Volume 306, number 2,3, 98–102 FEBS 11263 July 1992

Barley (1→3,1→4)-β-glucanase isoenzyme EI gene expression is mediated by auxin and gibberellic acid

Nada Slakeski and Geoffrey B. Fincher

Department of Biochemistry, La Trobe University, Bundoora, Victoria 3083, Australia

Received 12 March 1992; revised version received 11 May 1992

Treatment of young barley leaves with indole acetic acid (IAA) or gibberellic acid (GA_3) results in a dramatic increase in levels of (1→3,1→4)-β-glucanase isoenzyme EI transcripts. In young roots of comparable age, levels of isoenzyme EI mRNA are high; IAA inhibits expression while GA_3 has no effect on mRNA levels. The addition of both abscisic acid and GA_3 to leaves, roots and aleurone layers leads to higher levels of (1→3,1→4)-β-glucanase isoenzyme EI mRNA than is found with GA_3 alone. Little or no expression of (1→3,1→4)-β-glucanase isoenzyme EII is detected in vegetative tissues, but in isolated aleurone layers GA_3 enhances levels of isoenzyme EII transcripts, as does IAA. Thus, the two barley (1→3,1→4)-β-glucanase genes respond quite differently to phytohormone treatment, depending on the tissue and its stage of development.

Auxin; Barley; Gibberellic acid; (1→3,1→4)-β-Glucanase; Tissue-specific expression

1. INTRODUCTION

Two (1→3,1→4)-β-glucanases (EC 3.2.1.73), designated isoenzyme EI and EII, are differentially expressed in a variety of tissues in germinated barley grain and in developing seedlings [1,2]. In germinated grain the enzymes are synthesized de novo and secreted into the starchy endosperm where they depolymerize the (1→3,1→4)-β-glucans that constitute up to 70% of endosperm cell walls [3]. Expression of the two (1→3,1→4)-β-glucanase genes, as measured by the accumulation of their corresponding mRNAs, occurs in both the aleurone and scutellar epithelium [1, 4–7]. The expression of these two genes is subject to spatial and temporal co-ordination within the grain [1,5]. Expression of (1→3,1→4)-β-glucanase has also been detected in young leaves and roots of the barley seedling [1,7]. In contrast to the germinated grain, where both isoenzymes are detected, only isoenzyme EI appears to be expressed in these vegetative tissues [1,7]. The enzyme is presumably involved in cell wall metabolism, because the walls of leaves are known to contain significant levels of (1→3,1→4)-β-glucan, which decrease during development [8].

Thus, the (1→3,1→4)-β-glucanase genes are transcribed in several tissues, and expression of the genes is likely to be under tight hormonal control. Indeed, total (1→3,1→4)-β-glucanase mRNA levels for the two isoenzymes are increased by gibberellic acid (GA_3) in isolated aleurone layers [4,9] and this reflected in enhanced secretion of enzymic activity [2]. Auxins also mediate several physiological processes that are related to cell wall metabolism during plant development. These include cell elongation, vascular differentiation, division of cultured cells, and phototropic and geotropic responses [10,11]. In the present work we have examined the effects of plant growth regulators on the levels of mRNA encoding the individual (1→3,1→4)-β-glucanases in a variety of tissues from barley seedlings. The results indicate that the two genes are differentially regulated by hormones, and that their response to individual hormones is both time- and tissue-dependent.

Correspondence address: G.B. Fincher, Department of Biochemistry, La Trobe University, Bundoora, Victoria 3083, Australia. Fax: (61) (3) 479 2467.

2. EXPERIMENTAL

2.1. *Plant material*

Barley (*Hordeum vulgare* L., cv. Himalaya) aleurone layers were prepared from grain sections from which the embryo and distal ends had been removed [12]. The sections were surface-sterilized for 15 min in 1% (v/v) sodium hypochlorite, rinsed once with 0.1 M HCl and washed exhaustively with sterile water. The sections were soaked in sterile water for 2–4 h and spread on moist filter paper at 22°C for 3 days. Starchy endosperm was removed under aseptic conditions and the remaining aleurone layers incubated, with gentle shaking, in 10 mM $CaCl_2$ adjusted to pH 5.2 in the presence or absence of 2 μM GA_3, 20 μM abscisic acid (ABA), both GA_3 and ABA, and 5 μM indole acetic acid (IAA). In all experiments, 50 aleurone layers were incubated in 10 ml medium. After 20 h at 26°C, the incubation medium was removed and stored at −20°C prior to the determination of (1→3,1→4)-β-glucanase activity. Aleurone layers were rinsed with 10 mM $CaCl_2$ (adjusted to pH 5.2 with dilute HCl) and blotted dry before RNA extraction.

For the examination of hormonal induction of (1→3,1→4)-β-glucanase genes in young leaves or roots, barley grain was surface sterilized for 20 min with 0.2% (w/v) silver nitrate [1], washed with 0.5 NaCl, rinsed thoroughly and immersed for 16 h in sterile distilled water. Grain was germinated on moist vermiculite at 22°C under constant light. After 6 days, young leaves had emerged from the coleoptile and

 Published by Elsevier Science Publishers B.V.

were approximately 8 cm in length. The seedlings were sprayed with control or hormone solutions, each containing the surfactant Tween-20 diluted approximately 1 in 10^4. The control solution was 10 mM potassium citrate buffer, pH 6.0 and hormones (2.5 mM GA_3, 2.5 mM IAA, 5 mM ABA, 2.5 mM kinetin with 2.5 mM IAA, or 2.5 mM GA_3 with 5 mM ABA) were dissolved in the same buffer. Relatively high concentrations of exogenously applied hormones are required for induction of gene expression in intact tissues [13]. After 10 h, seedlings were rinsed with sterile water, leaves and roots were removed and frozen under liquid nitrogen, and stored at −80°C prior to RNA extraction. For (1→3,1→4)-β-glucanase induction in coleoptiles, grain was surface-sterilized, germinated as described above, and grown on moist vermiculite in the dark for 3 days. Intact seedlings were sprayed, under red light, with sterile water, 2.5 mM IAA, 2.5 mM 2,4-dichlorophenoxyacetic acid (2,4-D) or 2.5 mM GA_3, using Tween-20 as a surfactant. After 4 h, coleoptiles and roots were removed from the grain, frozen in liquid nitrogen and stored at −80°C. For induction in coleoptile sections, the tip (5 mm) of the coleoptile was removed and a 1.5 cm section dissected from the apical region. Sections [14] were floated in Petri dishes containing 5 ml 50 μM IAA or GA_3 in 10 mM potassium citrate buffer, pH 6.0, with gentle shaking at 26°C for 4 h. Sections were rinsed and stored at −80°C prior to RNA extraction.

2.2. *Enzyme activity*

Activity of (1→3,1→4)-β-glucanase in media surrounding isolated aleurone layers was measured viscometrically, using 40°C water-soluble (1→3,1→4)-β-glucan (Biocon Pty. Ltd., Cork, Ireland) as a substrate [15]. Specific activity is expressed as the change in the reciprocal of the specific viscosity ($\Delta 1/n_{sp}$) per minute per aleurone layer [2]. Specific activity is expressed as activity secreted per aleurone layer rather than on a protein basis because both secreted and intracellular proteins are rapidly metabolized in this tissue. Cellulase activity was measured viscometrically, using carboxymethyl cellulose (Edifas B50, ICI Australia Pty. Ltd.) as a substrate [16].

2.3. *Extraction of RNA and Northern analyses*

Total RNA was isolated from tissue ground to a fine powder under liquid N_2, using the hot phenol/LiCl procedure [17] for leaves and roots, or a modified phenol/sodium dodecylsulphate method [18] for aleurone. Samples (10 μg) were separated in 1% (w/v) agarose gels containing 2.2 M formaldehyde and transferred to nitrocellulose (Hybond C-Extra, Amersham) for probing. Filters were probed with ^{32}P-labelled DNA fragments corresponding to the 3′-untranslated regions of the (1→3,1→4)-β-glucanase isoenzyme, EI and EII, cDNAs, using hybridization conditions described previously [1,7].

3. RESULTS

3.1. *Induction in young leaves and roots*

Young leaves were harvested 6 days after the initiation of germination, which is approximately 2 days before (1→3,1→4)-β-glucanase mRNA is normally detected in leaves [1]. As expected, little (1→3,1→4)-β-glucanase mRNA was found in leaves 10 h after spraying with buffer. However, RNA extracted from the leaves sprayed with GA_3, IAA and GA_3/ABA contained relatively high levels of isoenzyme EI mRNA, and GA_3/ABA levels exceeded those for GA_3 alone (Fig. 1). No detectable increases in (1→3,1→4)-β-glucanase mRNA levels were found over the 10 h time period following treatment with ABA or kinetin/IAA (Fig. 1). No mRNA encoding isoenzyme EII was detected in young leaves after the hormone treatments (Fig. 1).

Northern analyses of total RNA preparations from young roots, also harvested at 6 days, revealed that significant levels of (1→3,1→4)-β-glucanase isoenzyme EI mRNA were present in control (buffer-treated) roots (Fig. 1). Treatment of the seedlings with GA_3, ABA and GA_3/ABA had little or no effect on isoenzyme EI mRNA levels in young roots, although IAA and kinetin/IAA treatments resulted in an apparent decrease in (1→3,1→4)-β-glucanase mRNA (Fig. 1). Again, no isoenzyme EII mRNA was found in the root extracts (Fig. 1).

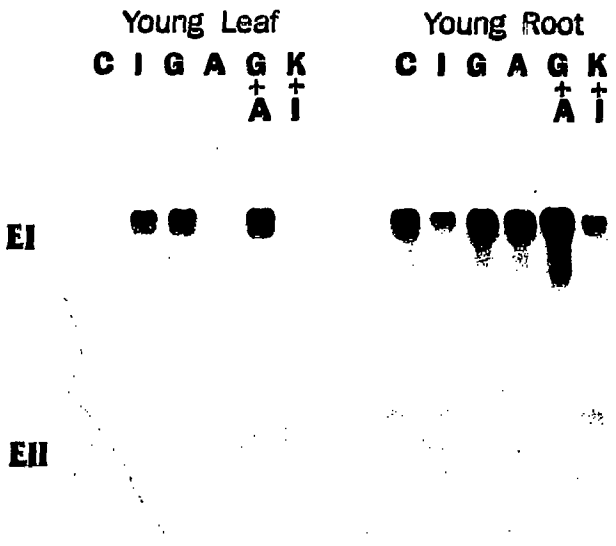

Fig. 1. (1→3,1→4)-β-Glucanase expression in young leaves and roots. The figure shows Northern analyses of RNA preparations from leaves and roots from 6-day-old barley seedlings. Seedlings were sprayed with buffer (C), IAA (I), GA_3 (G), ABA (A), GA_3+ABA (G+A) or kinetin + IAA (K+I) 10 h prior to RNA extraction. Approximately 10 μg RNA was loaded into each lane and the nitrocellulose filters were probed with cDNA probes corresponding to the 3′-untranslated regions of (1→3,1→4)-β-glucanase isoenzymes. EI or EII.

3.2. *Expression in coleoptiles*

Marked elongation of coleoptiles was observed 4 h after spraying with auxins. Thus, control coleoptiles were about 1.5 cm in length at this time, while IAA treatment caused elongation to 2.5–3.0 cm. 2,4-D-treated coleoptiles were 2.0-2.5 cm in length. No (1→3,1→4)-β-glucanase mRNA was ever detected in intact coleoptiles or in elongating coleoptile sections at this or other stages (data not shown).

3.3. *Expression in isolated aleurone layers*

In the untreated, control aleurone layers, significant levels of both isoenzyme EI and EII mRNAs were present (Fig. 2). While GA_3 treatment clearly resulted in an increase in isoenzyme EII mRNA, the hormone had little effect on levels of isoenzyme EI mRNA (Fig. 2). Treatment with ABA reduced levels of both mRNA species compared with controls, but combined ABA/GA_3 or IAA treatment led to higher levels of mRNAs

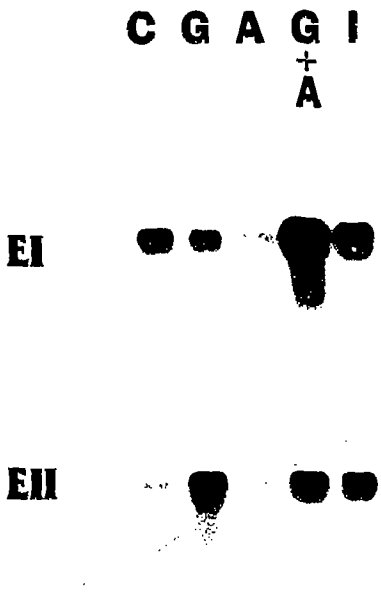

Fig. 2. (1→3,1→4)-β-Glucanase expression in hormonally treated aleurone layers. The figure shows Northern analyses of RNA from isolated aleurone layers treated with buffer (C), GA_3 (G), ABA (A), GA_3/ABA (G+A) or IAA (I). Approximately 10 μg RNA was loaded in each lane and filters were probed with isoenzyme EI- or EII-specific cDNAs, as described in Fig. 1.

for both isoenzymes, and ABA/GA_3 levels were higher than for GA_3 alone (Fig. 2).

When (1→3,1→4)-β-glucanase enzyme activity is measured in the incubation medium surrounding the isolated aleurone layers, it is apparent that GA_3 causes a 3-fold increase in activity, ABA treatment leads to a significant decrease in activity, while IAA and GA_3/ABA treatments cause a smaller, but significant increase in (1→3,1→4)-β-glucanase secretion compared with control aleurone layers (Table I). It should be noted that the values reflect activity contributed by both isoenzymes. No cellulase activity was detected.

Table I

(1→3,1→4)-β-Glucanase secretion from isolated aleurone layers

Treatment	Specific activity* (× 10^{-2})	% Control
Control (buffer only)	1.24	100
GA_3	3.78	305
ABA	0.48	39
GA_3 and ABA	2.31	186
IAA	1.90	153

*Specific activity is expressed as the change in the reciprocal of the specific viscosity ($\Delta 1/n_{sp}$) per minute per aleurone layer [2].

4. DISCUSSION

The expression of barley (1→3,1→4)-β-glucanase genes has been examined in a variety of tissues grown under different conditions and subjected to treatment with selected plant growth regulators. Intact seedlings were sprayed with phytohormone solutions, while isolated aleurone layers and coleoptile sections were incubated in the hormone solution, and RNA was isolated at selected times after the treatment. There are several variables when hormone solutions are applied exogenously to intact plant tissues in this way, including the concentration of the hormone solution required for a response, the rate of penetration of the solution into the tissue and therefore the actual hormone concentration at the cell surface, the possible requirement for additional cofactors, the stability of the hormone solution, the relative receptiveness of the tissue to the hormone at its particular developmental stage, and the time required for a maximal response to be observed [13,19,20]. Considerable controversy has therefore surrounded the effects of exogenously applied auxins to intact plant tissues, particularly as to whether the effects are due to auxin itself or to auxin-induced ethylene production [13,19]. Furthermore, expression levels in the present and other studies have been investigated by Northern analysis of total RNA isolated from the tissues and therefore represent steady- state levels of transcripts rather than the final expression of active, correctly targetted (1→3,1→4)-β-glucanase enzyme. Transcript levels are compared by examination of the relative intensities of bands on autoradiograms of Northern blots and, although a degree of standardization can be achieved by uniform loading and by checking the intensity of ribosomal RNA bands, the actual amounts of mRNA loaded may vary and the comparisons must be considered semi-quantitative.

Within the constraints imposed by these physiological and technical limitations, we have investigated the hormonal induction of the two genes encoding barley (1→3,1→4)-β-glucanase isoenzymes EI and EII (Figs. 1,2). Divergence of nucleotide sequences in the 3'-untranslated regions of the two genes enabled the design of gene-specific probes that can be used to monitor levels of mRNA encoding the individual isoenzymes [7]. Isoenzyme EII transcripts are detected almost exclusively in the aleurone, where they are enhanced by treatment with relatively low concentrations of both GA_3 and IAA, but inhibited by ABA. The gene encoding barley (1→3,1→4)-β-glucanase isoenzyme EI is also transcribed in isolated aleurone layers, but is not significantly enhanced by 2 μM GA_3 and is only marginally enhanced by IAA under the conditions used here. Again, ABA suppresses transcription. These results are consistent with the apparently preferential enhancement by GA_3 of (1→3,1→4)-β-glucanase isoenzyme EII secretion from isolated aleurone layers [2], and the sup-

pression of total translatable mRNA for (1→3,1→4)-β-glucanases by ABA [9]. However, the high levels of both mRNAs in aleurone layers treated with GA_3/ABA, compared with control and GA_3-treated levels, do not match the smaller increases in enzymic activity (Table I). Similar effects have been reported for the levels of α-amylase mRNAs in barley aleurone [21]. This may indicate that ABA induces an inhibitor of the (1→3,1→4)-β-glucanases or that it inhibits translation of (1→3,1→4)-β-glucanase mRNA.

In contrast to the isoenzyme EII gene, expression of isoenzyme EI is also detected in vegetative tissues indicating that although the two isoenzymes have the same substrate specificity against wall (1→3,1→4)-β-glucans, their tissue distribution, and therefore their participation in wall metabolism, is more specialized. The different responses of isoenzyme EI gene transcription to hormone treatment in these tissues also serve to emphasize that hormone responsiveness depends on growth conditions and the stage of development of the tissue (see [20]). Thus, isoenzyme EI mRNA levels increase dramatically in response to GA_3 treatment in young leaves, but are apparently unaffected in coleoptiles, roots and aleurone layers (Figs. 1,2). The natural auxin, IAA, enhances levels of isoenzyme EI mRNA in aleurone layers and leaves, but has no effect in roots of 3-day-old etiolated seedlings, and causes a significant decrease in mRNA levels in roots of 6-day-old seedlings. Similarly, ABA suppresses isoenzyme EI mRNA in aleurone layers but it has no apparent effect on levels in leaves and roots (Figs. 1,2). In the experiments with intact tissues, relatively higher concentrations of the hormones were required to elicit a response, as reported in earlier work [13].

Despite this variability between tissues, the pronounced induction in young leaves of the (1→3,1→4)-β-glucanase isoenzyme EI gene by both GA_3 and IAA (Fig. 1) represents an important observation with significant functional implications. At present the precise tissue or cellular location of (1→3,1→4)-β-glucanase in developing vegetative tissues is unclear, although the availability of monoclonal antibodies [22] and gene-specific DNA probes [7] will now enable the cellular sites of expression to be defined. The function of the (1→3,1→4)-β-glucanase in vegetative tissues is also uncertain, but it is likely to be related to wall metabolism. Auxin action has been linked to wall metabolism in actively growing plant tissues. Cell elongation and vascular differentiation [10,23] are likely to involve partial hydrolysis or turnover of cell wall polysaccharides and both auxin and GA_3 have been implicated in lignification of walls [24]. In elongating barley coleoptiles, auxin induces a marked decrease in (1→3,1→4)-β-glucan content of walls over a 6 h period [25]. However, we have been unable to detect any (1→3,1→4)-β-glucanase mRNA in elongating coleoptiles, and it is possible that the decreases in wall (1→3,1→4)-β-glucan observed in barley and other elongating coleoptiles are mediated by exo-β-glucanases [1,26].

The phytohormone GA also participates in stem elongation and wall extensibility in plants [27] but whether the induction of the (1→3,1→4)-β-glucanase isoenzyme EI gene by GA_3 in young leaves (Fig. 1) is related to this effect remains unknown. The (1→3,1→4)-β-glucan content of walls in young barley leaves decreases during development ([8], N. Sakurai, personal communication) and this could be related to the removal of unlignified primary wall material during auxin-mediated vascular differentiation [1,10]. Similar decreases in (1→3,1→4)-β-glucan content are induced by auxins and gibberellins in developing maize coleoptiles and leaves [28,29]. Of particular interest is the observation that the simultaneous addition of ABA and GA_3 increases (1→3,1→4)-β-glucanase isoenzyme EI mRNA in leaves and roots, as well as in aleurone, above levels detected with GA_3 alone (Fig. 1). A similar effect has been noted with β-glucanase expression in young roots of rice [30], yet in aleurone cells at least, ABA generally acts as an antagonist to GA_3 induction of gene expression [3]. At this stage, any connection between decreasing (1→3,1→4)-β-glucan levels, auxin, GA_3 or GA_3/ABA induction of the barley (1→3,1→4)-β-glucanase isoenzyme EI gene (Fig. 1) and vascular differentiation awaits definition of the precise tissue location of the enzyme.

Acknowledgements: This work was supported by grants from the Australian Research Council and the Barley Research Council of Australia. We thank Peilin Xu and Danny Doan for their advice and help with various aspects of the work. N.S. acknowledges receipt of a La Trobe University Postgraduate Research Award.

REFERENCES

[1] Slakeski, N. and Fincher, G.B. (1991) Plant Physiol. (in press).
[2] Stuart, I.M., Loi, L. and Fincher, G.B. (1986) Plant Physiol. 80, 310–314.
[3] Fincher, G.B. (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 305–346.
[4] Litts, J.C., Simmons, C.R., Karrer, E.E., Huang, N. and Rodriguez, R.L. (1990) Eur. J. Biochem. 194, 831–838.
[5] McFadden, G.I., Ahluwalia, B., Clarke, A.E. and Fincher, G.B. (1988) Planta 173, 500–508.
[6] Mundy, J., Brandt, A. and Fincher, G.B. (1985) Plant Physiol. 79, 867–871.
[7] Slakeski, N., Baulcombe, D.C., Devos, K.M., Ahluwalia, B., Doan, D.N.P. and Fincher, G.B. (1990) Mol. Gen. Genet. 224, 437–449.
[8] Buchala, A.J. and Wilkie, K.C.B. (1974) Phytochemistry 13, 1347–1351.
[9] Mundy, J. and Fincher, G.B. (1986) FEBS Lett. 198, 349–352.
[10] Aloni, R. (1987) Annu. Rev. Plant Physiol. 38, 179–204.
[11] Key, J. (1989) BioEssays 11, 52–58.
[12] Chrispeels, M.J. and Varner, J.E. (1967) Plant Physiol. 42, 398–406.
[13] Zurfluh, L.L. and Guilfoyle, T.J. (1982) Plant Physiol. 69, 338–340.
[14] Zurfluh, L.L. and Guilfoyle, T.J. (1982) Plant Physiol. 69, 332–337.

[15] Woodward, J.R. and Fincher, G.B. (1982) Eur. J. Biochem. 121, 663–669.
[16] Woodward, J.R. and Fincher, G.B. (1982) Carbohydr. Res. 106, 111–122.
[17] Verwoerd, T., Dekker, B.M.M. and Hoekema, A. (1989) Nucleic Acids Res. 17, 2362.
[18] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1987) Curr. Prot. Mol. Biol. 1, 4.3.1–4.3.4.
[19] Hall, J.L., Brummell, D.A. and Gillespie, J. (1985) New Phytol. 100, 341–345.
[20] Hensel, A., Brummell, D.A., Hanna, R. and Maclachlan, G.A. (1991) Planta 183, 321–326.
[21] Mozer, T.J. (1980) Cell 20, 479–485.
[22] Høj, P.B., Hoogenraad, N.J., Hartman, D.J., Yannakena, H. and Fincher, G.B. (1990) J. Cereal Sci. 11, 261–268.
[23] Lincoln, C., Britton, J.H. and Estelle, M. (1990) Plant Cell 2, 1071–1808.
[24] Aloni, R., Tollier, M.T. and Monties, B. (1990) Plant Physiol. 94, 1743–1747.
[25] Sukurai, N. and Masuda, Y. (1978) Plant Cell Physiol. 19, 1217–1223.
[26] Labrador, E. and Nevins, D.J. (1989) Physiol. Plant. 77, 479–486.
[27] Keyes, G., Sorrells, M.E. and Setter, T.L. (1990) Plant Physiol. 92, 242–245.
[28] Carpita, N.C. and Kanabus, J. (1988) Plant Physiol. 88, 671–678.
[29] Inouhe, M. and Nevins, D.J. (1991) Plant Physiol. 96, 285–290.
[30] Simmons, C.R., Litts, J.C., Huang, N. and Rodriguez, R.L. (1992) Plant Mol. Biol. 18, 33–45.