Expression of a Ribosomal Protein Gene in Axillary Buds of Pea Seedlings

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ABSTRACT

Axillary buds of intact pea seedlings (Pisum sativum L. cv Alaska) do not grow and are said to be dormant. Decapitation of the terminal bud promotes the growth of these axillary buds, which then develop in the same manner as terminal buds. We previously showed that unique sets of proteins are expressed in dormant and growing buds. Here we describe the cloning, sequencing, and expression of a cDNA clone (pGB8) that is homologous to ribosomal protein L27 from rat. RNA corresponding to this clone increases 13-fold 3 h after decapitation, reaches a maximum enhancement of about 35-fold after 12 h, and persists at slightly reduced levels at later times. Terminal buds, root apices, and elongating internodes also contain pGB8 mRNA but fully expanded leaflets and fully elongated internodes do not. In situ hybridization analysis demonstrates that pGB8 mRNA increases in all parts of the bud within 1 h of decapitation. Under appropriate conditions, growing buds can be made to stop growing and become dormant; these buds subsequently can grow again. Therefore, buds have the capacity to undergo multiple cycles of growth and dormancy. RNA gel blots show that pGB8 expression is reduced to dormancy levels as soon as buds stop growing. However, in situ hybridization experiments show that pGB8 expression continues at growing-bud levels in the apical meristem for 2 d after it is reduced in the rest of the bud. When cultured stems containing buds are treated with indoleacetic acid at concentrations ≥10 μM, bud growth and expression of pGB8 in the buds are inhibited.

Apical meristems of the shoot and root are the ultimate source of all cells in the plant body. In addition, shoot apical meristems play a direct role in the iterative formation of new stem tissues, leaves, and axillary buds (30). Several recent studies have characterized patterns of gene expression in meristematic tissues. A nonseed lectin gene is expressed at high levels in terminal buds of pea but not in expanded leaves (7). In barley, histone and ribosomal protein genes are expressed in vegetative and reproductive meristems (14). A histone gene is expressed in growing axillary buds of tomato, which occur near the apex, but not in dormant buds, which are found further from the apex (15). New meristem-specific gene expression is associated with the transition from the vegetative to the reproductive state of development (13, 21). A reporter gene fused to a histone promoter was used to show that this promoter is active in the flanks but not the most apical regions of shoot meristems in transgenic Arabidopsis plants (20). A similar approach was used to demonstrate the expression of a hydroxyproline-rich glycoprotein gene promoter in developing root meristems of transgenic tobacco plants (12).

Axillary buds of intact pea seedlings (Pisum sativum L. cv Alaska) are dormant. “Dormancy” may be defined based on the organ affected, the conditions that promote dormancy, or the source of signals that promote dormancy. We use the general definition of dormancy of Lang (16), which applies to “the temporary suspension of visible growth of any plant structure containing a meristem.” Decapitating the terminal bud promotes the development of dormant axillary buds. Auxin derived from the terminal bud is involved in maintaining this “apical dominance” (4, 32). Visible growth of pea buds can be detected within 8 h of decapitation (23). Under appropriate conditions, growing buds can reenter a dormant state and, subsequently, can be stimulated to grow again (this report). Seasonal cycles of growth and dormancy occur in overwintering buds of woody plants (25).

We previously analyzed protein expression in axillary buds of pea using two-dimensional gel electrophoresis (29; J.P. Stafstrom, unpublished results). Unique sets of proteins are expressed in dormant and growing buds of pea and in buds undergoing the transition from dormancy to growth and the transition from growth to dormancy. Therefore, at least two stable and two transition states can be defined by specific patterns of protein expression. To better understand gene expression in axillary buds of pea, we made a growing bud cDNA library. Here, we demonstrate using RNA gel blots and in situ hybridization techniques that expression of cDNA clone pGB8, which corresponds to a ribosomal protein gene, is associated with the growing state of axillary bud development. Growing floral meristems, root apices, and elongating stem internodes also express pGB8.

MATERIALS AND METHODS

Plant Material

Seedlings of pea (Pisum sativum L. cv Alaska) were grown as described previously (29). Seedlings were 7 d old at the beginning of all treatments. Tissues studied were axillary buds at the second node above the cotyledons, stem tissues from the second and fourth internodes above the cotyledons, fully expanded leaflets, and root apices (terminal 2 mm). Bud

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length was measured using a dissecting microscope with a calibrated ocular micrometer.

Library Construction and Screening

To isolate RNA, tissues were ground under liquid nitrogen, extracted with 4 M guanidine isothiocyanate and phenol/chloroform, and precipitated with 2 M LiCl (27). Poly(A)+ RNA from dormant buds and growing buds (collected from intact plants and from plants 24 h after decapitation, respectively) was purified by two rounds of chromatography on oligo(dT)-cellulose (27). A kit (Amersham) was used to synthesize cDNA from poly(A)+ RNA isolated from growing buds. First-strand synthesis was primed with an oligo(dT)/XbaI primer-adapter (Promega). The final products, which contained EcoRI and XbaI restriction sites at their 5' and 3' ends, respectively, were ligated to the directional cloning vector λ-GEM (Promega). A growing bud cDNA library was constructed by packaging the λ-DNA in vitro and infecting Escherichia coli strain LE 392. Differential plaque hybridization was performed using radiolabeled cDNA probes. pGEM-1 plasmids containing pea DNA inserts were isolated from λ-clones, ligated, and used to transform E. coli strain JM109 or DH5αF'. DNA inserts were isolated by restriction digestion with EcoRI and XbaI.

The temporal expression of pea genes was analyzed by RNA gel blotting. Total RNA (20 μg/lane) was separated by denaturing formaldehyde gel electrophoresis and transferred to Nyttran (Schleicher & Schuell). Blots were prehybridized in 50% formamide, 5x Denhardt’s reagent, 0.5% SDS, 5x SSPE, and 100 mg/mL of salmon sperm DNA for 2 h at 42°C. Blots were hybridized overnight to [α-32P]dCTP-oligolabeled probes (Prime-a-Gen kit, Promega) under the same conditions, washed in 0.2x SSC, 0.1% SDS at 55°C, and exposed to x-ray film at −70°C. Relative expression was quantified using a Bio-Rad video densitometer.

DNA Sequencing

Clone pGB8 was sequenced using Sequenase (United States Biochemical). Double-stranded sequencing was performed using SP6 and T7 primers. For single-stranded sequencing, the 0.5-kb EcoRI/XbaI insert was subcloned into M13 vectors mp18 and mp19, which were primed using the −40 universal primer. The GenBank data base was searched for similar sequences using the FASTA program (24).

In Situ Hybridization

mRNA corresponding to clone pGB8 was localized in tissues by in situ hybridization (17). Tissues were fixed in 5% formalin, 5% acetic acid, 50% ethanol, dehydrated through ethanol and tert-butyl alcohol series, and embedded in wax. Sections 8 μm thick were cut with a rotary microtome and mounted on glass slides coated with poly-o-lysine hydrobromide.

Mounted sections were deparaffinized with xylene and rehydrated through an ethanol series. Sections were incubated sequentially in: 0.2 M HCl for 20 min, 2x SSC for 30 min, 1 μg/mL of proteinase K (in 100 mM Tris-HCl [pH 8.0], 50 mM EDTA) for 30 min, PBS (10 mM sodium phosphate [pH 7.5], 150 mM NaCl), and 2 mg/mL of glycine in PBS. Sections were refixed in 4% paraformaldehyde in PBS and rinsed in PBS. The final incubation contained 0.1% acetic anhydride, 0.1 M triethanolamine, and 0.05 M HCl. The proteinase K incubation was at 37°C; all others were at room temperature.

Antisense RNA probes were made using EcoRI-digested pGB8 DNA and SP6 RNA polymerase (BRL) in the presence of [α-32P]UTP (New England Nuclear, 1000 Ci/mmol). For controls, sense-strand RNA probes were made using XbaI-digested pGB8 and T7 RNA polymerase (BRL). Treated sections were hybridized with RNA probes (2.2 × 106 dpm/mL) in 10 mM Tris-HCl (pH 6.8), 10 mM sodium phosphate (pH 6.8), 0.3 M NaCl, 10 mM DTT, 5 mM EDTA, 50% deionized formamide, 10% dextran sulfate, 1 mg/mL of yeast tRNA, and 0.5 mg/mL of poly(A) RNA. Hybridization was for 16 to 20 h at 50°C. The sections were washed at 50°C for 5 h in the same buffer without dextran sulfate, tRNA, or poly(A) RNA. The buffer was changed once during this time. Next, the sections were treated with 20 μg/mL of RNase A at 37°C for 30 min in 10 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10 mM DTT, and 1 mM EDTA and then washed several times in the same buffer. The sections were dehydrated through an ethanol series and air dried. Finally, the slides were dipped in Kodak NTB2 emulsion diluted 1:1 with water and exposed for 1 to 3 d. Dark-field images were recorded using a Nikon Optiphot microscope and Kodak TechPan film. The micrographs in each figure were from a single experiment. All sections of axillary buds and root apices were photographed and printed identically. Longer exposures of internode sections were necessary to localize pGB8 mRNA. Each experiment was repeated at least once and gave similar results.

Growth Regulation by Auxin

Stem segments (1 cm long) containing node-2 buds were floated on Murashige-Skoog medium (GIBCO) containing 3% sucrose. The medium also contained various concentrations of IAA. After 24 h, buds were measured and then prepared for in situ hybridization.

RESULTS

Kinetics of Axillary Bud Growth

There are four buds at the second node above the cotyledons on Alaska pea seedlings (29). These buds do not grow on intact plants. The largest bud (called "large" in this report) begins to grow 8 h after plants are decapitated just above this node (Fig. 1A). The second largest bud (called "small") grows for 2 d following decapitation and then ceases growing (Fig. 1B). When the shoot derived from the large bud was removed 5 d after the first decapitation, the small bud resumed growing.

Expression of cDNA clone pGB8

A cDNA library was synthesized using poly(A)+ RNA from growing axillary buds (collected 24 h after decapitation). This library was screened for clones whose corresponding mRNA
of the bp report, we growing. large RNA clone, was absent from dormant internodes of 7-d-old pea plants, which are fully elongated, do not express pGB8 (lane 4). In contrast, the fourth internodes of these plants, which measure about 1 cm and will elongate to about 10 cm, do contain pGB8 mRNA (lane 3).

DNA Sequence of pGB8

The DNA sequence of the EcoRI/XbaI fragment of pGB8 was determined (Fig. 3). A search of the GenBank data base indicated a high degree of similarity between the amino acid sequences of pGB8 and ribosomal protein L27 from rat (31). It is likely that the TG at the beginning of the nucleotide sequence is part of the ATG/Met start codon (see "Discussion"). There is 56% identity over 135 amino acids and 89% similarity when conserved amino acid substitutions are considered.

In Situ Expression of pGB8

pGB8 message was localized on sections of axillary buds and other tissues using a 35S-labeled antisense RNA probe, autoradiography, and dark-field microscopy to visualize silver grains. Very little signal was present in dormant buds on intact plants (Fig. 4A). Within 1 h of decapitation, an increase in signal intensity was apparent throughout the bud, especially in the apical meristem, young leaf primordia, the procambium, and a bud in the axil of the largest leaf primordium

was absent from dormant buds (from intact plants). In this report, we describe the expression of one growing bud-specific clone, called pGB8. An RNA gel blot containing total RNA from buds and other tissues was probed with the 500-bp EcoRI/XbaI fragment of pGB8 (Fig. 2A). A single band of about 600 bases was detected. Dormant buds on intact plants contained a small amount of pGB8 message (lane 1). This message increased 13-fold after 3 h, reached a maximal level of enhancement of about 35-fold after 12 h, and then was reduced slightly at later times (lanes 2–7). mRNA was present in terminal buds of the shoot and in root apices but was absent from fully expanded leaflets (lanes 8–10).

Expression of pGB8 in small buds was examined (Fig. 2B). Message was abundant when these buds were still growing 2 d after the terminal bud was removed (lane 2), reduced by 3 d (lane 3), and reduced further to dormancy levels thereafter (lanes 4–6). Removing the large bud promoted active expression of pGB8 1 d later (lane 7). Expression in growing and nongrowing internodes also was investigated (Fig. 2C). The

Figure 1. Growth kinetics of buds at node 2. A, Growth of large buds after decapitation. There is a lag of about 8 h before growth begins. Mean ± se; n = 25 (12 and 18 h) or 50. B, Large and small buds begin to grow following decapitation (closed arrow). Growth of the small buds is reduced at 3 d and ceases after 5 d. Removing the large buds after 5 d (open arrow) allows small buds to resume growing. Mean ± se; n = 25.

Figure 2. RNA gel blot analysis of pGB8 expression. Total RNA (20 μg) was separated by denaturing formaldehyde gel electrophoresis, transferred to Nytran, and hybridized with a radiolabeled pGB8 probe. A, RNA was isolated from axillary buds from intact plants (lane 1) and plants decapitlated 3, 6, 9, 12, 18, or 24 h before isolation (lanes 2–7, respectively); terminal buds of the shoot (lane 8); root apices (lane 9); and fully expanded leaflets (lane 10). B, RNA was isolated from small buds: from intact plants (lane 1); from plants decapitated 2, 3, 4, 5, or 6 d before isolation (lanes 2–6, respectively); or from plants 5 d after the first decapitation and 1 d after the large bud was removed (lane 7). C, RNA was isolated from dormant and growing axillary buds (lanes 1–2) and from fourth and second internode tissues (lanes 3 and 4, respectively).
Figure 3. Sequence analysis of pGB8. A. Nucleotide sequence of pGB8. The A at the beginning of the sequence was not present in the clone but is thought to be present in the gene (see text). The first in-frame stop codon is underlined. B. The derived amino acid sequence of pGB8 is compared to that of rat ribosomal protein L27 (31). Colons indicate amino acid identity; single dots indicate related amino acids, according to the criteria of Pearson and Lipman (24).

(Fig. 4B). After 6 and 24 h, pGB8 mRNA was abundant in all tissues (Fig. 4, C and D, respectively). Reduced levels of signal in the pith and cortex is probably due to the large vacuoles in these cells of these tissues. A control experiment in which a sense probe was used demonstrated a very low level of nonspecific labeling (Fig. 4E). A bright-field micrograph through the large and small buds at node 2 provides a more conventional view of these organs (Fig. 4F).

Figure 5 demonstrates in situ expression of pGB8 in small buds. Three days after the terminal bud was removed, pGB8 mRNA was somewhat reduced, but its overall distribution was similar to that in growing buds (Fig. 5A; cf. Fig. 4D). After 4 to 5 d, there was a substantial reduction in all areas of the bud except the apical meristem (Fig. 5B and C). After 6 d, labeling in the meristem also was gone (Fig. 5D). Removing the large buds after 5 d promoted expression of pGB8 1 d later in all areas of the small buds (Fig. 5E). This section includes developing flower primordia, which also contain pGB8 mRNA.

In situ expression of pGB8 in the root apex and stem tissues is shown in Figure 6. A near-medial section of a root apex shows that pGB8 mRNA was most abundant in the procambium of the central vascular cylinder and in the epidermis or outer cortex (Fig. 6A). Message was reduced or absent in the quiescent center of the root apical meristem. Refractile starch grains (statoliths) were apparent in the columella of the root cap, but this organ lacked specific signal. Growing fourth internodes (Fig. 6B) contained pGB8 message in the procambium (within and between the four central vascular bundles and in two peripheral vascular bundles), in developing xylem cells and phloem fibers, and in the outer cortex. The developing conducting phloem lacked this message. Nongrowing second internodes lacked pGB8 mRNA (Fig. 6C).

pGB8 Expression in Response to Auxin

Stem segments containing node-2 buds were floated on growth medium. The bract at this node served as an "outtrigger" and prevented contact between the bud and the medium. After 24 h, bud growth was maximal at ±0.1 μM IAA (about half as much growth would have occurred on decapitated plants), about half-maximal at 1.0 μM IAA, and inhibited completely at ±10 μM IAA (Fig. 7). Analysis of variance using the Student-Newman-Keuls multiple range test indicated two groupings (four lowest IAA concentrations versus the two highest) at P < 0.0001.

pGB8 expression in these buds was determined using in situ hybridization (Fig. 8). All growing buds, even those growing slowly in response to 1.0 μM IAA, contained pGB8 message in a pattern similar to that of growing buds on decapitated plants (Fig. 8, A–D; cf. Fig. 4). In nongrowing buds, only a low background signal was detected; the densely cytoplasmic apical meristem, young leaf primordia, and procambium lacked pGB8 message (Fig. 8, E and F).

DISCUSSION

Axillary Bud Growth

Axillary buds of Alaska pea do not grow on intact plants. A careful examination of individual buds indicated that growth begins 8 h after decapitation (23). We obtained the same result from a study of populations of buds (Fig. 1A). Thus, the developmental transition from dormancy to growth occurs rapidly. All node-2 buds begin to grow following decapitation, but only the largest bud continues growing (Fig. 1B). Other investigators have found that rapid growth of a bud can inhibit buds at higher and lower nodes in pea (11) and at opposite nodes of Alternanthera, a decussate plant (6). However, a detailed study of the transition from growth to dormancy, as we have described for small axillary buds of pea, has not been reported previously (Fig. 1B). This experiment demonstrates that buds can cycle rapidly and repeatedly between states of growth and dormancy. In contrast, the development of a vegetative meristem into a reproductive meristem is generally a unidirectional process (2).

Expression of a Ribosomal Protein Gene in Axillary Buds

Clone pGB8, which was isolated from a growing bud cDNA library, is homologous to ribosomal protein L27 from rat (Fig. 3; ref. 31). We used pGB8 as a probe to isolate three additional clones from a new cDNA library (S.B. Murfey and J.P. Stafstrom, unpublished observations). All clones contain ATG/Met codons in the predicted positions (Fig. 3). The nucleotide and amino acid sequences of the four clones are very similar but not identical. We are developing gene-
specific probes to determine the expression pattern of each member of this gene family. Ribosomal protein genes in plants and other organisms generally are expressed coordinately (9, 19), but exceptions have been noted (3).

The steady-state level of pGB8 mRNA in axillary buds increases before visible growth can be detected 8 h after decapitation. An RNA gel blot shows a 13-fold increase after 3 h, the shortest time tested (Fig. 2A). In situ hybridization analysis shows that message increases in all areas of the bud within 1 h (Fig. 4B). The latter result indicates that each bud cell is competent to respond to growth-promoting signals from the plant. Also, the absence of a proximal-distal gradient of pGB8 expression indicates that these signals must diffuse or be transported rapidly to all cells of the bud.

Expression of pGB8 is closely correlated with the growing state in small buds as they cycle between states of growth and dormancy (Figs. 1B and 2B). However, in situ hybridization analysis of these buds indicates that pGB8 message
GENE EXPRESSION IN AXILLARY BUDS OF PEA

Figure 5. In situ localization of pGB8 in small buds. Buds were from plants decapitated 3 d (A), 4 d (B), 5 d (C), 6 d (D), or 6 d (large bud removed 1 d earlier) (E) before fixation. The pattern of expression at 3 d is similar to that in growing buds. After 4 or 5 d, the signal occurs only in the apical meristem (am). After 6 d, pGB8 signal is absent in small buds if the large bud is present (D) or abundant if the large bud had been removed 1 d earlier (E). Flower primordia (fp) also are labeled in reactivated small buds. Bar, 500 μm.

persists at high levels in the apical meristem through 5 d after decapitation (Fig. 5). Therefore, cells of the meristem respond to growth-inhibiting signals from the plant differently than their neighbors. This cell autonomous pattern of expression could not have been predicted by RNA gel blots. This result demonstrates one way in which the transition from growth to dormancy is different from the transition from dormancy to growth. These transitions also can be distinguished on two-dimensional gels by specific patterns of protein expression (29; J.P. Stafstrom, unpublished results). It is quite possible that buds on plants in nature cycle repeatedly between states of growth and dormancy without any visible growth. For example, addition of auxin to the stems of decapitated pea plants completely inhibited bud growth. After 24 h, these buds contained dormancy-specific proteins, as expected. After 6 h, however, they contained elevated levels of growth-specific proteins and reduced levels of dormancy-specific proteins, indicating transient progress toward the growing state (29).

Increased expression of ribosomal protein genes has been demonstrated in other plant tissues and organs: auxin-treated hypocotyls of soybean (8, 9), developing maize kernels (18),...
cytokinin-treated soybean suspension culture cells (5), and developing barley meristems (14). The rate and extent of these increases were not as dramatic as those that occurred in pea buds. Significantly, all of these tissues and organs may be classified as "growing."

The addition of auxin in a lanolin paste to the stumps of decapitated plants inhibits development of axillary buds (32). However, auxin at a concentration of 1% (approximately 57 mM) is needed to inhibit bud growth completely (29, 32). Here, we showed that bud growth and pGB8 expression can be inhibited completely by 10 μM IAA (Figs. 7 and 8). Auxin is thought to inhibit bud growth by an indirect mechanism (10, 22, 28). At present, we do not know whether auxin in the stem might promote the synthesis of a secondary inhibitor or inhibit the synthesis, transport, or action of a growth promoter.

Expression of pGB8 in Other Organs

"Growth" implies an irreversible increase in size. Growth in meristematic tissues, including axillary buds, is due to both cell division and cell expansion. In dicotyledonous stems, there is little or no cell division. The presence of pGB8 message in growing pea stems implies that cell division is not necessary for its expression (Fig. 2C). In contrast, Baerson and Kaufman (1) found that rRNA transcription in Alaska peas is correlated with cell division but not with cell expansion. This discrepancy between our respective results may be due to the fact they studied transcription per se, whereas we looked at steady-state mRNA levels. Also, rRNA genes and ribosomal protein genes are regulated by different mechanisms (19).

Results of RNA gel blots and in situ hybridization experiments indicated that pGB8 is expressed in root apices (Fig. 6A). Message was detected in the cell division and expansion zones proximal to the root cap/root body junction (26). There is little or no expression in the quiescent center, where cell division is not inhibited by 10 μM IAA. Stem segments containing node-2 buds were cultured on Murashige-Skoog medium containing sucrose and various concentrations of IAA. Buds were measured after 24 h. Bud growth is inhibited at ≥10 μM IAA. Mean ± se; n = 15.
division occurs very infrequently, or in the root cap. Histone gene expression in pea root tips showed the same general pattern as pGB8 (15).

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LITERATURE CITED


