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Developmental Regulation of (1→3,1→4)-β-Glucanase Gene Expression in Barley[1]

Tissue-Specific Expression of Individual Isoenzymes

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ABSTRACT

Two genes encode (1→3,1→4)-β-D-glucan 4-glucanohydrolase (EC 3.2.1.73) isoenzymes EI and EII in barley (*Hordeum vulgare* L.). Specific DNA probes have been used in Northern analyses to examine the developmental regulation of individual (1→3,1→4)-β-glucanase genes in the aleurone and scutellum of germinated grain and in young leaves and young roots. In aleurone and scutella excised from germinated grain, mRNAs encoding both isoenzymes are present but developmental patterns differ between the two tissues. Thus, levels of both isoenzyme EI and EII mRNA increase significantly in the aleurone between 1 and 3 days after the initiation of germination. In the scutellum, isoenzyme EI mRNA predominates and decreases as germination proceeds. Isoenzyme EI mRNA appears in young leaves approximately 8 days after the initiation of germination and levels rise until about 20 days. Enzyme activity in leaf extracts parallels the development of isoenzyme EI mRNA. No isoenzyme EII mRNA is detected in the leaves in this period. Analysis of RNA from different leaf segments indicates that the isoenzyme EI mRNA is distributed relatively evenly along the length of the leaf. In young roots, mRNA encoding (1→3,1→4)-β-glucanase isoenzyme EI is detected at high levels 3 to 6 days after the initiation of germination; again, little or no isoenzyme EII mRNA is found. Overall, transcription of the (1→3,1→4)-β-glucanase isoenzyme EII gene appears to be restricted to the germinating grain, whereas isoenzyme EI is expressed in a wider range of tissues during seedling development.

During the germination of barley (*Hordeum vulgare* L.) hydrolytic enzymes secreted from the aleurone layer and scutellum depolymerize starch and reserve proteins stored in the cells of the starchy endosperm. An important preliminary event in this process is the removal of walls of the starchy endosperm cells because the wall acts as a physical barrier that restricts access of α-amylases, peptidases, and other hydrolases to substrates within the cell (7). The walls in the starchy endosperm of barley are composed of more than 70% by weight (1→3,1→4)-β-glucan. This polysaccharide is depolymerized by the action of two (1→3,1→4)-β-glucan 4-glucanohydrolases (EC 3.2.1.73) that have been purified and characterized and are known to be encoded by separate genes (19, 29, 30).

Both isolated aleurone layers and excised scutella secrete (1→3,1→4)-β-glucanase isoenzymes *in vitro* (26) and this has been confirmed *in vivo* by hybridization histochemical examination of the location of mRNA encoding the enzymes (21). Expression of (1→3,1→4)-β-glucanase mRNA is detected first in the scutellar epithelium. However, after 1 d (1→3,1→4)-β-glucanase mRNA levels in the scutellum decrease, but increase progressively in the aleurone from the proximal to the distal end of the grain (21). The cDNA probe used in these experiments (8) to monitor transcription of (1→3,1→4)-β-glucanase genes did not distinguish between the two individual genes, which exhibit 92% sequence identity in their protein-coding regions (24).

The genes encoding barley (1→3,1→4)-β-glucanase isoenzymes EI and EII have recently been isolated and characterized (18, 24, 28) together with near full-length cDNAs for both isoenzyme EI and EII (24). Although the two genes exhibit a high degree of sequence identity in their 5′ untranslated and protein-coding regions, their sequences diverge dramatically in the 3′ untranslated region (24). This permits the design of oligonucleotide and short DNA probes to specifically identify mRNA encoding the individual isoenzymes.

In the present work, we have used isoenzyme-specific probes to examine the differential expression of the two genes in the aleurone and scutellum of germinated grain. Further, the probes have been used to investigate the regulation of (1→3,1→4)-β-glucanase gene expression during the development of young barley leaves and in young roots and coleoptiles. The results indicate that transcription of the (1→3,1→4)-β-glucanase isoenzyme EII gene is "germination-specific," whereas isoenzyme EI is expressed both in the germinated grain and in developing vegetative tissues.

MATERIALS AND METHODS

Plant Material

Barley (*Hordeum vulgare* L., cv Clipper) was obtained from Joe White Maltings (Collingwood, Victoria, Australia) and from the John Innes Centre for Plant Science Research (Norwich, UK). For germination experiments, grains were surface-

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sterilized in 0.2% (w/v) silver nitrate for 20 min, rinsed with 0.5 M NaCl, washed exhaustively with sterile distilled water, and soaked for 16 h in sterile distilled water. Grain was subsequently germinated in sterile Petri dishes at 22°C in a growth cabinet, or on vermiculite in a glasshouse under ambient conditions of light and temperature. At selected times after the initiation of germination, tissues were harvested for RNA isolation and for the measurement of enzyme activity.

(1→3,1→4)-β-Glucanase Activity

Tissues were ground under liquid nitrogen and extracted with 50 mM sodium acetate buffer, pH 5.0 (containing 10 mM sodium azide, 10 mM EDTA, 10 mM DTT, and 3 mM PMSF) at 4°C for 10 min. Insoluble material was removed by centrifugation and the extract stored at −70°C prior to assay. Protein concentrations in the extracts were measured with Coomassie blue, using BSA as a standard. Enzyme activity was measured viscometrically, using water-soluble barley (1→3,1→4)-β-glucan (Biocon Pty. Ltd., Cork, Ireland) as substrate (29). A unit of activity is defined as the amount of enzyme causing, at 40°C, an increase of 1.0 in the reciprocal specific viscosity per min of a 5 mg/mL (1→3,1→4)-β-glucan solution in 50 mM sodium acetate buffer, pH 5.0 (containing 10 mM sodium azide and 400 μg/mL BSA) (29). Specific activity is calculated as the activity per milligram of protein in the extract. For the estimation of cellulase activity, carboxymethyl cellulose with a degree of substitution of 0.54 (Edifas B50; ICI Australia Pty. Ltd.) was used as a substrate in viscometric assays (30).

RNA Isolation and Northern Analysis

Total RNA was extracted from vegetative tissue ground to a fine powder under liquid nitrogen using hot phenol/LiCl (27). Total RNA samples from scutella and aleurone layers excised from intact grain 1 and 3 d after the initiation of germination were generously provided by Dr. Harri Ranki. The RNA samples (3–20 μg) were separated in 1% (w/v) agarose gels in 2.2 M formaldehyde (23) and transferred to nitrocellulose filters (Hybond-C Extra, Amersham). Control gels were stained with ethidium bromide for comparison of the relative intensities of ribosomal RNA bands and loadings were adjusted where necessary to ensure that approximately equal amounts of RNA were applied to each lane of the gel. Filters were prehybridized and hybridized with specific cDNA probes as described by Slakeski *et al.* (24). The probe sequences corresponded to the entire 3′ untranslated regions of the barley (1→3,1→4)-β-glucanase isoenzyme EI and EII genes (24), and their specificity was routinely checked by spotting isoenzyme EI and EII cDNA preparations onto the nitrocellulose filters before probing. The probes were prepared from the near full-length cDNAs by the PCR[2] using conditions described previously (24) and were approximately 400 and 180 base pairs in length for isoenzymes EI and EII, respectively. The fragments were recovered from agarose gels with GeneClean (Bio 101 Inc., La Jolla, CA) and labeled with α-[^{32}P]dCTP using random sequence hexanucleotides as primers (6). The specific activity of isoenzyme EI cDNA probes was usually 5- to 10-fold higher than the shorter isoenzyme EII probes.

[2] Abbreviation: PCR, polymerase chain reaction.

RESULTS

Expression in Germinated Grain

Isoenzyme EI mRNA of approximately 1500 nucleotides in length was found in RNA preparations from both aleurone layers and scutella that had been dissected from intact barley grain 1 or 3 d after germination was initiated (Fig. 1). After 1 d, levels of isoenzyme EI mRNA in the scutellum were relatively high, but decreased after 3 d. In contrast, isoenzyme EI mRNA in the aleurone RNA preparations increased significantly between 1 and 3 d (Fig. 1). Although comparisons of signal intensities allow the relative abundance of isoenzyme EI mRNA to be visually assessed in 1- and 3-d aleurone preparations, or in 1- and 3-d scutellar preparations, approximately four times more scutellar RNA was loaded on the

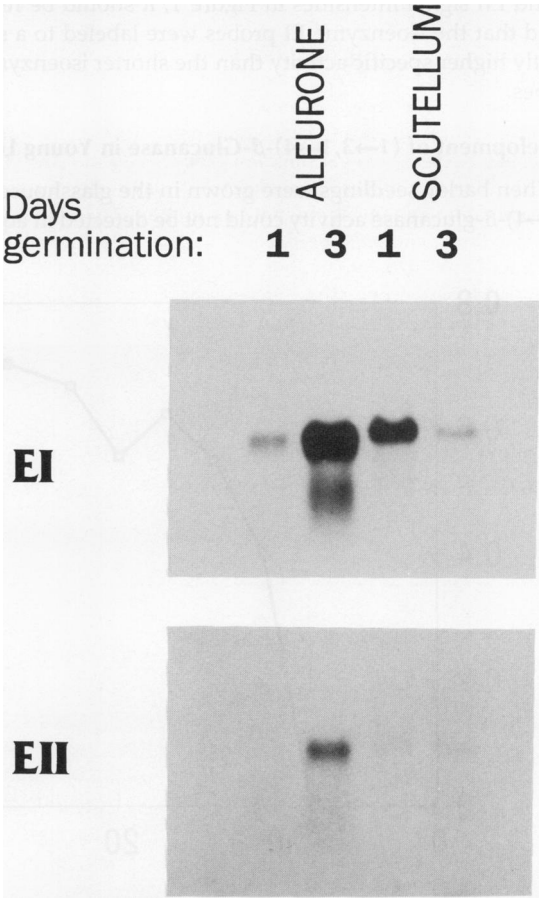

Figure 1. Northern analysis of RNA preparations from scutella and aleurone excised from intact, germinated barley grain 1 and 3 d after the initiation of germination. Approximately 3 μg aleurone and 12 μg scutellar RNA was loaded onto the gels. The nitrocellulose filter probed with the (1→3,1→4)-β-glucanase isoenzyme EI cDNA fragment was exposed for 18 h and the filter probed with the isoenzyme EII cDNA fragment for 48 h.

gel; therefore, care must be exercised in comparing the intensities of signals between the aleurone and scutellar preparations in Figure 1. This problem is compounded by the highly localized expression of (1→3,1→4)-β-glucanase genes in the single layer of epithelial cells in the scutellum (21), because mRNA from these cells will be diluted with mRNAs extracted simultaneously from metabolically active parenchymatous and vascular tissues of the scutellum (7). It is possible, therefore, that the abundance of isoenzyme EI mRNA in individual scutellar epithelial cells may be as high or even exceed corresponding levels in aleurone cells.

Careful examination of the northern blot (Fig. 1) reveals slight differences in the sizes of isoenzyme EI mRNAs from the scutellar and the aleurone extracts. The two transcripts presumably represent the products transcribed from different transcription start points on the gene (2, 24) or to differences caused by the use of multiple polyadenylation sites (5).

When the specific isoenzyme EII probe was used, an increase in binding of the probe to aleurone mRNA was again observed between 1 and 3 d (Fig. 1). Isoenzyme EII mRNA levels in the scutellum were relatively low and did not change significantly between 1 and 3 d. When comparing isoenzyme EI and EII signal intensities in Figure 1, it should be remembered that the isoenzyme EI probes were labeled to a significantly higher specific activity than the shorter isoenzyme EII probes.

Development of (1→3,1→4)-β-Glucanase in Young Leaves

When barley seedlings were grown in the glasshouse, (1→3,1→4)-β-glucanase activity could not be detected in aqueous extracts of young leaves until at least 8 d after the initiation of germination. However, activity increased rapidly between 8 and 15 d and thereafter increased more slowly (Fig. 2). Cellulases are also capable of hydrolyzing the (1→3,1→4)-β-glucan substrate used in this assay and are commonly found in plant tissues. Accordingly, the leaf extracts were assayed for cellulase activity using carboxymethyl cellulose as a substrate (30), but no activity was detected.

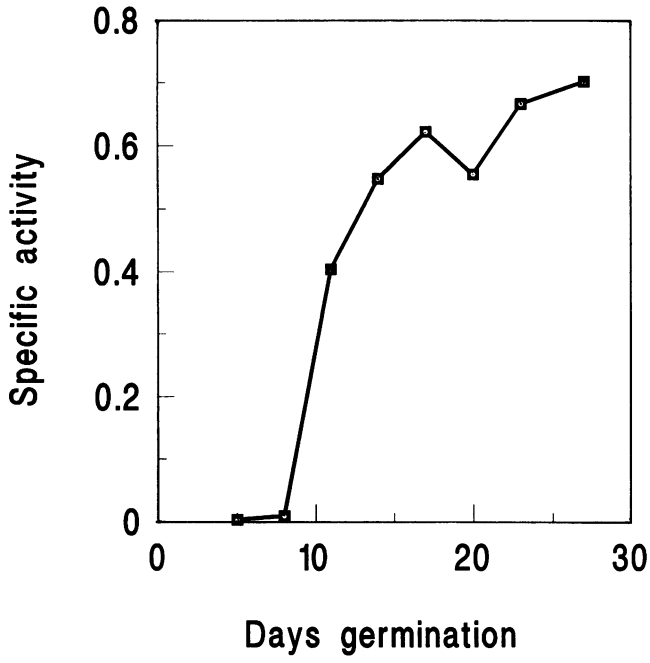

Figure 2. Development of (1→3,1→4)-β-glucanase enzyme activity in extracts of young leaves. The time scale represents the number of days after the initiation of germination. The first leaf emerged approximately 5 to 6 d after the initiation of germination and by 15 d, two to three leaves were visible.

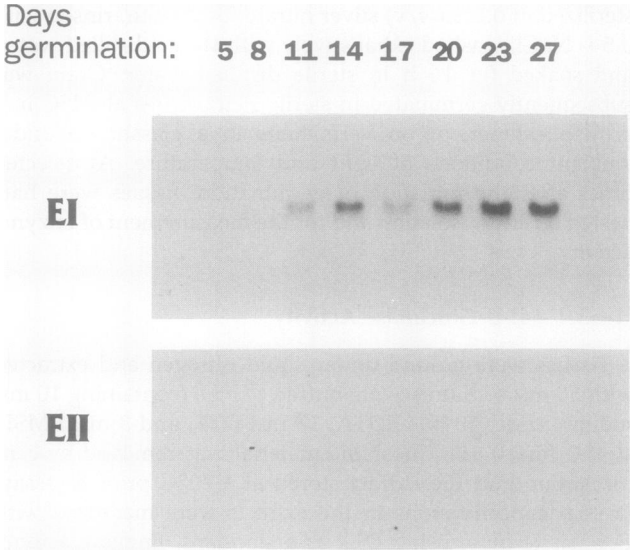

Figure 3. Northern blot showing the development of (1→3,1→4)-β-glucanase mRNA in young leaves at increasing times after the initiation of germination. The nitrocellulose filter probed with the (1→3,1→4)-β-glucanase isoenzyme EI-specific DNA was exposed for 18 h and the filter probed with the isoenzyme EII probe for 3 d. Approximately equal amounts of RNA (10 μg) were loaded in each lane, except for the 17-d preparation, where 6 μg was loaded.

When preparations of total RNA from leaves were subjected to northern analysis, isoenzyme EI mRNA was first detected at approximately 8 d after the initiation of germination. Levels of the isoenzyme EI mRNA then increased quickly up to 23 d and thereafter remained about the same (Fig. 3). No mRNA encoding (1→3,1→4)-β-glucanase isoenzyme EII was found in the leaf RNA preparations during the entire period of the experiment (Fig. 3).

Development of the leaves could be accelerated by growing the seedlings in a growth chamber under constant light; in leaf extracts from these plants, isoenzyme EI mRNA could be detected 6 d after the initiation of germination. It should be emphasized that the "young leaf" tissue from 8- to 15-d seedlings (Figs. 2 and 3) included all leaves in the seedling and, therefore, would include leaves at different developmental stages. To define the distribution of (1→3,1→4)-β-glucanase expression within a single leaf, leaves of 6-d-old, growth chamber-grown plants were carefully separated from the grain and the coleoptile and dissected into three approximately equal segments corresponding to the basal, medial, and apical portions of the leaves. Messenger RNA encoding isoenzyme EI was detected in all the extracts and, although it appeared to be slightly more abundant in the apical regions, this could be attributed to slight differences in the amount of

RNA loaded on this gel (Fig. 4). Further, in similar segments from 10-, 13-, and 16-d-old leaves, the mRNA was also distributed evenly along the leaf (not shown).

Expression in Coleoptiles

No mRNA corresponding to isoenzyme EI or EII could be detected in RNA from intact, 3-d-old coleoptiles (Fig. 5), or in RNA preparations of 4-, 5-, and 6-d-old etiolated coleoptiles (results not shown). Similarly, no expression was detected in intact coleoptiles or coleoptile sections after treatment with IAA (25).

Expression in Young Roots

In northern analyses of total RNA from young root tissue, mRNA for isoenzyme EI was detected at relatively high levels both 3 and 6 d after the initiation of germination (Fig. 5). Isoenzyme EII expression was not apparent in young roots at these developmental stages, even after prolonged autoradiography (Fig. 5). Two possible promoters of the (1→3,1→4)-β-glucanase isoenzyme EI gene, designated the distal and proximal promoter, have been identified (24) and transcription from these promoters was tested by PCR. When the young root RNA preparations were used as a template for the amplification of specific (1→3,1→4)-β-glucanase mRNAs by PCR (24), only one transcript was detected and this corresponded to expression from the distal promoter of the gene (data not shown). Thus, expression from the putative, proximal promoter (24) remains to be demonstrated.

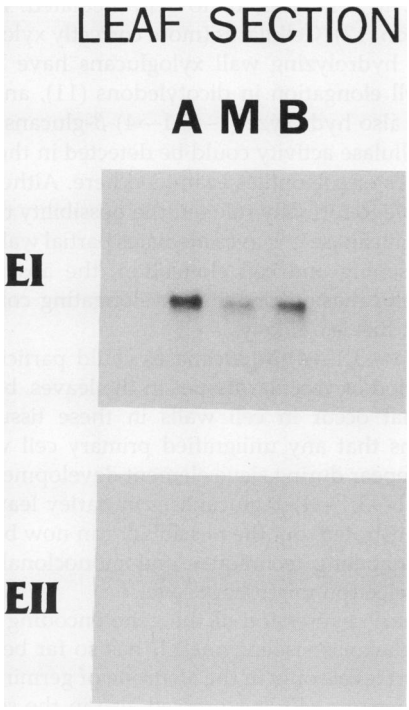

Figure 4. Location of (1→3,1→4)-β-glucanase isoenzyme EI mRNA in apical (A), medial (M), and basal (B) segments of young leaves of barley seedlings using northern analysis. Approximately 20 μg total RNA was loaded in each lane of the gel and autoradiography was for 2 d (isoenzyme EI probe) and 6 d (isoenzyme EII probe).

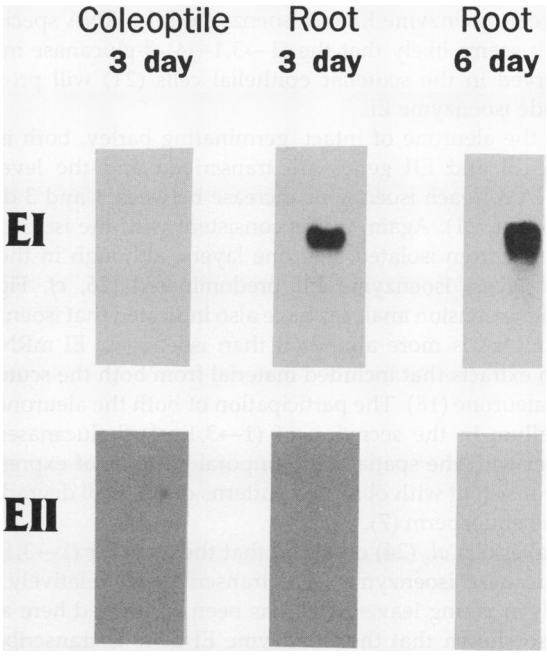

Figure 5. Northern blots of RNA from 3-d coleoptiles and from roots 3 and 6 d after the initiation of germination. In each case, 10 μg RNA was loaded onto the agarose gels and exposure times were 18 h for the filter probed with (1→3,1→4)-β-glucanase isoenzyme EI DNA and 2 d for filters probed with the isoenzyme EII cDNA fragment.

DISCUSSION

Transcription of genes encoding the two (1→3,1→4)-β-glucanase isoenzymes in germinated barley grain has been demonstrated by northern analysis using probes specific for the individual gene products. In intact grain, isoenzyme EI mRNA is detected in the scutellum, with levels apparently decreasing between 1 and 3 d after germination begins (Fig. 1). Relatively low levels of isoenzyme EII gene transcription are detected in the scutellum (Fig. 1). These results with intact, germinated grain are consistent with earlier observations that excised scutella, incubated *in vitro* at 25°C, secrete (1→3,1→4)-β-glucanases into the surrounding medium and that isoenzyme EI predominates (26). The identity of individual isoenzymes in the early work was based on relative electrophoretic mobility and on the binding of polyclonal antibodies on western blots (26). Thus, the secretion of individual (1→3,1→4)-β-glucanase isoenzymes from isolated scutella (26) appears to accurately reflect the transcription patterns of the corresponding genes in the scutellum of intact, germinated grain (Fig. 1). It is clear, however, that the temporal patterns of expression differ. In isolated scutella, isoenzyme EI is secreted for 3 to 4 d (26), whereas in intact grain the level of mRNA for isoenzyme EI in the scutellum is much higher 1 d after the initiation of germination than at 3 d (Fig. 1). Similarly, examination of (1→3,1→4)-β-glucanase mRNA by hybridization histochemical methods showed that transcriptional activity of the genes in the scutellum decreased dramatically after 1 d (21). Although the cDNA probe used in the *in situ* hybridization experiments did not discriminate

between isoenzyme EI and isoenzyme EII mRNA species (8, 21), it seems likely that the (1→3,1→4)-β-glucanase mRNA observed in the scutellar epithelial cells (21) will prove to encode isoenzyme EI.

In the aleurone of intact, germinating barley, both isoenzyme EI and EII genes are transcribed and the levels of mRNA for each isoenzyme increase between 1 and 3 d (Fig. 1 and ref. 21). Again, this is consistent with the isoenzymes secreted from isolated aleurone layers, although in the isolated layers isoenzyme EII predominated (26, *cf.* Fig. 1). Primer extension analyses have also indicated that isoenzyme EII mRNA is more abundant than isoenzyme EI mRNA in grain extracts that included material from both the scutellum and aleurone (18). The participation of both the aleurone and scutellum in the secretion of (1→3,1→4)-β-glucanases, together with the spatial and temporal patterns of expression, are consistent with observed patterns of cell wall degradation in the endosperm (7).

Slakeski *et al.* (24) observed that the gene for (1→3,1→4)-β-glucanase isoenzyme EI is transcribed at relatively high levels in young leaves. This has been confirmed here and it is also shown that the isoenzyme EI gene is transcribed in young roots (Figs. 3 and 5). In these tissues, only the gene encoding (1→3,1→4)-β-glucanase isoenzyme EI is expressed; no isoenzyme EII mRNA is detected (Figs. 3–5). The development of isoenzyme EI mRNA in young leaves parallels the development of (1→3,1→4)-β-glucanase enzyme activity (Figs. 2 and 3) and expression levels appear to be essentially similar along the length of the young leaf (Fig. 4). The functional role of the (1→3,1→4)-β-glucanases in germinated barley grain is almost certainly to degrade the (1→3,1→4)-β-glucans of the cell walls during endosperm mobilization. However, the function of the enzyme in developing leaves and roots (Figs. 2–4) is not yet clear, but is likely to be related to cell wall metabolism. The walls of young leaves contain approximately 16% (w/w) (1→3,1→4)-β-glucan (N. Sakurai, personal communication). The high levels of (1→3,1→4)-β-glucanase mRNA in developing barley leaves have prompted speculation that the enzyme participates in the formation of intercellular airspaces necessary for diffusion of carbon dioxide, oxygen, and water vapor in parts of the leaf that initially have no direct access to the atmosphere (24); airspace formation is prerequisite for the development of an efficient photosynthetic system in young leaves. In preliminary experiments, monoclonal antibodies specific for (1→3,1→4)-β-glucanase isoenzyme EI (13) have been used in gold-labeling studies to locate the enzyme in sections of developing barley leaves (B. Wells, K. Roberts, G.B. Fincher, unpublished). Although airspace formation was clearly evident, no (1→3,1→4)-β-glucanase antigen could be detected in the walls of separating mesophyll cells, and we conclude that the enzyme is not involved in the formation of these airspaces.

Another possible role for the (1→3,1→4)-β-glucanase in developing leaves could be in the "loosening" of cell wall polysaccharides during cell elongation. The process of cell elongation has been examined in great detail in elongating coleoptiles of barley and maize (14, 22). The (1→3,1→4)-β-glucan content of cell walls from 4-d-old barley coleoptiles is approximately 19% by weight (22) but the level decreases progressively during coleoptile growth. Similarly, the (1→3,1→4)-β-glucan content of young barley leaves and oat coleoptiles decreases during development (3). In maize coleoptile walls, levels of the polysaccharide initially increase (4) but then decrease from 14 to 3% (w/w) between 5 and 10 d (20). The removal of (1→3,1→4)-β-glucans from maize coleoptile walls has been attributed to an exo-β-glucanase that is capable of degrading the polysaccharide to glucose (17), although recent work in which cell elongation was inhibited by polyclonal antibodies raises the possibility that endohydrolases may also be involved (15). Presumably, glucose released during (1→3,1→4)-β-glucan degradation in these tissues, which would require the concerted action of (1→3,1→4)-β-glucan endohydrolases, exohydrolases, and possibly β-glucosidases (7, 8), provides an energy source to support seedling growth.

In barley, no (1→3,1→4)-β-glucanase transcription was detected in coleoptiles (24). In the present study, we have reexamined several RNA preparations from intact coleoptiles and coleoptile segments (Fig. 5 and other data not shown), together with RNA from coleoptiles in which cell elongation was significantly exhanced by auxin treatment (25), but can find no evidence for the transcription of (1→3,1→4)-β-glucanase genes, even at low levels, for up to 8 d after the initiation of germination. This finding, coupled with the detection in barley seedling extracts of exo-β-glucanases (J. Wang, P.B. Høj, G.B. Fincher, unpublished data), suggests that the (1→3,1→4)-β-glucan endohydrolases may not mediate in auxin-mediated cell elongation in barley coleoptiles and that the removal of (1→3,1→4)-β-glucan from the walls of elongating cells may be attributable to the β-glucan exohydrolases, to cellulases, or to acid-mediated, nonenzymic solubilization (12). Cellulases (more correctly xyloglucanases) capable of hydrolyzing wall xyloglucans have been implicated in cell elongation in dicotyledons (11), and these enzymes will also hydrolyze (1→3,1→4)-β-glucans (10). However, no cellulase activity could be detected in the extracts of young leaves or coleoptiles examined here. Although we are as yet unable to formally rule out the possibility that the (1→3,1→4)-β-glucanase in leaves mediates partial wall hydrolysis during loosening and cell elongation, the absence of (1→3,1→4)-β-glucanase expression in elongating coleoptiles indicates that this is unlikely.

Finally, (1→3,1→4)-β-glucanases could participate in the differentiation of vascular tissues in the leaves, based on the changes that occur in cell walls in these tissues and on observations that any unlignified primary cell walls in the xylem disappear during sieve element development (9). Such a role for (1→3,1→4)-β-glucanases in barley leaves remains to be demonstrated, but the possibility can now be addressed using gold-labeling techniques and monoclonal antibodies (13) at the electron microscope level.

In summary, expression of the gene encoding barley (1→3,1→4)-β-glucanase isoenzyme EII has so far been detected at significant levels only in the aleurone of germinating grain, whereas isoenzyme EI is expressed also in the scutellum, in leaves, and in roots. This raises the question as to why this pattern of tissue-specific expression has evolved. Both isoenzymes exhibit essentially the same substrate specificity (29), so there appears to be no reason for differential expression based on enzyme action patterns. It is worth noting that

Table I. *(1-3,1-4)-β-Glucanase Expression in the Germinated Grain and Developing Vegetative Tissues*

Tissue	mRNA Transcript Levels	
	EI	EII
Germinated grain		
Aleurone 1 day	++	trace
Aleurone 3 day	++++	++++
Scutellum 1 day	++	++
Scutellum 3 day	+	++
Vegetative tissue		
6-d leaf	trace	−
6-d root	++++	−
3-d coleoptile	−	−
3-d root	++++	−

the aleurone is triploid, whereas the other tissues examined here are diploid, but again there is no indication that this difference is related to the expression patterns. It has also been observed that the promoter of the gene encoding isoenzyme EI contains a 150-nucleotide pair insertion that is absent in the isoenzyme EII promoter and that this segment could be responsible for the differential expression patterns (28). This can now be investigated.

We have attempted to summarize the mRNA levels in a semi-quantitative fashion in Table I. Given the role of GA_3 in the induction of (1→3,1→4)-β-glucanase and other proteins in tissue from germinating grain (7, 26), together with the central importance of auxin in plant vascular differentiation (1) and in cell extension (16), the tissue-specific regulation of individual barley (1→3,1→4)-β-glucanase genes by phytohormones has now been examined and is reported elsewhere (25).

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