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Induction of (1→3,1→4)-β-D-glucan hydrolases in leaves of dark-incubated barley seedlings

Abstract When seedlings of barley (*Hordeum vulgare* L.) were transferred from a natural light/dark cycle into darkness, (1→3,1→4)-β-D-glucan endohydrolase (EC 3.2.1.73) activity in leaf extracts increased 3- to 4-fold after 2 days. Activity decreased to normal levels within a day if the light/dark cycle was restored. Although there are two (1→3,1→4)-β-D-glucan endohydrolase isoenzymes in barley, the increased enzyme activity in dark-grown seedlings was attributable entirely to increases in isoenzyme EI. Northern hybridization analyses confirmed that mRNA transcripts encoding (1→3,1→4)-β-D-glucan endohydrolase isoenzyme EI accumulated in the leaves of dark-incubated seedlings; no isoenzyme EII mRNA was detected. Activity of β-D-glucan glucohydrolases also increased 10-fold after 2 days of dark treatment. The latter, broad-specificity enzymes release glucose from (1→3,1→4)-β-D-glucans and from β-D-oligoglucosides released by (1→3,1→4)-β-D-glucan endohydrolases. Consistent with the activity patterns of these enzymes, the (1→3,1→4)-β-D-glucan content of leaf cell walls decreased by about 30% when barley seedlings were transferred into darkness. Soluble sugars in the leaves decreased by about 60% during the same period. Because no measurable leaf elongation was detected during the various light/dark treatments, the enzymes were unlikely to be participating in wall loosening and cell elongation. Instead, the results suggest that cell wall (1→3,1→4)-β-D-glucans can be re-mobilized in the non-elongating, dark-incubated leaves and the glucose so generated could serve as an energy source under conditions of sugar depletion.

Keywords Cell wall · Dark induction · (1→3,1→4)-β-D-Glucan · (1→3, 1→4)-β-D-Glucan endohydrolase · β-D-Glucan glucohydrolase · *Hordeum* (cell wall)

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Introduction

The (1→3,1→4)-β-D-glucans are major polysaccharide constituents of cell walls in the Poaceae (Carpita and Gibeaut 1993). The extended yet irregular structures of (1→3,1→4)-β-D-glucans are well-adapted to their role in the primary wall, where they are components of a hydrophilic gel-like matrix that allows the apoplastic diffusion of water, phytohormones and low-molecular-weight metabolites. Cellulosic microfibrils that are embedded in the matrix phase strengthen the cell wall as a whole. Cell walls in higher plants are dynamic structures that are modified during normal cell expansion, during secondary thickening, during the formation of cells with specialized functions, or in response to changing environmental conditions. The changes to cell walls that occur during plant growth and development involve the modification of both matrix-phase and fibrillar components of the wall. An array of hydrolytic and oxidative enzymes, together with proteins that have no apparent enzymic activity, effect changes not only in the overall composition of the wall, but also in the size and fine structure of individual wall components, and in the physical properties of the wall (Cosgrove 1999).

The (1→3,1→4)-β-D-glucans are similarly subjected to modification during normal growth and development (Carpita and Gibeaut 1993). In young leaves, (1→3,1→4)-β-D-glucans appear to be partly degraded. Thus, (1→3,1→4)-β-D-glucans account for about 16% of the wall in young barley leaves, but progressively

decrease in abundance as the leaves mature (Buchala and Wilkie 1974). Similarly, (1→3,1→4)-β-D-glucans in maize seedlings are turned over during growth (Inouhe et al. 1997) and auxin-induced elongation of maize and barley coleoptiles is associated with a decrease in (1→3,1→4)-β-D-glucan content in the walls (Kotake et al. 2000). In rice coleoptiles, (1→3,1→4)-β-D-glucan turnover is suppressed during light-induced inhibition of elongation (Chen et al. 1999). Here, we have focused on the influence of light on (1→3,1→4)-β-D-glucan turnover, but have used fully expanded secondary leaves of young barley seedlings, rather than coleoptiles, because interpretation of the results will not be complicated by the participation of enzymes in the cell wall loosening that occurs during rapid cell elongation in coleoptiles.

Several enzymes that are involved in (1→3,1→4)-β-D-glucan hydrolysis have been characterized in detail (Hrmova and Fincher 2001). Thus, two (1→3,1→4)-β-D-glucan endohydrolases (EC 3.2.1.73) purified from extracts of germinated barley grain hydrolyze (1→4)-β-glucosyl linkages which are adjacent, on the reducing terminal side, to (1→3)-β-glucosyl linkages; the two enzymes have been designated isoenzymes EI and EII (Woodward and Fincher 1982). The (1→3,1→4)-β-D-glucan endohydrolases release (1→3,1→4)-β-D-oligoglucosides containing three to four glucosyl residues as the major products. These enzymes may be contrasted to another group of (1→3,1→4)-β-D-glucan endohydrolases from elongating maize coleoptiles, which release much longer oligosaccharide products from the polysaccharide (Thomas et al. 1998).

β-D-Glucan exohydrolases, more correctly referred to as β-D-glucan glucohydrolases, are also found in germinated cereal grains and elongating vegetative tissues (Labrador and Nevins 1989; Hrmova et al. 1996; Kim et al. 2000; Kotake et al. 2000; Harvey et al. 2001). Barley β-D-glucan glucohydrolases catalyze the hydrolytic removal of single, non-reducing terminal glucose units not only from (1→3,1→4)-β-D-glucans, but also from a range of other β-D-glucans and β-D-oligoglucosides. The broad specificity of these enzymes can be readily explained in terms of their three-dimensional structure (Varghese et al. 1999), but precludes their unequivocal classification in any of the existing EC groups of enzymes (Hrmova et al. 1996).

Thus, enzymic modifications of cell wall (1→3,1→4)-β-D-glucans are physiologically important during normal growth and development of the graminaceous monocotyledons. In the work described here, we have compared levels of enzymes capable of modifying (1→3,1→4)-β-D-glucans in non-expanding leaves of barley seedlings subjected to extended dark periods, or to a normal light/dark cycle. In the dark-incubated leaves, the concentration of soluble sugars quickly decreases. At the same time, dark induction of specific enzymes is observed, together with decreases in the abundance of (1→3,1→4)-β-D-glucans in cell walls.

Materials and methods

Plant material

Barley (*Hordeum vulgare* L. cv. Clipper) grains, provided by Professor Andrew Barr, University of Adelaide, were germinated at 20 °C in the dark for 2 days on wet filter paper and transferred to composted soil. Seedlings were grown in the greenhouse under natural light at 22 ± 1 °C during the day (approx. 12 h) and at 17 ± 1 °C during the night for 2–3 weeks. When the secondary leaf was fully expanded, plants were either kept under the natural light/dark cycle or placed in complete darkness. Experiments were always started in the middle of the light period and were carried out several times. No measurable leaf elongation could be detected during the different treatments. Secondary leaves were harvested at different times after the start of the dark-incubation, immediately frozen in liquid nitrogen and stored at –70 °C.

Enzyme extraction

Leaves were homogenized in 4 ml (g FW)$^{-1}$ 50 mM sodium acetate buffer (pH 5.0) containing 10 mM EDTA, 10 mM sodium azide, 3 mM β-mercaptoethanol and 3 mM phenylmethylsulfonyl fluoride, using a Polytron mixer (PT 2000; Kinematica, Littau, Switzerland) for 2×30 s at full speed. After 30 min incubation on ice with occasional mixing, insoluble material was removed by centrifugation and the extract was stored at –70 °C.

To examine the possibility that enzymes remained associated with cell walls after extraction, the pelleted insoluble material was washed exhaustively with homogenization buffer and water. After each wash, walls were allowed to settle under gravity because centrifugation resulted in their contamination with membranous material. The final wall preparations were concentrated by centrifugation, and resuspended in homogenization buffer to the initial extraction volume prior to analysis by immunoblotting.

Enzyme assays

(1→3,1→4)-β-D-Glucan endohydrolase activity was measured viscometrically, using water-soluble barley (1→3,1→4)-β-D-glucan (medium viscosity; Megazyme, Bray, Ireland) as a substrate. One unit of activity is defined as the amount of enzyme causing, at 40 °C, an increase of 1.0 in the reciprocal specific viscosity ($\Delta 1/\eta_{sp}$) per min of a 3 mg ml^{-1} (1→3,1→4)-β-D-glucan solution in 50 mM sodium acetate buffer (pH 5.0) containing 5 mM sodium azide and 400 μg ml^{-1} BSA (Woodward and Fincher 1982). For the estimation of cellulase activity, carboxymethyl cellulose with a degree of substitution of 0.4 (medium viscosity; Megazyme) was used as a substrate in viscometric assays.

β-D-Glucan glucohydrolase activity was determined with the GOD-Perid method (formerly obtained from Boehringer Mannheim, Germany) by measuring the glucose released, at 37 °C, from a 3 mg ml^{-1} (1→3,1→4)-β-D-glucan solution in 50 mM sodium acetate buffer (pH 5.25) containing 2.5 mM sodium azide and 160 μg ml^{-1} BSA. One unit of activity is defined as the amount of enzyme required to release 1 μmol of glucose per min. Analyses were carried out in duplicate and mean values were used for the calculation of the activities.

SDS-PAGE and immunoblotting

Gel electrophoresis was carried out according to Laemmli (1970) using 1.5-mm-thick slab gels (12%). Cell wall preparations or soluble extracts of leaves were diluted with sample buffer [196 mM Tris-HC1 buffer (pH 6.8) containing 6.3% (w/v) SDS, 16% (v/v) β-mercaptoethanol, 32% (v/v) glycerol and 0.02% (w/v) bromophenol blue] in a ratio 2:1 (v/v). Soluble extracts were heated at 95 °C for 5 min, while cell wall preparations were pre-heated at

65 °C for 10 min, followed by 95 °C for 5 min. Each lane was loaded with an amount of extract equivalent to 2.5 mg leaf FW. After electrophoresis, gels were blotted onto nitrocellulose membranes (0.45 μm; BioRad, Glattbrugg, Switzerland) for the immunodetection of the (1→3,1→4)-β-D-glucan endohydrolase isoforms. Immunoblotting was performed as described by Mitsuhashi and Feller (1992) except that the membranes were exposed overnight at 4 °C to the primary monoclonal antibodies raised in mice against barley (1→3,1→4)-β-D-glucan endohydrolase isoenzymes EI and EII (Høj et al. 1990). The membranes were subsequently incubated for 2 h with goat anti-mouse IgG (M-8642; Sigma, Buchs, Switzerland) diluted 1:1,000 (v/v) with 1% (w/v) ovalbumin in 50 mM Tris-HCl buffer (pH 7.5) containing 0.2 M NaCl, and later for 2 h with mouse peroxidase-anti-peroxidase complex (P-3039; Sigma) diluted 1:400 (v/v) in the same buffer. The primary monoclonal antibodies were diluted 1:500 (v/v). Two replicate samples were analyzed for each experiment.

RNA extraction and Northern analysis

Barley leaves were ground to a fine powder under liquid nitrogen. Total RNA was extracted using the hot phenol/LiCl procedure (Verwoerd et al. 1989). The RNA samples (10 μg) were separated on 1.2% (w/v) agarose gels containing 1.2 M formaldehyde and transferred to nylon membranes (Hybond-N^+; Amersham Pharmacia) for probing. To test for approximately equal loadings, control gels were stained with ethidium bromide for comparison of the relative intensities of ribosomal RNA bands. Specific DNA fragments corresponding to the 3′-untranslated regions of cDNAs encoding the barley (1→3,1→4)-β-D-glucan endohydrolase isoenzymes EI and EII were amplified by the polymerase chain reaction according to Slakeski et al. (1990) and labeled with γ-[^{32}P]dCTP using random sequence hexanucleotides (Amersham Pharmacia). Filters were prehybridized in 1.5× high-salt buffer [1× HSB: 30 mM Pipes buffer (pH 6.6) containing 0.9 M NaCl and 7.5 mM Na_2-EDTA] containing 30× Denhardt's III solution [0.6% (w/v) gelatine, 0.6% (w/v) Ficoll 400 and 1.5% (w/v) tetrasodium diphosphate], 3% (w/v) SDS, 0.25 mg ml^{-1} denatured salmon sperm DNA and 7.5% (w/v) dextran sulphate for 4 h at 65 °C. Hybridization was performed at 65 °C for 16 h in the same buffer. Filters were washed to a final concentration of 0.1× standard saline citrate (1× SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0) containing 0.1% SDS at 65 °C prior to autoradiography on Hyperfilm-MP (Amersham Pharmacia) at –80 °C for 48 h.

Chlorophyll, soluble protein and soluble sugar determinations

Following homogenization of the leaves and filtration through Miracloth, the filtrate (20 μl) was mixed with 1 ml of 80% (v/v) acetone and the chlorophyll was quantified according to Strain et al. (1971). Soluble protein was determined in the filtrate, after centrifugation, by the method of Bradford (1976) using BSA as a standard. Soluble sugars were measured as described by Stieger and Feller (1994), using glucose as a standard.

Measurement of (1→3,1→4)-β-D-glucan content

The tissue that served for the extraction of the enzymes (approx. 500 mg per sample) was thawed to room temperature and exhaustively extracted in a screw-top centrifuge tube with boiling 80% (v/v) methanol (5×5 ml), until no reducing sugar was detected in the soluble material. The residue was suspended in 1 ml water using a high-speed homogenizer (Polytron PT-100; Kinematica) and autoclaved for 20 min at 121 °C. The cooled suspension was saturated with N_2 and an equal volume of 8% (w/v) KOH, also saturated with N_2, was added. The suspension was shaken overnight and centrifuged for 10 min at 10,000 *g*. The supernatant was recovered and neutralized with glacial acetic acid. The residue was treated with 5 ml 24% (w/v) KOH (saturated with N_2) for a further 18 h with shaking. After centrifugation for 10 min at 10,000 *g*, the supernatant was recovered, added to the material solubilized in 4% (w/v) KOH and the pooled solutions neutralized with acetic acid to pH 5.8. The final volume was made up to 20 ml with distilled water. This solution gave a negative iodine/iodide test for starch. Aliquots (2×1 ml) were incubated for 12 h at 25 °C with 1 mg of a mixture of (1→3,1→4)-β-D-glucanase purified from a crude α-amylase preparation (Novo 1000S, batch P1 1103) from *Bacillus subtilis* (B.A. Stone, School of Biochemistry, La Trobe University, Bundoora, Australia; personal communication), and a β-glucosidase from almond emulsin (Fluka, Buchs, Switzerland). The glucose produced was determined in samples (2×0.2 μl) by the GOD-Perid method (formerly obtained from Boehringer Mannheim, Germany).

Results

Enzyme activities

Enzyme activities were measured in extracts of the secondary leaves of barley seedlings exposed to three different regimes, namely a normal 12 h/12 h light/dark cycle, complete darkness, or 3 days in the dark before their return to the light/dark cycle. Both the (1→3,1→4)-β-D-glucan endohydrolases and β-D-glucan glucohydrolases from barley are secreted enzymes that are not bound to cell walls under the extraction conditions used here (Slakeski and Fincher 1992a, b; Harvey et al. 2001). Thus, the majority of enzyme activity is likely to be located in the intercellular space. Activities were expressed on a fresh-weight basis, because no detectable changes occurred in the fresh weight of the leaves or in their size during the experiments. In contrast, protein and soluble sugar levels varied appreciably, as described later, and the expression of activity on a dry-weight or protein basis could therefore be misleading.

The (1→3,1→4)-β-D-glucan endohydrolase and β-D-glucan glucohydrolase activities followed generally similar temporal patterns during these treatments (Fig. 1). Enzyme activities increased markedly after the seedlings were transferred into darkness. Activities of both enzymes remained high for up to 9 days in the dark, but declined to control levels when the seedlings were returned to the light/dark growth conditions (Fig. 1a, b). However, several important differences were observed between (1→3,1→4)-β-D-glucan endohydrolase and β-D-glucan glucohydrolase activities during dark incubation. The increase in (1→3,1→4)-β-D-glucan endohydrolase activity was detected after 1 day in darkness (Fig. 1a), but the increase in β-D-glucan glucohydrolase activity was not apparent until after 2 days in darkness (Fig. 1b). In addition, (1→3,1→4)-β-D-glucan endohydrolase activity returned to control levels within a day of re-exposure to light/dark cycles (Fig. 1a), while β-D-glucan glucohydrolase activity declined more slowly after re-exposure to light/dark cycles (Fig. 1b). In the experiments summarized in Fig. 1a, the (1→3,1→4)-β-D-glucan endohydrolase activity was somewhat lower in the dark/light-incubated plants than in plants held in the

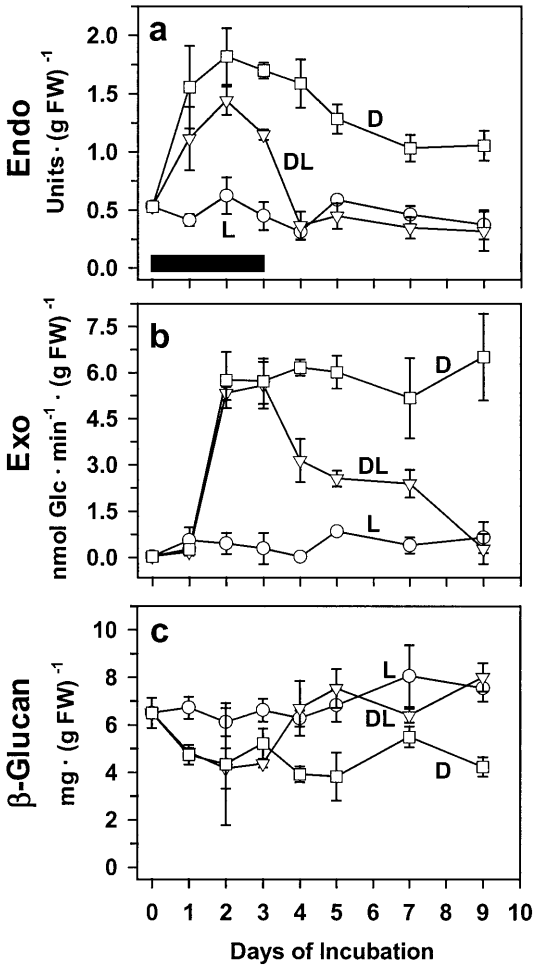

Fig. 1a–c Time course of accumulation of (1→3,1→4)-β-D-glucan endohydrolase activity (**a**), β-D-glucan glucohydrolase activity (**b**), and of (1→3,1→4)-β-D-glucan content (**c**) in secondary leaves of barley (*Hordeum vulgare*) plants incubated under a natural light/dark cycle (*circles*; *L*), for the first 3 days in complete darkness (indicated by the *black bar*) followed by the natural light/dark cycle (*triangles*; *DL*), or in complete darkness throughout (*squares*; *D*). Data are presented as means ± SE of four independent experiments, except for **c** where values are the means of two replicate experiments. Experiments and sampling of the leaves were always started in the middle of the light period

dark throughout the experiment. The final difference between the two classes of enzyme was that (1→3,1→4)-β-D-glucan endohydrolase activity increased 3- to 4-fold within 2–3 days of dark incubation; in the same period the activity of β-D-glucan glucohydrolases increased about 10-fold (Fig. 1).

Because cellulases can also hydrolyze (1→3,1→4)-β-D-glucans (Høj and Fincher 1995) and because the activities of cellulases, or xyloglucanases, increase in etiolated pea hypocotyls (Maclachlan 1988), it was necessary to ensure that these enzymes were not interfering with the assay. Leaf extracts were therefore assayed viscometrically for cellulase activity, using carboxymethyl cellulose as a substrate, but no activity was detected (data not shown).

(1→3,1→4)-β-D-Glucan content

Although the measurements of (1→3,1→4)-β-D-glucan contents of the secondary leaves of barley seedlings showed some variability between replicates, it is clear that transfer of the seedlings into darkness resulted in a decrease of 30% or more in (1→3,1→4)-β-D-glucan content (Fig. 1c). Levels of the polysaccharide in leaves of seedlings grown in the light/dark cycle showed no appreciable change over 9 days. In seedlings grown in the dark for 3 days before return to the light/dark conditions there was an initial decrease in (1→3,1→4)-β-D-glucan content in the dark followed by an increase in (1→3,1→4)-β-D-glucan in the presence of light (Fig. 1c). It was observed that after 5–6 days in complete darkness, the leaves of the barley seedlings became somewhat brittle, especially in the basal region of the leaf blade. This apparent change in leaf texture might be indicative of alterations in cell wall composition and structure.

Chlorophyll, soluble protein and soluble sugars

Exposing plants to a prolonged dark period represents a convenient means for artificially promoting premature leaf senescence. Common symptoms of dark-induced senescence include a reduction in chlorophyll and soluble protein contents in the senescing leaves (Smart 1994; Gan and Amasino 1997). In addition, complete darkness should lead to a rapid depletion in soluble sugars in the leaves. When barley seedlings were incubated in darkness for up to 9 days, chlorophyll levels declined slightly and appeared relatively slow to recover when seedlings were transferred back to the normal light/dark regime (Fig. 2a). Soluble protein levels in leaves decreased in seedlings incubated in continuous darkness and, to a lesser extent, in seedlings grown in the normal light/dark cycle (Fig. 2b). In contrast to chlorophyll, soluble protein content rapidly returned to control levels upon re-exposure of the seedlings to light (Fig. 2b). Soluble sugars decreased quickly during the dark treatment (about 60% decline after 1 day) but returned to initial levels within a day of re-exposure to light (Fig. 2c).

Western blot analysis

Leaf extracts were subjected to SDS-PAGE and separated polypeptides were transferred to nitrocellulose filters. The filters were probed with monoclonal antibodies that are specific for (1→3,1→4)-β-D-glucan endohydrolase isoenzymes EI or EII (Høj et al. 1990). The specificities of the antibodies were confirmed using purified enzymes, and it was clear that only isoenzyme EI was synthesized in the leaves of the barley seedlings (Fig. 3). Visual comparisons of band intensities showed that levels of the (1→3,1→4)-β-D-glucan endohydrolase

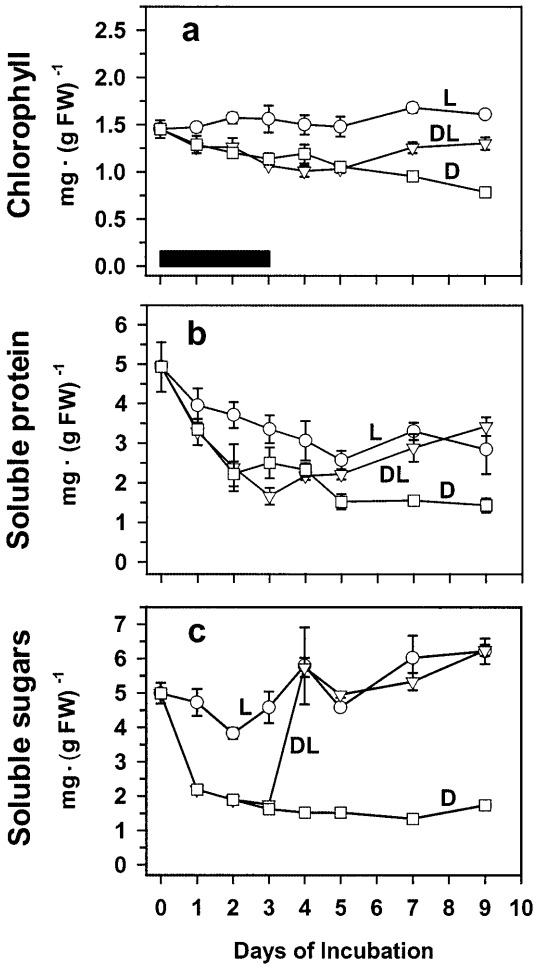

Fig. 2a–c Time course for changes in chlorophyll (**a**), soluble protein (**b**) and soluble sugars (**c**) in secondary leaves of barley plants incubated under a natural light/dark cycle (*circles*; *L*), for the first 3 days in complete darkness (indicated by the *black bar*) followed by the natural light/dark cycle (*triangles*; *DL*), or in complete darkness throughout (*squares*; *D*). Data are presented as means ± SE of four independent experiments

isoenzyme EI rapidly increased in the dark, but decreased again to control levels within a day when the seedlings were returned to light/dark growth conditions (Fig. 3a, b).

To ensure that large proportions of the enzymes did not remain bound to cell walls, walls were prepared after extraction of the tissues and wall-associated proteins were separately subjected to Western blot analysis. Some (1→3,1→4)-β-D-glucan endohydrolase isoenzyme EI was detected in the walls, but this was usually less than about 10% of extracted enzyme (Fig. 3a, b). The low levels of wall-associated enzyme appeared to parallel the much higher levels of soluble enzyme in all tissue samples and were presumably subject to the same light/dark regulatory mechanisms (Fig. 3a, b). This result is similar to that of Harvey et al. (2001), who reported that the great majority of β-D-glucan exohydrolase could be extracted from barley coleoptiles with dilute aqueous buffers; high salt buffers are not needed.

Thus, the results obtained in the Western blot analyses (Fig. 3) were entirely consistent with direct measurements of (1→3,1→4)-β-D-glucan endohydrolase activity (Fig. 1a) and further showed that enzyme activity could be attributed exclusively to isoenzyme EI.

Northern hybridization analysis

Although the genes encoding the two barley (1→3,1→4)-β-D-glucan endohydrolase isoenzymes share about 92% sequence identity at the nucleotide level (Slakeski et al. 1990), gene-specific probes can be prepared from the divergent 3′-untranslated regions of the corresponding cDNAs (Slakeski and Fincher 1992a). These probes confirmed that only the (1→3,1→4)-β-D-glucan endohydrolase isoenzyme EI gene was transcribed in the young barley leaves, and that dark treatment resulted in greatly elevated levels of mRNA transcripts of the isoenzyme EI gene (Fig. 4). The accumulation of (1→3,1→4)-β-D-glucan endohydrolase isoenzyme EI transcripts was clearly apparent 6 h after the transfer of the seedlings to darkness, the transcripts reached maximal levels by 12 h, but appeared to decrease after prolonged incubation in the dark (Fig. 4). The latter effect may be related to the senescence status of the leaves. Upon re-exposure of the seedlings to light, (1→3,1→4)-β-D-glucan endohydrolase isoenzyme EI transcripts disappeared within 1 day (Fig. 4).

Discussion

When barley seedlings are transferred from light to darkness, steady-state levels of mRNA encoding (1→3,1→4)-β-D-glucan endohydrolase isoenzyme EI increase markedly. The complete absence of isoenzyme EII mRNA or enzyme is consistent with earlier work from Slakeski and Fincher (1992a), who reported that the barley (1→3,1→4)-β-D-glucan endohydrolase isoenzyme EII gene is expressed only in the aleurone layer of germinated grain. While the elevated levels of isoenzyme EI mRNA in dark-incubated seedlings could reflect an increase in transcription rates from the corresponding gene (Fig. 4), they could equally represent increased mRNA stability (Chan and Yu 1998; Shiina et al. 1998). The higher levels of isoenzyme EI mRNA are manifested both in increased amounts of the (1→3,1→4)-β-D-glucan endohydrolase isoenzyme EI protein (Fig. 3), and in increased enzyme activity (Fig. 1a). Taken together, the results suggest that these responses are related to changes at the steady-state levels of mRNA, and that inhibitors or activators of enzyme activity are unlikely to play a role in the process. This may be contrasted with auxin-stimulation of cell wall (1→3,1→4)-β-glucanase activities in maize coleoptiles, where a non-enzymic protein activator has been implicated in the process (Inouhe and Nevins 1997).

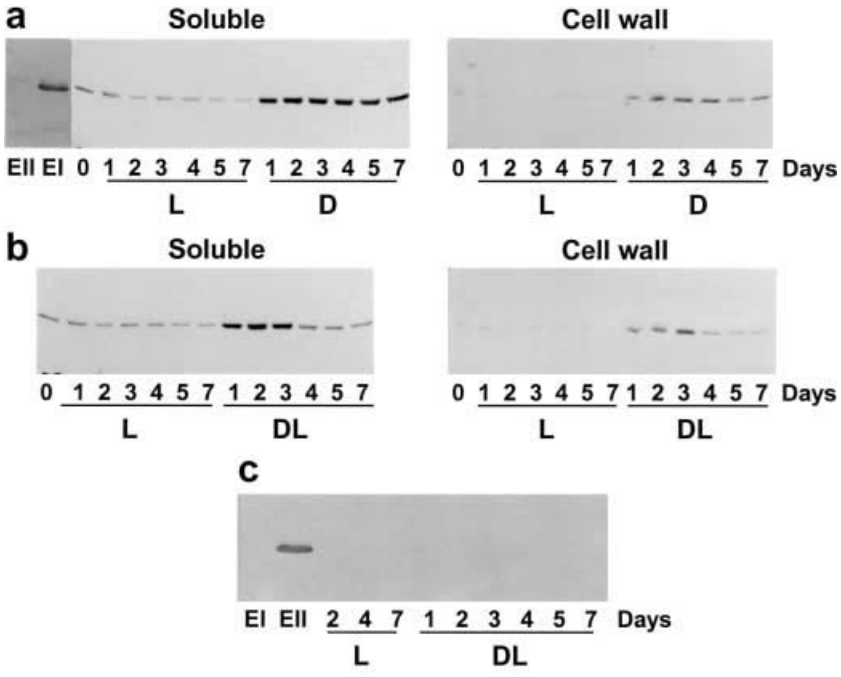

Fig. 3a–c Dark-induced accumulation of (1→3,1→4)-β-D-glucanase isoform EI. Barley plants were incubated under a natural light/dark cycle (*L*), or kept in complete darkness during the first 3 days (*DL*), or placed under continuous darkness throughout (*D*). Changes in the levels of (1→3,1→4)-β-D-glucanase isoenzymes EI (**a, b**) and EII (**c**) in extracts from secondary leaves (*Soluble*) were monitored by immunoblotting with specific monoclonal antibodies. Following extraction, cell walls were isolated from the insoluble material and levels of (1→3,1→4)-β-D-glucanase isoenzyme EI associated with the walls were visualized by immunoblotting (*Cell wall*). Purified preparations of the enzymes (EI and EII) were kindly provided by Dr. M. Hrmova and were used to check the specificity of the antibodies

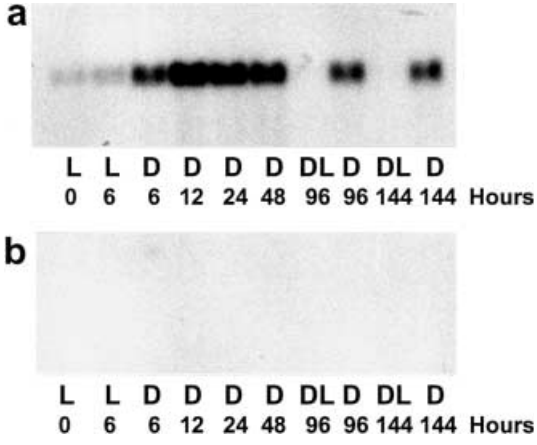

Fig. 4a, b Changes in the steady-state transcript levels for (1→3,1→4)-β-D-glucanase isoenzymes EI (**a**) and EII (**b**) in secondary leaves of barley seedlings grown under a natural light/dark cycle (*L*), in complete darkness throughout (*D*) or switched from darkness to natural light cycle after 3 days (*DL*). The experiment was started in the middle of the light period. Filters were probed with DNA fragments specific for the genes of isoenzymes EI and EII. Approximately equal amounts of RNA (10 μg) were loaded in each lane

It is not clear at this stage if the accumulation of isoenzyme EI mRNA results directly from dark-induction of gene expression, or from the release of light-repression of the same gene. Examination of the promoter of the barley (1→3,1→4)-β-D-glucan endohydrolase isoenzyme EI gene (Slakeski et al. 1990) revealed the sequence motif GCTTTTACAGA (Fig. 5), which differs at only two positions from the GATTTTACAGT sequence that has been implicated in phytochrome-mediated down-regulation of the small GTPase gene *pra2* from peas (Inaba et al. 1999), and sequences that are very similar to the C/AAACGG and AATATTTTTTCT motifs that have been linked with photoregulation in maize (Shiina et al. 1998). The putative light repression motif GCTTTTACAGA is located about 20 bp upstream from the proposed transcription start point for the barley (1→3,1→4)-β-D-glucan endohydrolase isoenzyme EI gene (Slakeski et al. 1990) and, perhaps more importantly, is part of a 15-bp region of the isoenzyme EI promoter that is not present in the isoenzyme EII gene promoter (Wolf 1991; Fig. 5). In addition, certain sequence similarities with *cis*-elements that are believed to regulate light-induced repression of the pea *AS1* gene (Ngai et al. 1997) were also detected in the barley (1→3,1→4)-β-D-glucan endohydrolase isoenzyme EI promoter (data not shown). Thus, the presence of a light repression motif in the isoenzyme EI promoter, but not in the isoenzyme EII gene promoter, is consistent with the dark-induction of the isoenzyme EI gene and with the absence of any apparent effects of light on expression of the isoenzyme EII gene (Figs. 3, 4). In rice, light-induced inhibition of coleoptile growth has been linked with the suppression of (1→3,1→4)-β-D-glucan turnover and the inhibition of cell wall (1→3,1→4)-β-glucanase activities (Chen et al. 1999). However, the nature of the mechanisms involved has not been determined.

In parallel with the increases in (1→3,1→4)-β-D-glucan endohydrolase activity observed when young barley seedlings were incubated in the dark was an increase in activity of the broad-specificity β-D-glucan glucohydrolase, although the increase in levels of the latter, exo-acting enzyme were observed 1–2 days later than the increase in endohydrolase activity (Fig. 1). Both enzyme types are secreted from cells in which they are synthesized, and would therefore have access to cell wall

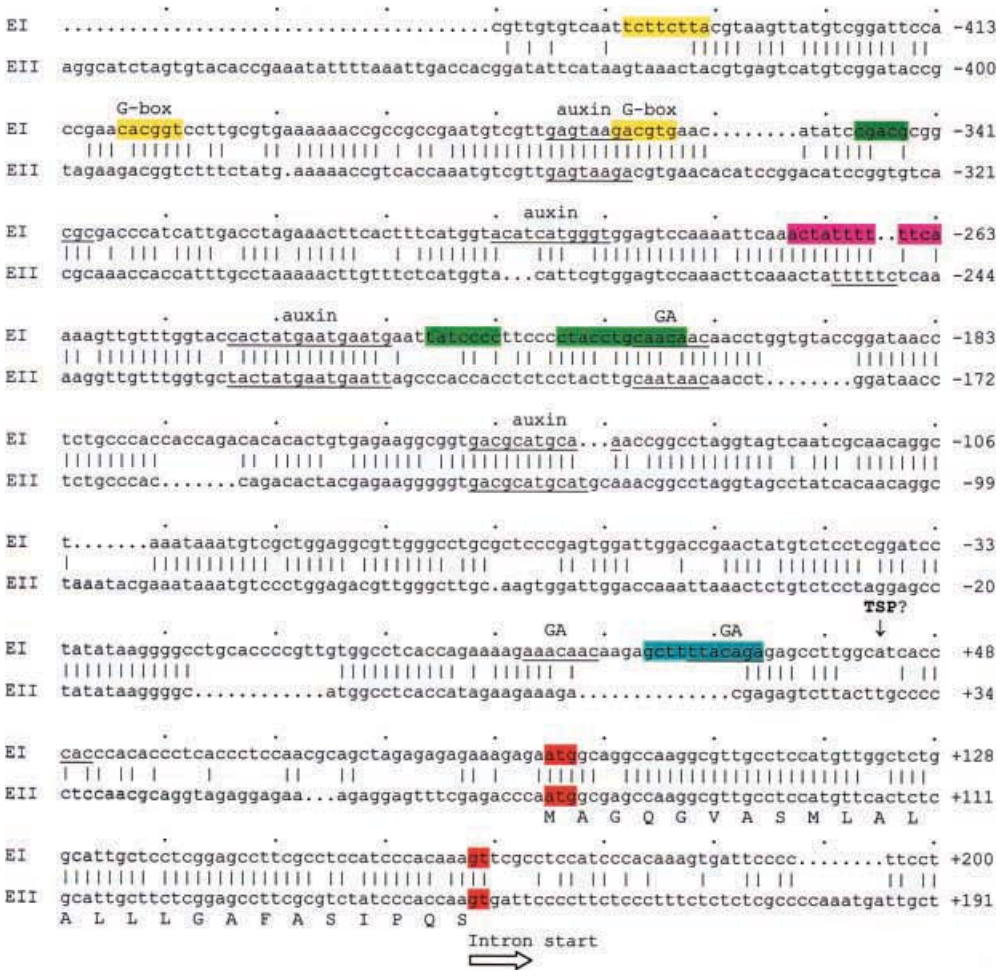

Fig. 5 Alignment of promoter sequences for genes encoding barley (1→3,1→4)-β-D-glucanase isoenzymes EI and EII. The transcription start point (*TSP*) for the isoenzyme EI gene is indicated by an *arrow*, the ATG translational start codons are in *red*, and the start of the single large intron is indicated. Sequences associated with gibberellin induction (*GA*), auxin induction (*auxin*), light repression (*blue*), photoregulation (*pink*), sugar repression (*yellow*) and enhanced expression under sugar starvation (*green*) indicate that most of these *cis*-acting regulatory elements are found in the promoter of the isoenzyme EI gene

(1→3,1→4)-β-D-glucans (Slakeski and Fincher 1992a, b; Hrmova et al. 1996). The endo- and exohydrolases can be easily distinguished in leaf extracts because the endohydrolase rapidly reduces the viscosity of (1→3,1→4)-β-D-glucan solutions, without releasing glucose (Woodward and Fincher 1982), while the exohydrolase releases glucose from (1→3,1→4)-β-D-glucans, without having any pronounced effect on the viscosity of the (1→3,1→4)-β-D-glucan solutions. There are several isoforms of the barley β-D-glucan glucohydrolase (Hrmova et al. 1996) but gene-specific probes are not yet available (Harvey et al. 2001). Thus, the identification of specific β-D-glucan glucohydrolase genes that might be up-regulated in dark-grown seedlings is not yet possible. It is noteworthy that a β-D-glucan glucohydrolase gene from maize (accession number AF064707) appears to be subject to photoregulation, although the details have not yet been published (L. Bogorad, Department of Cellular and Molecular Biology, Harvard University, Cambridge, Mass., USA; personal communication).

The physiological functions in young leaves of dark induction, or light repression, of genes encoding (1→3,1→4)-β-D-glucan endohydrolases and the β-D-glucan glucohydrolases are not known, but there are at least two possibilities. Firstly, the enzymes may play a role in cell wall loosening and tissue elongation during the etiolation process. It is widely held that partial "endo-hydrolysis" of matrix-phase polysaccharides in higher-plant cell walls reduces covalent or non-covalent 'cross-linking' of cellulosic microfibrils by the matrix polysaccharides, and that this 'loosening' of the wall allows turgor pressure-driven cell expansion (Cosgrove 1999). However, we were unable to detect any elongation or expansion of the fully opened second leaves of barley seedlings in the present study, and therefore conclude that the elevated enzyme levels in dark-treated leaves were not related to cell expansion.

The second possible physiological function of increased (1→3,1→4)-β-D-glucan hydrolase activity in dark-grown barley seedlings might be linked to the release of glucose from cell wall polysaccharides and thereby might be required for the maintenance of metabolic activity and tissue growth in leaves where photosynthetic rates are low for prolonged periods. Thus, the action of (1→3,1→4)-β-D-glucan endohydrolases could partially depolymerize cell wall (1→3,1→4)-β-D-glucans and the β-D-glucan glucohydrolase would convert released β-D-oligoglucosides to glucose. It must be emphasized that wall (1→3,1→4)-β-D-glucan was not completely degraded under the conditions used here;

levels of wall (1→3,1→4)-β-D-glucan decreased by about 30% during the dark treatment (Fig. 1c). The released glucose would be available for immediate metabolism in leaves, or could be translocated to other parts of the plant for use as an energy source. Whether or not the depolymerization of cell wall-bound (1→3,1→4)-β-D-glucan is coordinated with the mobilization of fructans is not known at this stage.

By releasing glucose from wall (1→3,1→4)-β-D-glucans, the 'sugar starvation status' and hence the dark-induced senescence program (Chung et al. 1997; Gan and Amasino 1997; Yu 1999) might be delayed. The delay could prevent the irreversible damage of leaf tissue and thereby enhance its chances of survival. Two senescence indicators, namely the chlorophyll content and the abundance of soluble protein, were examined here in leaves of dark-incubated barley seedlings. Both indicators decreased slightly for up to 9 days of dark treatment (Fig. 2) and it may be concluded that senescence did not proceed very far in the dark-treated leaves.

If the accumulation of (1→3,1→4)-β-D-glucan endohydrolases and β-D-glucan glucohydrolases in dark-grown barley leaves truly reflects a response to the absence of photosynthesis and hence to lower sugar levels, one might expect that the increases in enzyme activity would be accompanied by sugar depletion. Indeed, changes in the enzyme levels were paralleled by reciprocal changes in the soluble carbohydrate content of the leaves, which were rapidly depleted of sugars upon dark treatment (Fig. 2c). Soluble sugars would also include any fructans present in the leaves. Sugar-mediated regulation of gene expression occurs commonly in higher plants and has been implicated in the plant's response to changes in nutrient availability (Koch 1996; Lalonde et al. 1999). Inspection of the promoter region of the barley (1→3,1→4)-β-D-glucan endohydrolase isoenzyme EI gene (Fig. 5) revealed sequences very similar to, or identical with, the TATCCAT, CGACG and CTAGTGGCA motifs that have been detected in rice α-amylase genes that are expressed at high levels under sugar starvation (Hwang et al. 1998). Again, the sequences are detected in the isoenzyme EI promoter but not in the isoenzyme EII promoter (Fig. 5).

In conclusion, the increased activities of (1→3,1→4)-β-D-glucan endohydrolase isoenzyme EI and β-D-glucan glucohydrolases in the leaves of dark-incubated barley seedlings would be expected to result in the release of some glucose from cell wall (1→3,1→4)-β-D-glucan, and released glucose could provide a source of energy that would sustain respiration and other metabolic processes in darkness. Whether the actual trigger for increased transcription of the corresponding β-D-glucan hydrolase genes is dark induction, the release of light repression, or reduced sugar status in the cells can now be investigated. Moreover, the observations made here and elsewhere do suggest that feedback loops interconnect responses to light intensity, sugar status and cell wall metabolism in young barley leaves. The primary event that elicits the changes, photoreceptors or other cellular molecules that might be involved, and the molecular and cellular mechanisms through which feedback loops are connected and regulated during homeostasis of leaf function, remain to be demonstrated.

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