Characterization of an Endo-β-1,4-Glucanase Gene Induced by Auxin in Elongating Pea Epicotyls

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A gene (EGL1) encoding an endo-β-1,4-d-glucanase (EGase, EC 3.2.1.4) of pea (Pisum sativum) has been cloned and characterized. EGL1 encodes a 486-amino acid polypeptide, including a 24-mer putative signal peptide. The mature protein has a calculated molecular mass of 51.3 kD and an isoelectric point of 9.1. This pea EGase shares significant similarity with EGases from other plant species, but it appears to be distinct from the EGases associated with abscission and fruit ripening. Although EGL1 transcripts are detected in all parts of pea plants, they are relatively abundant in flowers and young pods undergoing rapid growth and most abundant in elongating epicotyls of etiolated seedlings. When epicotyl segments (6 mm long, 4 mm from the apical hook) are incubated in a 5 µM solution of the synthetic auxin analog 2,4-dichlorophenoxyacetic acid, the concentration of EGL1 mRNA increases about 10-fold when the segments elongate most rapidly.

Plant cell walls are intimately involved in plant growth, development, differentiation, and defense against invading pathogens (McNeil et al., 1984; Varner and Lin, 1989; Carpita and Gibeaut, 1993). The metabolism of cell walls participates in a number of physiological processes, including elongation and division of cells, abscission of leaves and flowers, and fruit ripening (Maclachlan and Carrington, 1991). For example, considerable breakdown of cell walls occurs during abscission and fruit ripening (Sexton and Roberts, 1982; Fischer and Bennett, 1991). On the other hand, it has been hypothesized but not proven that wall polymers are cleaved and perhaps rejoined during cell elongation and division (Albersheim, 1975; Carpita and Gibeaut, 1993; Cosgrove, 1993; Fry, 1993). Cell-wall polymer cleavage is also involved in the generation of oligosaccharins, oligosaccharide signal molecules believed to be involved in regulating plant development, cell elongation, and defense reactions (reviewed by Darvill et al., 1992).

Primary cell walls are composed of cellulose microfibrils embedded in a matrix of xyloglucans, arabinoxylans, pectic polysaccharides, and proteins (McNeil et al., 1984; Carpita and Gibeaut, 1993). It is generally believed that EGase, which cleaves the internal β-1,4-linkages of the glucosyl backbone of xyloglucan and probably other glucans as well, plays a role in cell-wall metabolism (Hayashi, 1989; Maclachlan and Carrington, 1991). Indeed, EGases or their mRNAs have been associated with a variety of physiological processes, including fruit ripening of avocado and tomato (Christoffersen et al., 1984; Cass et al., 1990; Fischer and Bennett, 1991; Lashbrook et al., 1994) and abscission of leaves and floral organs (Tucker et al., 1988, 1991; Bonghit et al., 1992; Kemmerer and Tucker, 1994; Lashbrook et al., 1994; Taylor et al., 1994). EGases are also expressed in young styles and anthers (Del Campillo and Lewis, 1992), adventitious roots (Kemmerer and Tucker, 1994), drought-stressed tissues (Huberman et al., 1993), and cultured cells and tissues (Trudseau and Wyndale, 1991; Nakamura and Hayashi, 1993; Yoshida and Komaie, 1993). Those studies revealed the existence of multiple forms of EGases with different molecular weights, pl's, and expression patterns (Byrne et al., 1975; Kanellis and Kalaitzis, 1992; Kemmerer and Tucker, 1994; Lashbrook et al., 1994). Sequence analysis of EGase cDNAs has established that plant EGases are encoded by a small gene family or families. EGases from different plant species are conserved within structural domains, although the degree of similarity varies (Tucker and Milligan, 1991; Kemmerer and Tucker, 1994; Lashbrook et al., 1994). Based on their expression profiles, plant EGases may be divided into two divergent groups, one associated with fruit ripening and the other with abscission, although overlapping expression can sometimes be observed (Lashbrook et al., 1994).

The possible involvement of EGase activity in auxin-stimulated cell enlargement was made evident in studies of etiolated epicotyls of pea (Pisum sativum) conducted more than 20 years ago by Maclachlan and his associates (Fan and Maclachlan, 1966, 1967; Verma et al., 1975). They purified two EGases, a cell-wall-bound 75-kD protein and a Golgi-located 15-kD protein, from pea epicotyls that had been sprayed with a toxic concentration (5 mM) of 2,4-D (Byrne et al., 1975; Bal et al., 1976). The toxic level of 2,4-D resulted in accelerated ethylene production and swelling of stem tissues. An extension of these studies by Hayashi and Ohsumi (1994) found a 51-kD EGase rather than the 15- and 75-kD EGases. These efforts led to the conclusion that some EGases in etiolated pea seedlings are induced by auxin and function in the cleavage of xyloglucan (Hayashi et al., 1984; Hayashi, 1989; Hoson et al., 1993). Meanwhile, other stud-

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ies have demonstrated that in vitro generated xyloglucan oligosaccharides inhibit the 2,4-D-stimulated elongation of pea stems (York et al., 1984; Emmerling and Seitz, 1990; McDougall and Fry, 1990, 1991; Augur et al., 1992; Darvill et al., 1992; Fry, 1993). It has been theorized that xyloglucan fragments generated in vivo by EGase during auxin-stimulated cell elongation participate in the regulation of cell elongation (York et al., 1984; McDougall and Fry, 1991; Darvill et al., 1992; Fry, 1993). We now describe the cloning and characterization of an EGase gene obtained from pea. This EGase gene is predominantly expressed in elongating pea epicotyls and is induced by treating excised epicotyl segments with 2,4-D.

**MATERIALS AND METHODS**

**Plant Materials**

Seeds of pea (Pisum sativum L. cv Alaska; Athens Seed Co., Athens, GA) were washed several times and soaked in water for 36 h at 21°C in the dark before being planted in vermiculite. Pea plants were grown in a growth chamber at 21°C with a RH of 65% and with 14 h of light and 10 h of dark. Leaves, tendrils, stems, and roots were harvested separately from 15-d-old green plants. Flower buds, flowers, seed pods at different developmental stages, and floral abscission zones were harvested from 20- to 50-d-old plants. Etiolated seedlings were harvested after 8 to 10 d of growth in total darkness at 21°C and 65% RH.

**Cloning of EGases from Pea**

Degenerate oligonucleotides, GGNGGNTAYTAYGAY-GCNGG and GGNGTRTCCATRTCYTNGG, corresponding to two conserved domains of plant EGases (Tucker and Milligan, 1991; Lashbrook et al., 1994), were synthesized and used as a primer set to amplify by PCR an EGase-specific DNA fragment from pea genomic DNA. This PCR product was used as a DNA probe to screen for pea EGase genes from a genomic library constructed in the λ vector, EMBL3, by Clontech Laboratories (Palo Alto, CA). The DNA inserts of positive clones were mapped by restriction endonucleases, subcloned into pBluescript II phagemid vector (Stratagene), and sequenced from both strands by the University of Georgia Molecular Genetics Instrumentation Facility (Athens, GA). DNA sequences were analyzed and manipulated with Wisconsin Package, version 8, software (Genetics Computer Group, Madison, WI).

**Pea Stem Elongation Assay**

The pea stem elongation bioassay is a modification of the process described by York et al. (1984). Epicotyl segments (6 mm long starting 4 mm from the apical hook) were excised with razor blades from the third internode of 9-d-old etiolated pea seedlings. About 1000 stem segments were washed for 30 min in 200 mL of 5 mM potassium phosphate, pH 6.0. One hundred segments were then transferred to each of ten 45-mL Falcon tubes, five of which contained 20 mL of control buffer (5 mM potassium phosphate, pH 6.0), and the other five contained an assay solution (5 μM 2,4-D in control buffer). The tubes were placed horizontally on a gyratory shaker (50 rpm at 21°C) for specified times. At each time (0, 5, 10, 15, and 20 h), one lot each of control and 2,4-D-treated stem segments was removed from the shaker, drained through a double layer of cheesecloth, and rinsed twice with sterile water. Eighty of the 100 stem segments from each treatment were frozen in liquid nitrogen for RNA isolation, and the lengths of the remaining 20 were measured on a Nikon profile projector (model 6C). All experimental manipulations were performed in a darkroom under dim red light.

**Total RNA Isolation**

Large-scale RNA samples were prepared from various organs of pea plants according to the method of Cathala et al. (1983). RNA minipreps from auxin-treated pea stem segments were prepared as follows: 20 to 30 pea stem segments (approximately 0.5 g) were placed in a 13-mL polyethylene Falcon tube and 5 mL of lysis buffer (5 mM guanidinium isothiocyanate, 0.1 M sodium acetate, pH 7.0, 20 mM EDTA) were added. The stem segments were homogenized for 1 min at speed 8 with a Polytron homogenizer (Brinkmann). The homogenate was transferred to a 40-mL polyethylene centrifuge tube containing 0.5 mL of 3 M sodium acetate, pH 4.0, 5 mL of water-saturated phenol, and 1 mL of a mixture of chloroform:isoamyl alcohol (24:1). The suspension was vortexed for 2 min, followed by centrifugation for 10 min at 10,000 rpm in a JA-20 rotor (Beckman). Total RNA in the clear aqueous phase was precipitated by mixing it with 5 mL of isopropanol. The RNA pellet was recovered by centrifugation, washed with 70% ethanol, air dried, and dissolved in RNase-free water.

**First-Strand cDNA Synthesis**

First-strand cDNAs were synthesized from total RNA at 39°C for 1 h in a 50-μL reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 1 mM each of dATP, dCTP, dGTP, and dTTP, 2 μM universal oligo(dT) primer [5'-GCTCGAGCAGGTGCACG(T)₆₀] or a gene-specific primer, 8 units of RNase inhibitor (Boehringer Mannheim), and 300 units of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL).

**Cloning of Full-Length EGL1 cDNA**

Poly(A⁺) mRNA, purified from 100 μg of total RNA from etiolated pea seedlings by Oligotex-Dt chromatography (Qiagen Inc., Chatsworth, CA), was used as the RNA template for first-strand cDNA synthesis as described above. The full-length cDNA of EGL1 transcript was amplified by PCR as described by Wu et al. (1995). The EGL1-specific primers used in PCR amplifications were as follows:

- **Primer I:** 5'-CAGTTTGTTATTGTTGTTGTTGTTGCA
- **Primer II:** 5'-CACAGGATCCTTGGCTGTTGAAAATGGTT
- **Primer III:** 5'-CTGTCGACCCCTTAGTTGGGTATAGACGG
- **Primer IV:** 5'-CTCGAGGATCCTTGGCTGTTGAAAATGGTT
- **Primer V:** 5'-GAACGACGGATTTCCGGATG
- **Primer VI:** 5'-CTGGCAGCAGTGTTGTTGTTGTTGCA

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- **Primer II:** 5'-CACAGGATCCTTGGCTGTTGAAAATGGTT
- **Primer III:** 5'-CTGTCGACCCCTTAGTTGGGTATAGACGG
- **Primer IV:** 5'-CTCGAGGATCCTTGGCTGTTGAAAATGGTT
- **Primer V:** 5'-GAACGACGGATTTCCGGATG
- **Primer VI:** 5'-CTGGCAGCAGTGTTGTTGTTGTTGCA

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- **Primer V:** 5'-GAACGACGGATTTCCGGATG
- **Primer VI:** 5'-CTGGCAGCAGTGTTGTTGTTGTTGCA
Mapping of 5' End of EGL1 mRNA

The 5' end of EGL1 mRNA was determined by rapid amplification of cDNA ends and primer extension as described by Wu et al. (1995). EGL1-specific primer I (see above), complementary to the sequence immediately upstream from the putative start codon, was used as the extending primer.

RNA Quantitation

Quantitation of EGL1 mRNA was conducted by competitive RT-PCR (Gilliland et al., 1990) with an added "hot start" modification (Chou et al., 1992). First-strand cDNA samples (5 μL; see above) were added to 100-μL master reaction cocktails containing 5 x 10^-11 nmol cloned EGL1 genomic DNA fragment, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.0 mM MgCl₂, 0.001% (w/v) gelatin, 0.25 mM each dATP, dCTP, dGTP, and dTTP, 0.2 μM each EGL1-specific primers IV and VI (see above), and 3 units of Taq DNA polymerase (Perkin-Elmer Co.). PCR was performed for 30 repeated cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. An aliquot (15 μL) of each PCR product was subjected to electrophoresis on a 1.2% agarose gel. After electrophoresis, the gel was stained in a 0.5-μg/mL ethidium bromide solution for 30 min and then photographed. The negative film (New Type 665; Polaroid Co., Cambridge, MA) was scanned, and DNA bands were quantitated on a personal densitometer (Molecular Dynamics, Inc., Sunnyvale, CA).

RESULTS

Isolation and Characterization of an EGase Gene from Pea

A 260-bp DNA fragment was synthesized by PCR from pea genomic DNA based on two conserved domains, GGYYDAG and PEDMDTP, present in EGases from avocado, bean, and tomato (Tucker and Milligan, 1991; Lashbrook et al., 1994). The 260-bp PCR product has an open reading frame that encodes an 87-amino acid peptide possessing about 60% identity with the corresponding segments of other plant EGases (data not shown). This DNA fragment was used to detect, in the pea genome, a multi-gene EGase family consisting of about 10 sequences (Fig. 1). The same probe was used to isolate 11 positive clones from a pea genomic library. One of the clones, EGL1, encodes an intact EGase gene. The other clones are either truncated EGase sequences or pseudogenes (data not shown). Based on the DNA sequence of EGL1 (Fig. 2), the full-length cDNA of EGL1 was amplified by RT-PCR and sequenced (Fig. 2). The exons of EGL1 encode a protein (EGL1) of 486 amino acids, including a 24-mer hydrophobic, signal peptide-like sequence (Fig. 3). The putative mature protein has a predicted molecular mass of 51.3 kD and a pi of 9.1. EGL1 does not contain N-linked glycosylation motifs (N-X-S/T).

Comparison of EGL1 with Other Plant EGases

EGL1 is similar to other plant EGases in that it possesses their characteristic conserved domains (Fig. 3). However, the overall similarity varies sharply depending on the particular plant EGase EGL1 is being compared to. For example, EGL1 has 76% identity with an Arabidopsis EGase (clone p96), about 60% identity with EGases associated with fruit ripening, but only about 50% identity with those predominantly expressed in abscission zones. A phylogenetic tree constructed from a limited numbers of plant EGases is shown in Figure 4. Based on this model, two groups of EGases, exemplified by the bean abscission EGase (BAC) and an avocado fruit EGase (Cell), were probably derived from a common ancestor before the divergence of dicotyledonous species. EGL1 and the Arabidopsis EGase form a distinct branch within the group of EGases associated with fruit ripening, implying that they have a distinct expression profile and/or function.

Quantitiation of EGL1 Transcripts

The relative concentration of EGL1 transcripts in various parts of pea plants was determined by competitive RT-PCR (Gilliland et al., 1990). In addition to its high sensitivity, RT-PCR can be performed with high specificity by using gene-specific PCR primers. The specificity provided by RT-PCR is required to distinguish between the expression patterns of different members of the pea EGase gene family (Fig. 1). We chose primers IV and VI (Fig. 2) for the RT-PCR

Figure 1. Samples of pea genomic DNA (10 μg) were cleaved by restriction enzymes BamHI (B), EcoRI (E), or HindIII (H). The digested DNA samples were separated in a 1% agarose gel and blotted onto a Hybond-N filter (Amersham Life Science). The filter was probed with the 260-bp EGase-specific PCR product under moderate stringency (hybridization in 5X SSC at 55°C and wash in 0.5X SSC at 55°C).
analysis because primer IV resides in a poorly conserved region of plant EGases (Fig. 3) and primer VI is located in a nonconserved 3' untranslated region of EGL1 (Fig. 2). The IV/VI primer pair amplifies a 457-bp product (cEGLZ) from the target EGL1 cDNA and a 546-bp product (gEGLZ) as shown in Figure 5A, lane S, both the 457-bp and the 546-bp fragments hybridize to the internal oligonucleotide probe V (Fig. 2), confirming that they were amplified.

The IV/VI primer pair amplifies a 457-bp product (cEGLZ) region of plant EGases (Fig. 4) through pod maturation (15 d postflowering). EGLl transcripts are barely detectable in the abscission zone of stem segments (Fig. 6A). After 10 h, the elongation continues at a much slower pace and almost ceases after 20 h (Fig. 6B). The increase in the pods are about 5.5 cm long and are no longer elongating. The concentration of EGLZ transcripts remains low throughout pod maturation (15 d postflowering). EGLl transcripts are barely detectable in the abscission zone of stem segments (Fig. 6A). After 10 h, the elongation continues at a much slower pace and almost ceases after 20 h (Fig. 6B).

**EGL1 is Induced by 2,4-D in Elongating Epicotyls**

Since EGL1 is highly expressed in etiolated pea seedlings, which are largely dominated by elongating epicotyls (Fig. 5), we wondered whether there is a correlation between the rate of stem elongation and EGL1 mRNA accumulation. The synthetic auxin 2,4-D, which mimics the action of the plant auxin IAA by inducing Egase expression (Fan and Maclachlan, 1966, 1967; Byrne et al., 1975; Verma et al., 1975; Hayashi and Ohsumi, 1994) and stem elongation of etiolated seedlings (York et al., 1984), was used to examine this question. Indeed, a 5 μM solution of 2,4-D stimulates stem elongation (Fig. 6A). After 10 h, the elongation continues at a much slower pace and almost ceases after 20 h (Fig. 6B). The synthetic auxin 2,4-D, which mimics the action of the plant auxin IAA by inducing Egase expression (Fan and Maclachlan, 1966, 1967; Byrne et al., 1975; Verma et al., 1975; Hayashi and Ohsumi, 1994) and stem elongation of etiolated seedlings (York et al., 1984), was used to examine this question. Indeed, a 5 μM solution of 2,4-D stimulates stem elongation (Fig. 6A). After 10 h, the elongation continues at a much slower pace and almost ceases after 20 h (Fig. 6B). The increase in synthesis of Egl1 transcripts continues during the emergence and rapid growth of seed pods. However, the concentration of Egl1 transcripts declines rapidly in pods 6 DAF, i.e. when the pods are about 5.5 cm long and are no longer elongating. The concentration of Egl1 transcripts remains low throughout pod maturation (15 d postflowering). Egl1 transcripts are barely detectable in the abscission zone of stem segments (Fig. 6A). After 10 h, Egl1 transcripts continue at a much slower pace and almost ceases after 20 h (Fig. 6B).
**Endo-β,1,4-Glucanase Gene of Pea**

**Figure 3.** Plant EGase sequences were manipulated and compared to EGL1 using the programs of the Wisconsin Package (Genetics Computer Group, Madison, WI). Identical amino acid residues are shown in black and conserved ones in gray backgrounds. Cell is an EGase from avocado fruit (Cass et al., 1990) and BAC is an EGase from the bean abscission zone (Tucker and Milligan, 1991). The vertical arrowhead points at the putative cleavage site of the signal peptide.

**Figure 4.** Plant EGase sequences retrieved from GenBank were edited, before evaluation, to exclude the phylogenetically illegitimate signal peptides and truncated sequences. The evolutionary relationships of the EGases were calculated pairwise using the Kimura method (Kimura, 1983). The illustrated phylogenetic tree was constructed by the neighbor-joining method (Swofford and Olsen, 1990). In addition to the EGases shown in Figure 3, the following sequences were used for the phylogenetic analysis: p96 of Arabidopsis (GenBank entry No. U17888, submitted by R. J. Ferl, Horticultural Sciences, University of Florida, Gainesville), Cel2-T and Cel2-T of tomato (Lashbrook et al., 1994), and JET1 of common elder (Taylor et al., 1994). The descriptions “Fruit Ripening” and “Abscission” were assigned to the upper and the lower main branches of the tree because EGases included in these two branches, except EGL1 and p96, had been shown to be overwhelmingly expressed in ripening fruits and in abscission zones, respectively (see the introduction and “Results”). The expression pattern and function of the subbranch that includes EGL1 and the Arabidopsis EGase are unknown; thus this subbranch was labeled with a question mark.

The stem segments, after being placed in 5 μM 2,4-D, exhibit a 10-fold increase of EGL1 transcripts in 5 h, a 12-fold increase in 10 h, and a 13-fold increase in 15 h. The abundance of EGL1 transcripts declines thereafter (Fig. 6B). Segments incubated in the absence of added 2,4-D elongate at about half the rate of those treated with exogenous 2,4-D, indicating the presence of endogenous growth regulator(s). The concentration of EGL1 transcripts does not change significantly during growth in the absence of 2,4-D (Fig. 6B).

**DISCUSSION**

We have described the cloning and characterization of EGL1, an EGase from pea. EGL1 has significant similarity with all of the plant EGases cloned to date (Fig. 3), is auxin inducible (Fig. 6), and is presumably an extracellular or cell-wall-bound protein being targeted by a putative signal peptide (Fig. 3). EGL1 is closely related to a known pea EGase purified by Hayashi and Ohsumi (1994) from etiolated epicotyls that had been sprayed with 2,4-D. The first 10 amino acids in the N terminus of mature EGL1 are nearly identical with those of the purified EGase, differing only in that EGL1 has a Lys rather than a Ser as the N-terminal amino acid (Fig. 3; Hayashi and Ohsumi, 1994).

The amino acid sequence of EGL1 differs considerably from abscission zone-associated EGases (Fig. 4). Indeed, EGL1 transcripts are barely detectable in the abscission zones of floral pedicels and are 2,4-D induced, whereas abscission-related EGases are most abundant in abscission zones and are auxin suppressed (Tucker et al., 1988). EGL1 is phylogenetically closer to fruit ripening-associated than abscission zone-associated EGases. However, EGL1 also differs from fruit-ripening EGases in several respects, including significant phylogenetic differences (Fig. 4). For example, EGL1 differs in number and size of introns from those of the avocado fruit EGase gene; the avocado EGase gene has two more introns than EGL1 (Cass et al., 1990). Whereas the tomato fruit EGase contains one potential N-linked glycosylation site and the avocado fruit EGase has two, EGL1 does not possess any. Furthermore, the expression profile of EGL1 differs from that of fruit ripening EGases, namely, EGL1 is predominantly ex-

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Figure 5. A, Agarose gel electrophoresis of competitive RT-PCR products. RNA samples used in these experiments were treated with RNase-free DNase (Ambion, Inc., Austin, TX) for 30 min at 37°C to eliminate possible genomic DNA contamination, followed by inactivating the DNase at 95°C for 10 min. First-strand cDNA was synthesized from the DNA-free RNA samples (20 μg) using EGL1-specific primer VI. One-tenth (5 μL) of the cDNA sample was used as a target DNA template in a 100-μL PCR reaction. A constant amount (5 × 10⁻¹¹ nmol), which is approximately 100 times less than the amount required to reach the PCR plateau (data not shown), of the EGL1 genomic clone (Fig. 2) was added to each reaction as a competitive template. The quantitative PCR reactions were carried out for 30 thermal cycles. Contents of lanes are as in C; lane S, gel blot analysis of lane 13 with the 32P-labeled primer V (see Fig. 2) as a probe. B, Northern blot analysis of 5 μg of total RNA samples as in A with a 32P-labeled β-tubulin (TUB) cDNA of pea (Liaud et al., 1992) as a probe. The results indicated that the concentration of the RNA templates used in the competitive RT-PCR experiments were correctly measured, since the overall content of β-tubulin mRNAs is known to be constant in various tissues (Cleveland and Sullivan, 1985). Contents of lanes are as in C. C, The EGL1 PCR products shown in A were scanned and quantified on a densitometer, and the density ratio of the cDNA-derived product (cEGL1) over the genomic DNA-derived product (gEGL1) was plotted. Lane 0, Blank control; lane 1, leaf; lane 2, tendril; lane 3, stem; lane 4, root; lane 5, floral bud 1 d prior to opening; lane 6, flower; lanes 7 to 11, seed pods 2, 4, 6, 10, and 15 DAF; lane 12, floral abscission zone including flanking pedicel segments (about 3 mm from both sides harvested approximately 20 DAF); and lane 13, aerial portion of etiolated pea seedling.

Figure 6. A, Segments (20 per sample) of etiolated pea epicotyls were incubated in assay solutions for 0, 5, 10, 15, and 20 h. The lengths of stem segments were measured at each time. •, 5 μM 2,4-D solution; ○, control buffer. SDS (n = 20) are as indicated at each point. B, Competitive RT-PCR and quantitation of EGL1 transcripts are as described in Figure 5, except that the templates for RT-PCR contained 5 μg rather than 20 μg of total RNA samples. Symbols are pressed in elongating epicotyls, whereas fruit ripening EGase genes are most abundantly expressed in ripening fruit. Thus, EGL1 is likely the first representative of a third class of EGase genes whose function has yet to be defined.

The synthetic auxin 2,4-D induces both pea epicotyl elongation and the expression of EGL1 (Fig. 6). The data do not establish a cause-and-effect relationship between EGL1 expression and the rate of stem elongation. Furthermore, we did not observe an obvious increase in EGL1 transcripts throughout the 20-h incubation period in the control stem segments, although these control segments exhibited significant elongation with kinetics similar to those treated with 2,4-D (Fig. 6). However, in favor of a relationship between EGL1 and elongation growth, the concentration of EGL1 mRNAs does increase during the rapid elongation of seed pods and decreases when the longitudinal growth of the pods stops (Fig. 5).

The exact function of EGases in plants is unknown, although plant EGases characterized so far are always associated with physiological processes in which cell walls are markedly degraded, such as fruit ripening, abscission, and pith autolysis (Fischer and Bennett, 1991; Tucker et al., 1991; Huberman et al., 1993). It has been suggested that auxin-induced EGases may be involved in the regulation of cell elongation by generating xylol glucan oligosaccharins or by cleaving xylol glucan chains that cross-link cellulose microfibrils (Albersheim, 1975; Hayashi, 1989; Maclachlan and Carrington, 1991; Darvill et al., 1992; Fry, 1993; Hoson et al., 1993). Whether EGL1 belongs to such an EGase gene family is largely unknown. Nevertheless, that EGL1 is expressed most abundantly in tissues undergoing rapid elongation and is quickly induced following exposure to 2,4-D supports the hypothesis that EGL1 is associated with a
developmental process different from other cloned plant EGases. Construction of transgenic pea plants with effector-controlled reduction or elevation of EGL expression should provide information about the role of EGL in elongation.

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LITERATURE CITED


Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in the flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J 3: 1–30


Dawson DM (1989) Endo-p-1,4-Glucanase Gene of Pea 169


Lashbrook CC, Gonzalez-Bosch C, Bennett AB (1994) Two divergent endo-1,4-glucanase genes exhibit overlapping expression in ripening fruit and abscissing flowers. Plant Cell 6: 1485–1493


Yoshida K, Komae K (1993) Dissociation of cortical cell walls and enhancement of cellulase activity during the emergence of callus from rice roots in the presence of 2,4-D. Plant Cell Physiol 34: 507-514