter Assay System (Promega) and a luminometer (Luminescencer-JNR AB-2100; Atto, Tokyo, Japan) according to the manufacturer's recommendations after the frozen sections were ground to powder by a homogenizer.

Results

Isolation of a cDNA clone

By screening about 160,000 colonies by differential hybridization, we isolated seven cDNAs of auxin-regulated genes. Of these, four were cDNAs of the *Aux/IAA* gene family

(Hashimoto and Yamamoto 1997), one was a cDNA of an auxin down-regulated mRNA (Hashimoto and Yamamoto 1998), and the remaining two, DS260 and DS297, were cDNAs of new auxin up-regulated genes. In the present study, we report on DS260, whose corresponding gene is named *ARG8.* DS260 consists of 783 bp, of which a 53-bp portion at its 3' end was a poly(A) tail (Fig. 1). The first ATG triplet is found at positions 72 – 74, and is followed by the open reading frame that encodes 169 amino acid residues, equivalent to a protein of 18.9 kDa. Because the reading frame extends to the 5' end of the cDNA, DS260 might not be a full-length cDNA. However, genomic cloning of the 5'-flanking region and 5'-RACE analysis (described below) showed that an in-frame nonsense codon, TAA, is present 115 – 117 bp upstream of the ATG codon, and that no ATG triplets are found in the upstream region (Fig. 3, the Pro-2 sequence). These results indicate that DS260 is a full-length cDNA of *ARG8,* and the site of initiation of translation is tentatively assigned to the ATG codon at positions 72 – 74.

The nucleotide sequence of *ARG8* cDNA and its predicted amino acid sequence were examined for similarities in the DNA databases. No related sequences were found except for four and five expressed sequence tags of *Arabidopsis* and rice, respectively, and a genomic clone of *Arabidopsis.* The hydropathy analysis of the predicted ARG8 protein shows a long hydrophobic region near each of the amino and carboxy termini (Fig. 1).

Isolation of the promoter region

Genomic DNA was digested with each of five restriction enzymes, and hybridization was performed under low-stringency conditions using the cDNA as a probe (Fig. 2). Neither enzyme cut the cDNA, and a single hybridization signal was observed when the digested DNA was hybridized with the cDNA probe. The 5'-flanking region of *ARG8* cDNA was isolated by inverse PCR of the genomic *Eco*RI fragment of 3.9 kb (Fig. 2), using the sense (P1 primer) and antisense primers (AP1 primer) that were located within the *ARG8* cDNA, as shown in Fig. 1. To establish the nucleotide sequence, nine independent PCRs were carried out against genomic DNA, taking advantage of the sequence information obtained from the inverse PCR. One clone of the PCR products was isolated for each PCR. Determination of the nucleotide sequences of the nine clones revealed two slightly different sequences, Pro-1 and Pro-2 (Fig. 3): eight clones yielded the Pro-1 sequence, and one clone yielded the Pro-2 sequence. Using the *Eco*RI fragment of genomic DNA, we also carried out single-sided PCR once to obtain the *ARG8* promoter. The Pro-2 sequence was recovered in this case. The results suggested that the Pro-2 sequence was not just an artifact of PCR. The mung bean population used

in the present study did not seem genetically homogeneous, and might contain two kinds of the *ARG8* gene that are closely related to each other.

5'-RACE analysis

A 5'- RACE analysis was carried out to determine the 5' end of *ARG8* mRNA. After nested PCR, gel electrophoresis of the products revealed a 220-bp sized band with a smear. Determination of the nucleotide sequences of a few clones in each of the three independent PCRs showed that the 5' end of the longest cDNA corresponded to adenine at position −221 relative to the translation start site (marked with a solid triangle in Fig. 3). Thymines were located immediately 5'of the adenine. A poly(A) tail was added to the first cDNA strand in the 5'-RACE procedure used in the present study. Therefore, the adenine at position −221 is not necessarily the 5' end of *ARG8* cDNA. It is also possible that the thymines immediately 5' of the adenine is the 5' end of the cDNA. No TATA-like elements were found upstream of the putative transcription start site.

The two sequences, Pro-1 and Pro-2, were obtained for the 5'-flanking region of *ARG8* cDNA, as described above. They were different from each other in the transcribed region: cytosine at position −106 of the Pro-2 sequence was missing in the Pro-1 sequence (Fig. 3). All the cDNA sequences obtained through the 5'-RACE procedure agreed with the Pro-2 sequence, which is a second indication of the authenticity of the Pro-2 sequence.

Gene expression

RNA gel-blot hybridization was performed to study the expression of *ARG8* using the *ARG8* cDNA, DS260, as a probe (Fig. 4). A single band of about 1,000 nucleotides was detected under these conditions, which was consistent with the cDNA length calculated from the results of the 5'-RACE analysis described above. First, we measured the distribution of *ARG8* mRNA in 3-day-old, dark-grown seedlings, which were about 4.5 cm tall (Fig. 4A). The steady state level of the *ARG8* transcript was highest in 1-cm-long root-tip sections; upper portions of the hypocotyl also contained a considerable amount of the transcript. These results suggested that the *ARG8* mRNA was abundant in elongating tissues.

Next, the effects of various chemical or physical treatments of hypocotyl sections was measured (Fig. 4B). When 1-cm-long sections of hypocotyl were incubated in KPSC buffer at 29°C, the *ARG8* mRNA level gradually decreased and reached a basal level that was about 30% of the initial level 6 h after the sectioning (data not shown). Incubation with 10 μ*M* IAA for 1 h after the 8 h long preincubation increased the *ARG8*

mRNA level about 5-fold. FC at the same concentration showed a similar effect. Treatment with other plant hormones, cycloheximide (CHX), cadmium ion, or heat shock had no significant effects.

The kinetics of accumulation of the *ARG8* mRNA were determined (Fig. 4C). An increase in abundance of the *ARG8* mRNA was first detected after incubation with 10 μ*M* IAA for 60 min. This response was slower than that of an *Aux/IAA* gene (*ARG12*; Hashimoto and Yamamoto 1997), whose mRNA level was increased after a 30-min treatment.

Finally, we checked the effects of osmolarity of the incubation medium on the IAA-induced increase in the mRNA level (Fig. 4D). The presence of 0.3 *M* mannitol decreased the basal mRNA level of both *ARG8* and the *Aux/IAA* genes by half. It inhibited the IAA- and FC-induced increase in the *ARG8* mRNA level by half again. In contrast, IAA inducibility of the *Aux/IAA* gene was not significantly affected by 0.3 *M* mannitol, and its mRNA level was not affected by FC.

Transient expression assay of *ARG8* promoter

In order to determine whether regulation of *ARG8* expression is at the transcriptional level, we made recombinant constructs linking the 5'-untranslated region of the *ARG8* gene with a reporter gene, firefly *LUC.* These constructs were introduced to hypocotyl tissues of etiolated seedlings of mung bean using the particle bombardment technique, and a transient expression assay was carried out (Fig. 5). *Renilla LUC* under control of a modified CaMV 35S promoter was cotransfected as an internal control, and firefly LUC activity was standardized against *Renilla* LUC activity. The *as-1* element of the CaMV 35S promoter, which had been shown to be responsive to auxin treatment (Liu and Lam 1994), was removed from the modified CaMV 35S promoter, so that activity of the reference reporter gene was not affected by auxin.

Activities of both the Pro-1 promoter (−747 to −1 relative to the translation start site) and the Pro-2 promoter (−750 to −5) were about a few tenths of those of the modified 35S promoter (Fig. 5A). They were increased a few fold when excised sections of hypocotyl were treated with 10 μ*M* IAA for 3 h. Constructs with three successive 5'-deletion mutants for each of the two promoters were checked for their activities. Pro-1-Δ4 (−328 to −1) and Pro-2-Δ3 (−403 to −5) directed the relative expression of the firefly *LUC* and auxin inducibility that were similar to those of Pro-1 and Pro-2, respectively, while neither Pro-1-Δ6 (−214 to −1) nor Pro-2-Δ5 (−274 to −5) functioned as a promoter.

The same functional assay was carried out to determine the effects of an

increase in osmolarity in the incubation buffer; transfected sections of hypocotyl were incubated in the presence of 0.3 *M* mannitol (Fig. 5B). No auxin-induced increase in the relative firefly LUC activities was observed in this case. Finally, we measured the effects of treatment with 1 μ*M* FC for 3 h on the promoter activities of the Δ3 – Δ6 constructs (Fig. 5C). The FC treatment showed results similar to those of IAA: Pro-1-Δ4 and Pro-2-Δ3 functioned as a promoter and showed FC inducibility, while Pro-1-Δ6 and Pro-2-Δ5 lost their promoter activities.

Discussion

In the present study, we identified a new mung bean gene, *ARG8,* whose expression is dependent on elongation growth of hypocotyl tissue. *A RG8* was initially obtained as an auxin-induced gene. However, the auxin-induced gene expression of *ARG8* was inhibited by the presence of 0.3 *M* mannitol in the incubation medium (Fig. 4D), and gene expression of *ARG8* was induced by treatment with FC as well as auxin (Fig. 4B). These results indicate that it is not auxin but elongation that affects the gene expression of *ARG8.* In contrast, auxin inducibility of early auxin-inducible genes, the *Aux/IAA* genes, was not influenced by treatment with mannitol (Fig. 4D; Theologis et al. 1985), and the *Aux/IA A* genes were not FC inducible (Fig. 4D; Yamamoto 1994).

We determined the promoter activity of the 5'-flanking region of the *ARG8* gene by the transient LUC assay. Two slightly different sequences, Pro-1 and Pro-2, were obtained for the 5'-flanking region of *ARG8,* and three successive 5' deletion constructs were made for each promoter sequence. Both of the 5'-flanking sequences directed expression of the LUC reporter gene, which was increased a few fold by auxin treatments (Fig. 5A). The relatively high error rates are probably resulting from the low number of cells actually transformed by the ballistic method. The response to auxin disappeared when mannitol was added to the incubation medium (Fig. 5B), suggesting that the *ARG8* promoters responded to elongation. The LUC activities of the six deletion constructs were not comparable with each other in the strictest sense because of small but significant differences between the Pro-1 and Pro-2 sequences used for the deletion constructs. However, these results strongly suggest that the 53-bp region from positions −328 to −276 of the Pro-1 sequence (Fig. 3) is most responsible for its promoter activity and inducibility by elongation. Deletion constructs that contained this segment also responded to treatment with FC (Fig. 5C), supporting the hypothesis that this region receives one or more signals controlling elongation. The 53-bp region contains a tandem

repeat of CGTGTTTG (seven out of eight matching) separated by 2 bp. The antisense sequence of CGTGTTTG includes an abundant seed storage-protein element, CAAACAC, which is conserved in the upstream sequence of many seed storage-protein genes (Ellerstöm et al. 1996, Stälberg et al. 1996). This repeat structure may play an important role in the promoter activity and growth inducibility of the 5'-flanking region of the *ARG8* gene.

The predicted amino acid sequence of the ARG8 protein suggests nothing about its function due to the absence of sequence similarity to known proteins. However, the presence of basic amino acid residues near the amino terminal and the following hydrophobic region suggests that the amino-terminal portion is a cleavable signal peptide for the ER (Nakai and Kanehisa 1992). Many eukaryotic cell-surface proteins are anchored to the membrane by a carboxy-terminal linkage to glycosylphosphatidylinositol (GPI) (Udenfriend and Kodukula 1995). All the GPI-anchored proteins have an amino-terminal signal sequence for translocation across the ER membrane and the carboxy-terminal GPI signal peptide including a second hydrophobic domain. The amino acid sequence of the predicted ARG8 protein agrees well with these structural requisites for GPI-anchored proteins (Udenfriend and Kodukula 1995). In yeast, a GPI-anchor is required for transportation of major cell wall proteins to the plasma membrane and their incorporation into the cell wall, and the availability of GPI-dependent cell wall proteins determines the rate of cell wall construction and limits the growth rate (Vossen et al. 1997).

The responses of the growth-dependent genes mentioned in Introduction are generally slow. Exceptions are *TCH4*, which encodes xyloglucan endotransglycosylase (W. Xu et al. 1995), and *EXP4*, which encodes expansin (Cho and Kende 1997). The mRNA levels of both of these genes change within 10 – 30 min after exposure to various stimuli. Changes in gene expression of the other genes are only detectable at least 3 h and often 24 h after the start of the treatment. Changes in the *ARG8* expression were observed 1 h after the start of the auxin or FC treatment, which is rather fast among the growth-dependent genes. Therefore, the *ARG8* product may play an intermediary role in complex growth-controlling processes.

Gene expression is usually regarded as growth-dependent when it is not controlled specifically by a single hormone or environmental stimulus, and is correlated with growth upon growth modulation by multiple hormones and/or environmental signals. The growth response is an integrated output of a complex network into which the effects of various hormones and environmental stimuli are fed. Therefore, growth-regulated gene expression often means that we do not know the true factors

controlling gene expression. Expression of the *ARG8* gene may reflect changes in physical and chemical parameters associated with elongation, such as tensile strength or composition of cell wall polymers, surface tension of the plasma membrane, and so on. The current study should be a starting point for a future study to elucidate the physical or chemical factors that directly control gene expression of *ARG8* through the putative 53-bp regulatory region of its promoter in the complex network of growth regulation.

The nucleotide sequence data reported will appear in the GenBank, EMBL and DDBJ Nucleotide Sequence Databases under the accession number AB013853 (cDNA), AB013854 (Pro-1), and AB013855 (Pro-2).

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Figure legend

Fig. l. Nucleotide sequence and the deduced amino acid sequence of the *ARG8* cDNA. The latter sequence is shown below by single-letter abbreviations. Hydrophobic regions of the predicted ARG8 protein estimated by the method of Kyte and Doolittle (1982) are double-underlined. AP1 and P1: location of the primers used for inverse PCR to isolate a genomic clone; AP1 and AP2: location of the primers used for 5'-RACE analysis.

Fig. 2. Southern blot analysis of the *ARG8* gene. Genomic DNA (2 μg) was digested with the indicated restriction enzymes, separated on a 0.8% agarose gel, blotted onto a nylon membrane and hybridized with a ^{32}P-labeled *ARG8* cDNA at low stringency (in 2 x SSPE (Sambrook et al., 1989) and 0.1%(w/v) SDS at 65°C). The migration of the size markers is shown at the right.

Fig. 3. Two nucleotide sequences, Pro-1 and Pro-2, of the 5'-flanking region of *ARG8* gene. Asterisks indicate the nucleotides identical to the Pro-1 sequence; hyphens show introduction of a gap. The putative translation start site is set at +1. The 5' ends of the cDNA and the 5'-RACE products are denoted by an open and a closed arrowhead, respectively. The tandem repeat discussed in the text is shown by arrows. The 5' ends of the series of the deleted promoter regions used for reporter fusion constructs are also shown (Δ1 — Δ6).

Fig. 4. RNA blot hybridization analysis of *ARG8* expression. (A) Distribution of *ARG8* mRNA in 3-day-old, dark-grown seedlings. Plumules, hooks, and 1-cm-long sections of root tip were excised from the seedlings. Six sequential sections, each of which was 5 mm long, were cut from the hypocotyl immediately below the hook. One is the top section and six is the bottom section. (B) Effects of various chemical and physical treatments on the steady state level of *ARG8* mRNA. After 1-cm-long hypocotyl sections were preincubated in KPSC buffer for 8 h at 29°C, they were incubated in KPSC buffer containing IAA, 6-benzylaminopunne (BA), GA3, abscisic acid (ABA), FC, or cycloheximide (CHX) at a final concentration of 10 μ*M* for 1 h at the same temperature. For cadmium ions, 100 μ*M* $CdCl_2$ was added. Heat shock consisted of exposure to 40°C for 1 h after preincubation. (C) Time course of the increase in the steady state level of *ARG8* mRNA. After the preincubation described above, the hypocotyl sections were

incubated in 10 μ*M* IAA for the indicated period of time at 29°C. (D) Effects of the presence of 0.3 *M* mannitol in the incubation medium on the abundance of *ARG8* mRNA. After preincubation with 10 m*M* potassium phosphate, pH 6.8, for 6 h, the hypocotyl sections were incubated in buffer containing 0.3 *M* mannitol with or without 10 μ*M* IAA or FC for 1 h. In (C) and (D), the mRNA level of a mung bean *Aux/IAA* gene (*ARG12*; Hashimoto and Yamamoto 1997) was also measured for comparison. In each case, 10 μg of total RNA was loaded into each lane.

Fig. 5. Transient assay of *ARG8* promoter/firefly *LUC* fusion gene in hypocotyl sections of etiolated mung bean seedlings. The portions of the *ARG8* promoter used for driving the firefly *LUC* gene are shown at the left. Hypocotyl sections were incubated with (solid bar) or without (open bar) IAA (A, B) or FC (C). (A) Effects of IAA. The bombarded regions of the hypocotyls were excised into 1-cm-long sections, preincubated in 10 m*M* potassium phosphate, pH 6.8, for 6 h at 29°C, and incubated with the same buffer containing 10 μ*M* IAA for 3 h at the same temperature. The firefly *LUC* activity was measured relative to activity of *Renilla LUC* driven by modified CaMV 35S promoter. Mean and SD of 5 – 8 measurements are shown. (B) Effects of mannitol. The transfected hypocotyl sections were incubated in 10 m*M* potassium phosphate, pH 6.8, containing 0.3 *M* mannitol with or without 10 μ*M* IAA for 3 h. Four independent measurements were done. (C) Effects of FC. Incubation medium consisting of 1 μ*M* FC and 10 m*M* potassium phosphate, pH 6.8, was used in this experiment. Data consist of the results of two independent trials.

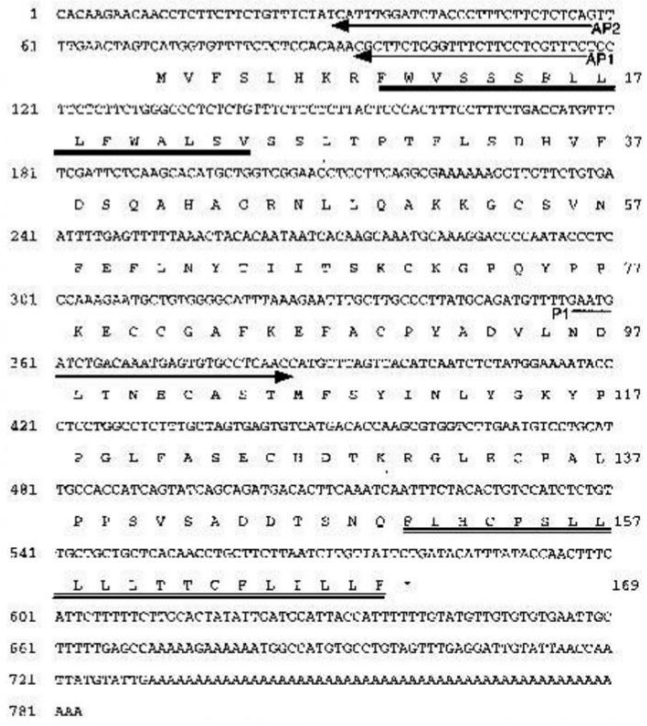

AP2
AP1
P1

Fig. 1

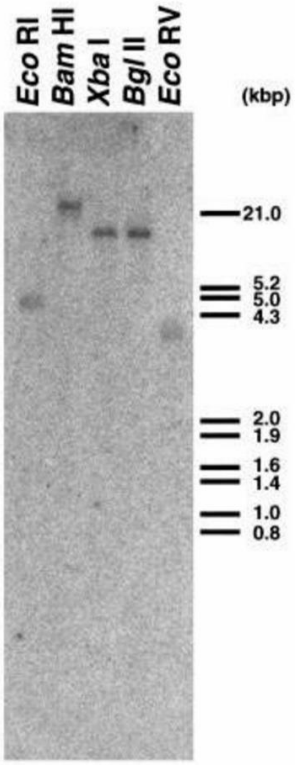

Eco RI
Bam HI
Xba I
Bgl II
Eco RV
(kbp)
21.0
5.2
5.0
4.3
2.0
1.9
1.6
1.4
1.0
0.8

Fig. 2

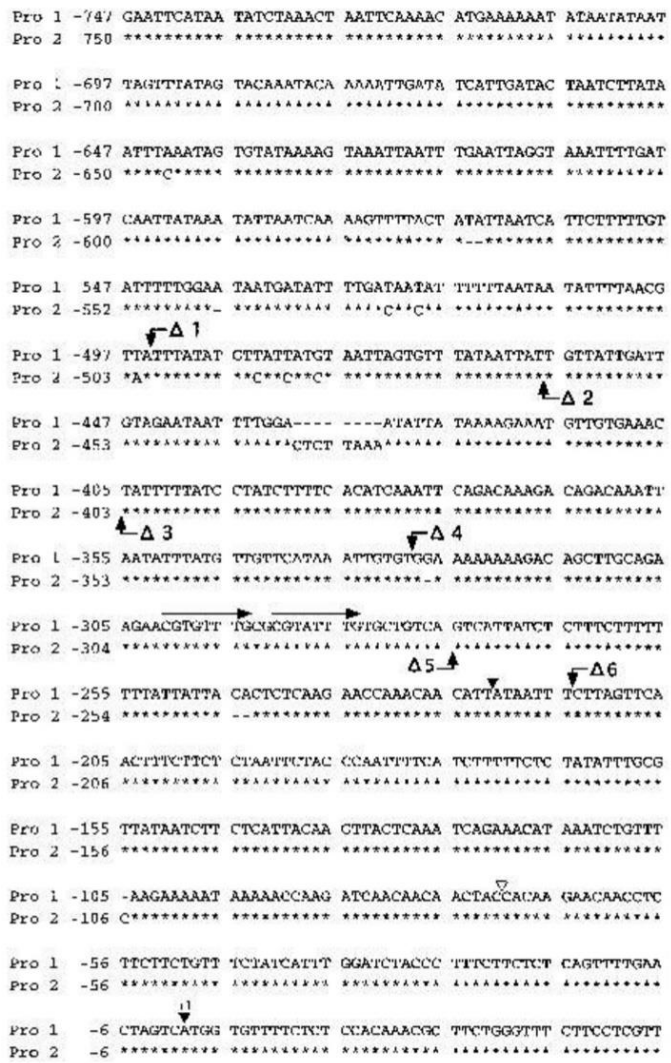

Pro 1 -747 GAATTCATAA TATCTAAACT AATTCAAAAC ATGAAAAAAT ATAATATAAT
Pro 2 750
Pro 1 -697 TAGTTTATAG TACAAATACA AAAATTGATA TCATTGATAC TAATCTTATA
Pro 2 -700
Pro 1 -647 ATTTAAATAG TGTATAAAAG TAAATTAATT TGAATTAGGT AAATTTTGAT
Pro 2 -650
Pro 1 -597 CAATTATAAA TATTAATCAA AAGTTTTACT ATATTAATCA TTCTTTTTGT
Pro 2 -600
Pro 1 547 ATTTTTGGAA TAATGATATT TTGATAATAT TTTTTAATAA TATTTTAACG
Pro 2 -552
Δ 1
Pro 1 -497 TTATTTATAT CTTATTATGT AATTAGTGTT TATAATTATT GTTATTGATT
Pro 2 -503
Δ 2
Pro 1 -447 GTAGAATAAT TTTGGA---- ----ATATTA TAAAAGAAAT GTTGTGAAAC
Pro 2 -453
Pro 1 -405 TATTTTTATC CTATCTTTTC ACATCAAATT CAGACAAAGA CAGACAAATT
Pro 2 -403
Δ 3
Δ 4
Pro 1 -355 AATATTTATG TTGTTCATAA ATTGTGTGGA AAAAAAAGAC AGCTTGCAGA
Pro 2 -353
Pro 1 -305 AGAACGTGTT TGCGCGTATT TGTGCTGTCA GTCATTATCT CTTTCTTTTT
Pro 2 -304
Δ 5
Δ 6
Pro 1 -255 TTTATTATTA CACTCTCAAG AACCAAACAA CATTATAATT TCTTAGTTCA
Pro 2 -254
Pro 1 -205 ACTTTCTTCT CTAATTCTAC CCAATTTTCA TCTTTTTCTC TATATTTGCG
Pro 2 -206
Pro 1 -155 TTATAATCTT CTCATTACAA CTTACTCAAA TCAGAAACAT AAATCTGTTT
Pro 2 -156
Pro 1 -105 -AAGAAAAAT AAAAACCAAG ATCAACAACA ACTACCACAA GAACAACCTC
Pro 2 -106
Pro 1 -56 TTCTTCTGTT TCTATCATTT GGATCTACCC TTTCTTCTCT CAGTTTTGAA
Pro 2 -56
+1
Pro 1 -6 CTAGTCATGG TGTTTTCTNT CCACAAACGC TTCTGGGTTT CTTCCTCGTT
Pro 2 -6

Fig. 3

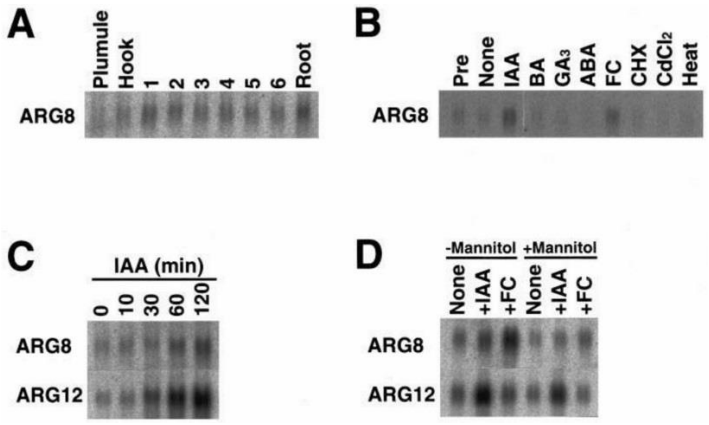

A
Plumule
Hook
1
2
3
4
5
6
Root
ARG8
B
Pre
None
IAA
BA
GA3
ABA
FC
CHX
CdCl2
Heat
ARG8
C
IAA (min)
0
10
30
60
120
ARG8
ARG12
D
-Mannitol
+Mannitol
None
+IAA
+FC
None
+IAA
+FC
ARG8
ARG12

Fig. 4

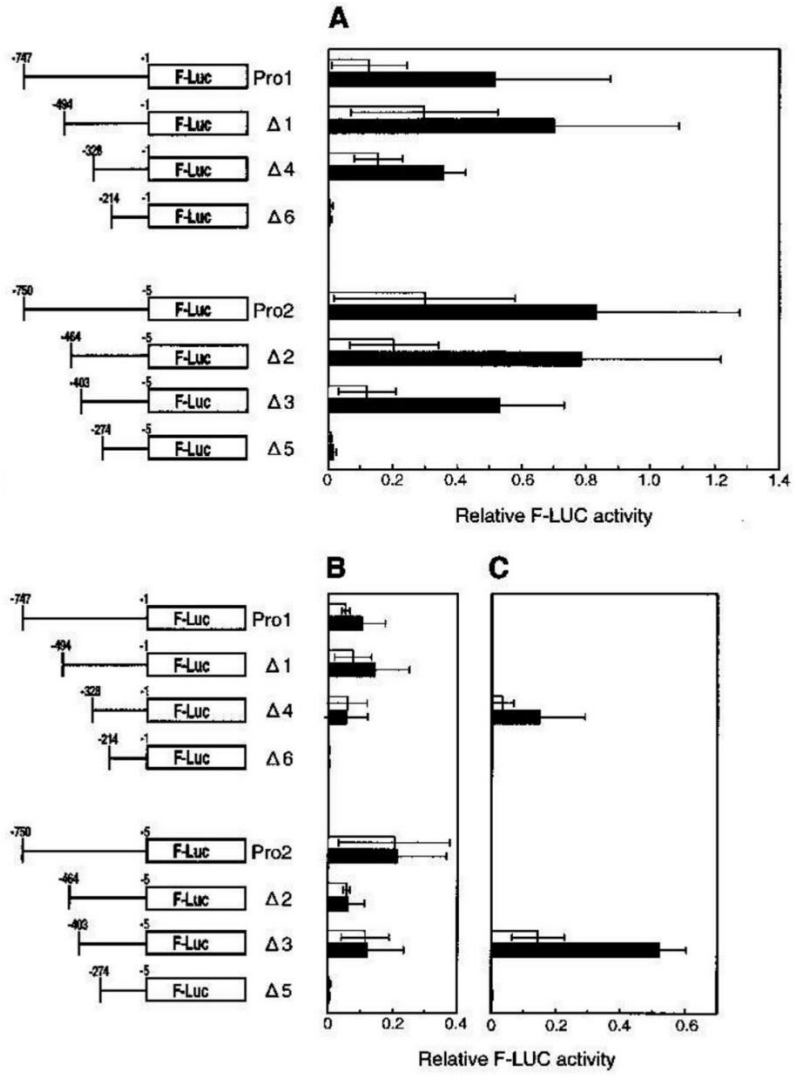

A
-747
-1
F-Luc
Pro1
-494
-1
F-Luc
Δ1
-328
-1
F-Luc
Δ4
-214
-1
F-Luc
Δ6
-750
-5
F-Luc
Pro2
-464
-5
F-Luc
Δ2
-403
-5
F-Luc
Δ3
-274
-5
F-Luc
Δ5
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Relative F-LUC activity
B
C
-747
-1
F-Luc
Pro1
-494
-1
F-Luc
Δ1
-328
-1
F-Luc
Δ4
-214
-1
F-Luc
Δ6
-750
-5
F-Luc
Pro2
-464
-5
F-Luc
Δ2
-403
-5
F-Luc
Δ3
-274
-5
F-Luc
Δ5
0
0.2
0.4
0
0.2
0.4
0.6
Relative F-LUC activity

Fig. 5