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Auxin-Induced Elongation Growth and Expressions of Cell Wall-Bound Exo- and Endo-β-Glucanases in Barley Coleoptiles

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When auxin stimulates rapid cell elongation growth of cereal coleoptiles, it causes a degradation of 1,3:1,4-β-glucan in hemicellulosic polysaccharides. We examined gene expressions of endo-1,3:1,4-β-glucanase (EI) and exo-β-glucanase (ExoII), of which optimum pH are about 5, and molecular distribution of hemicellulosic polysaccharides in barley (*Hordeum vulgare* L.) coleoptile segments treated with or without IAA. IAA (10^{-5} M) stimulated the gene expression of EI, while it did not affect that of ExoII. IAA induced gene expression of EI after 4 h and increased wall-bound glucanase activity after 8 h. The molecular weight distribution of hemicellulosic polysaccharides from coleoptile cell walls was shifted to lower molecular weight region by 2 h of IAA treatment. Fusicoccin (10^{-6} M) mimicked IAA-induced elongation growth and the decrease in molecular weight of hemicellulosic 1,3:1,4-β-glucan of coleoptiles in the first 4 h, but it did not promote elongation growth thereafter. These facts suggest that acidification of barley cell walls by IAA action enhances pre-existing cell wall-bound glucanase activity in the early first phase of IAA-induced growth and the late second phase involves the gene expression of EI by IAA.

Key words: Acid growth — Barley (*Hordeum vulgare*) coleoptile — Fusicoccin — Glucanase — IAA.

Abbreviations: ExoII, an isozyme of barley exo-β-glucanase II; EI and EII, isozymes of barley endo-1,3:1,4-β-glucanase I and II, respectively; FC, fusicoccin.

Introduction

Auxin induces rapid elongation growth of higher plants. The mechanism of auxin action in the elongation growth acts on the cell walls, since mechanical properties of the cell walls change as early as IAA induces elongation. There are two hypotheses for the mechanism; one is acid-growth theory (Cleland 1971) and the other is enzyme-linked cell wall loosening (Tanimoto and Masuda 1968). Auxin enhances proton extrusion by activating a membrane H^+-ATPase (Hager et al. 1991) or stimulating its gene expression (Frias et al. 1996). The action of proton is thought to break so-called acid-labile bonds of cell wall constituents that bear turgor pressure generated by osmotic potential of cytoplasm, but the actual and responsible bond has been poorly understood. The enzyme-linked loosening mechanism claims that auxin stimulates gene expressions of some glycanase(s) that catalyze the hydrolyses of cell-wall polysaccharides. However, there has been little direct evidence of expression of enzymes involved in cell wall loosening.

The enzyme-linked mechanism was strengthened by the finding that IAA treatment decreased β-glucan content of the non-cellulosic polysaccharides (Loescher and Nevins 1972). The addition of cycloheximide and cordycepin (Sakurai and Masuda 1979) and a β-glucanase inhibitor (Sakurai et al. 1977) inhibited the decrease in the β-glucan content as well as auxin-induced elongation. Furthermore, IAA caused a decrease in molecular weight of 1,3:1,4-β-glucan in the cell walls (Sakurai et al. 1979). Using antibodies against 1,3:1,4-β-glucan, Hoson and Nevins (1989) indicated direct association of β-glucan degradation with cell wall loosening responsible for auxin-induced elongation growth.

Huber and Nevins (1980) first released exo- and endo-β-glucanase activity from cell walls of maize coleoptiles with 3 M lithium chrolide. Inouhe and Nevins (1991) indicated that both exo- and endo-β-glucanase activities were required for auxin-induced elongation growth of coleoptiles. These wall-bound glucanases are thought to be involved in the auxin-induced change in mechanical properties of the cell walls. The detail properties, however, of the glucanases remained to be characterized.

Endo-1,3;1,4-β-glucanase (EI) was first purified from barley seeds and the biochemical properties were determined (Woodward and Fincher 1982). Endo-1,3;1,4-β-glucanase hydrolyses 1,3:1,4-β-glucan specifically in endo-manner. Hrmova et al. (1996) first, biochemically characterized exo-β-glucanase (β-glucan exohydrolase) isoenzyme ExoI and ExoII and cloned ExoII cDNA from barley seedlings. We have purified and characterized a wall-bound type of exo-β-glucanase from barley seedlings, and reported that the purified wall-bound-β-glucanase was ExoII (Kotake et al. 1997). The ExoII released monomeric glucose from 1,3:1,4-β-glucan. It is thought to reduce the amount of 1,3:1,4-β-glucan, while EI to be responsible for a decrease in its molecular weight during auxin-induced elongation growth of barley coleoptiles.

Slakeski and Fincher (1992a) and Slakeski and Fincher

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(1992b) reported that two barley endo-1,3:1,4-β-glucanases, namely EI and EII were expressed in the germinated seed, young leaf and root but not in 3-day-old coleoptile, and that gene expressions of EI in aleurone layer of seed and young leaf were stimulated by IAA, while that in young root was suppressed by IAA. However, to date, the gene expressions of glucanases in auxin-induced elongation growth of coleoptiles remained to be studied.

Here we report the gene expression of endo-1,3:1,4-β-glucanase (EI) and exo-β-glucanase (ExoII) in IAA-induced elongation growth of coleoptiles, since the enzyme characterizations and full lengths of transcripts had been reported. We also clarified an influence of acidification of cell walls on 1,3:1,4-β-glucan degradation in coleoptiles, using fusicoccin.

Materials and Methods

Plant material

Barley seeds (*Hordeum vulgare* L. cv. Himalaya) were imbibed in tap water for 8 h, sown on moist vermiculite and incubated in the dark at 25°C. The coleoptiles (3–4 cm) were harvested after 3 d with special care of removal of young leaves, roots and seeds. Five-mm segments were excised from the region 5–10 mm below coleoptile tip. The segments were treated with IAA or FC in 10 mM citrate-phosphate-potassium buffer (pH 6.6) in the dark for 8 h. The segment lengths were measured with a binocular microscope.

Preparation of ExoII and EI probes

cDNA clone of endo-1,3:1,4-β-glucanase EI was a kind gift of Prof. Fincher of Adelaide University in Australia. It encoded 3′ untranslated region of EI cDNA (Slakeski and Fincher 1992a, Slakeski and Fincher 1992b). Partial cDNA sequence of exo-β-glucanase ExoII (1.5 kb) was amplified by reverse transcription-PCR with RNA LA PCR kit (Ver.1.1, Takara, Tokyo, Japan). The primers were designed according to the cDNA sequence of ExoII reported by Hrmova et al. (1996) (ExoII-sense; 5′-CCTCATGTTCTGCTTGGCGG-3′ and ExoII-antisense; 5′-CCGACCACCACGATSGCGTAGTC-3′). The amplified DNA fragment was cloned into pGEM vector with a TA-cloning kit (Promega, Madison, U.S.A.), and sequenced with an autosequencer (373A, ABI). The ^{32}P-labeled probes were prepared with a BcaBest labeling kit (Takara, Tokyo, Japan).

Northern blot analysis

Total RNA was extracted from etiolated barley tissues with a help of ISOGEN (Nippon gene, Tokyo, Japan). The total RNA was separated on 1.2% formaldehyde-agarose gel and blotted onto nitrocellulose membrane (Nitro Bind, Micron Separation Inc., Westborough, MA, U.S.A.). The blotted membrane was baked at 80°C for 2 h and hybridized with ^{32}P-dCTP labeled ExoII or EI probe in 50% formamide, 5× SSPE (750 mM sodium chloride, 50 mM phosphate, 1 mM EDTA, pH 7.4), 5× Denhardt's (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.1% SDS and 0.1 mg ml^{-1} salmon sperm DNA at 42°C for 16 h. Then the membrane was washed twice with 2× SSC (300 mM sodium chloride, 20 mM sodium citrate) and 0.1% SDS at 55°C for 10 min and twice with 0.5× SSC (75 mM sodium chloride, 5 mM sodium citrate) and 0.1% SDS at 65°C for 15 min. The washed membrane was exposed and analyzed on a bioimaging analyzer (BAS-2000, Fuji Xerox, Tokyo, Japan).

Measurement of wall-bound glucanase activity

Cell wall proteins of IAA- or FC- treated coleoptile segments were released with 3 M lithium chloride as described before (Kotake et al. 1997). Thirty segments were treated with 10^{-5} M IAA or 10^{-6} M FC for 4 and 8 h in the dark and immediately frozen with liquid nitrogen. The segments were homogenized in 5 mM sodium chloride containing 100 μM Pefabloc SC (Merck, Germany) and centrifuged at 1,000×*g* for 10 min. Resultant precipitate was washed twice with deionized water and suspended in 3 M lithium chloride containing 10 μM Pefabloc SC for 24 h at 4°C. The suspension was centrifuged at 1,000×*g* for 10 min and used for enzyme assay. Glucanase activity was measured as increase in the reducing terminals of liberated sugars by a neocuproin method (Dygert et al. 1965). The reaction medium employed during the enzyme purification steps consisted of 20 μl of enzyme and 200 μg of β-glucan from barley (Sigma, St Louis, MO, U.S.A.), and incubated for 2, 4 or 6 h at 25°C.

Preparation and analysis of hemicellulose B from coleoptile cell walls

Fifty 5-mm segments of barley coleoptiles were incubated for 4 h in 10 mM citrate-phosphate-potassium buffer (pH 6.6) with or without 10^{-5} M IAA or 10^{-6} M FC. The coleoptile segments were fixed in boiling 80% ethanol and homogenized in deionized water by mortar and pestle. Cell walls were collected by centrifugation (1,000×*g* for 10 min), treated with 400 unit of α-amylase (porcine pancreas, Boehringer Mannheim, Germany) in 50 mM sodium acetate buffer (pH 6.5) for 4 h at 37°C, then extracted twice with hot water for 10 min at 100°C and three times with 50 mM EDTA (pH 6.8) for 15 min at 100°C. Hemicellulose was extracted from the resultant precipitate with 17.5% sodium hydroxide containing 0.02% sodium borohydride for 20 h at 4°C. After neutralization with glacial acetic acid, the hemicellulose was dialyzed against deionized water for 2 d at 4°C and centrifuged at 1,000×*g* for 10 min to remove hemicellulose A. The soluble hemicellulose B fraction was applied to a gel filtration column (G5000PW, Tosoh, Tokyo, Japan) in 20 mM sodium acetate (pH 5.4) as eluant. The eluted polysaccharides were detected by a refractive detector (RID-300, Jasco, Tokyo, Japan) and fractionated at 1 min intervals. Polysaccharides of each fraction were hydrolyzed and reduced by the method of Albersheim et al. (1967) and acetylated by the method of Blackeny et al. (1983). The neutral sugar composition was determined by a gas-liquid chromatograph (GC-7A, Shimadzu, Kyoto, Japan) equipped with a capillary column (DB-23, J & W Scientific, Folsom, CA, U.S.A.).

Results

Spatial gene expressions of ExoII and EI in etiolated barley seedlings

Northern blot analysis equally detected transcript of ExoII in coleoptiles, young leaves and roots of 3-day-old etiolated seedlings but failed to detect mRNA of EI in any region of coleoptiles (Fig. 1). Slakeski and Fincher (1992a) have already examined the gene expressions of EI by Northern analysis and reported that mRNA of EI was not detected in 3-day-old coleoptiles. However, we detected mRNA of EI but not EII by specific reverse transcription-PCR (data not shown). Therefore, EI gene may be expressed in coleoptiles at very low level even in the actively growing region. EI transcript was intensely detected in root as reported (Slakeski and Fincher 1992a).

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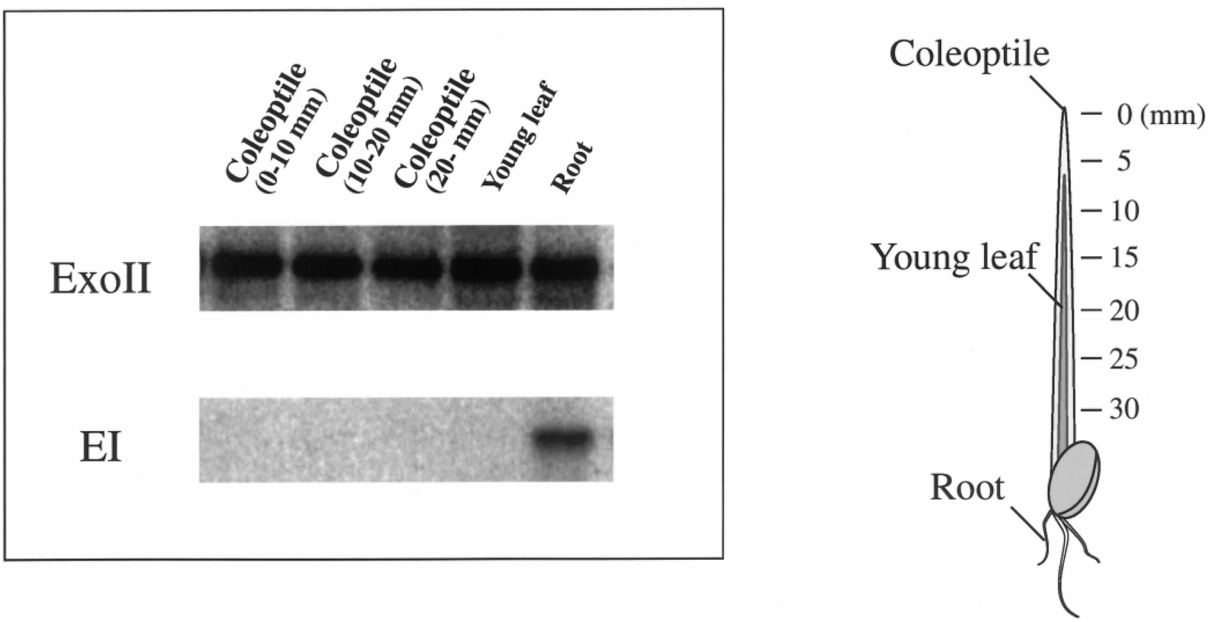

Fig. 1 Northern blot analysis of ExoII and EI in different parts of barley seedlings. Total RNA extracted from different parts of coleoptiles, first leaves and roots of etiolated barley seedlings was hybridized with ^{32}P-labeled ExoII or EI probe. Intensity of 25S rRNA bands as disclosed by staining with methylene blue was similar in all the treatment.

Effect of auxin on induction of gene expressions of β-glucanases

Effects of IAA on the gene expressions of ExoII and EI were evaluated by Northern analysis (Fig. 2). The ExoII gene was intensely expressed at 0 h (Fig. 2A). IAA treatment did not affect the amount of ExoII mRNA until 8 h. Ratio of ExoII expression of IAA-treated segments to that of control segments remained constant up to 8 h (Fig. 2B), indicating that the ExoII gene was constitutively expressed and not regulated by IAA. Gene expression of EI was not detected in the initial coleoptile segments (0 h), but detected in IAA-treated segments at 4 h and more intensely at 8 h. IAA treatment for 4 h increased amount of EI transcript by about 5 times (Fig. 2B). Control segments exhibited promotion of EI expression at 8 h. Nevertheless, ratio of EI expression of IAA-treated segments to that of control segments was still about 5 at 8 h. Treatment of ethylene precursor, 1-aminocyclopropane-1-carboxylate (10^{-4} M), for 8 h did not stimulate induction of EI expression (data not shown), indicating that IAA induced-gene expression of EI is not mediated by ethylene evolved by exogenous IAA treatment. FC did not promote EI expression up to 8 h of incubation (data not shown).

The effect of different concentrations of IAA on ExoII and EI expression in barley coleoptiles was examined (Fig. 3A). From 0.1 μM to 100 μM IAA did not enhanced gene expression of ExoII up to 8 h (Fig. 3B). EI expression was induced even by 0.1 μM of IAA and saturated by 1 μM (Fig. 3B). The EI induction corresponded to the effect of IAA on the elongation growth of coleoptile segments, which was also saturated at around 1 μM (Fig. 3C).

Elongation growth of coleoptile segment by fusicoccin

The effect of fusicoccin (FC) at 10^{-6} M on elongation growth of barley coleoptiles was similar to that of IAA in the first 4 h of incubation (Fig. 4). FC-induced growth slightly declined after 2 h, then completely ceased after 4 h. The effect of FC on EI expression was examined by Northern analysis. FC did not affected the amount of EI mRNA up to 8 h of treatment (data not shown).

IAA enhanced amount of wall-bound glucanase activity

Total β-glucanase activity of cell wall proteins extracted with 3 M lithium chloride was measured using barley β-glucan as substrate. The amount of activity was increased by 4 and 8 h of IAA treatment (Fig. 5). FC (10^{-6} M) did not showed promotive effect on the wall-bound glucanase activity both at 4 and 8 h.

Molecular weight distribution of hemicellulose B in IAA- or FC-treated coleoptiles

1,3:1,4-β-Glucan and arabinoxylan are major components of hemicellulosic polysaccharides of cereal plant. Molecular weight distribution of hemicellulose B of IAA- or FC-treated coleoptiles was examined by a gel filtration column with a refractive index detector (Fig. 6). FC-treatment decreased the

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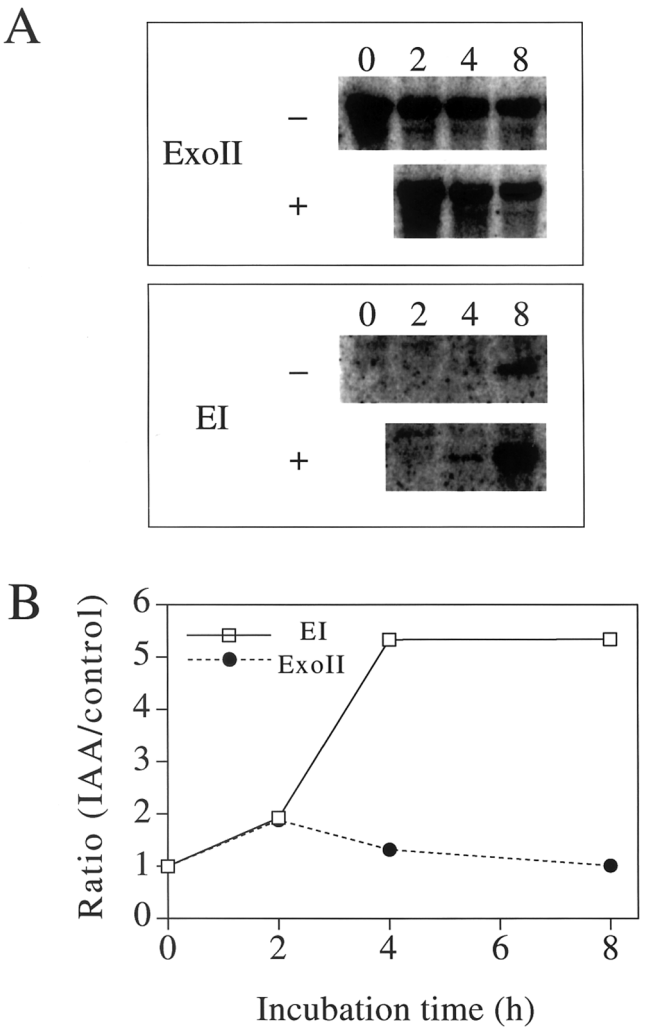

Fig. 2 Effect of IAA on the amount of ExoII and EI transcripts in barley coleoptile segments. A, coleoptile segments excised from 5–10 mm region from the tip were incubated with or without 10^{-5} M IAA in 10 mM citrate-phosphate-potassium buffer (pH 6.6) for 8 h in the dark. The gene expressions of ExoII and EI were examined by Northern hybridization. B, the signal intensity was expressed in ratio of IAA-treated segment to control segment.

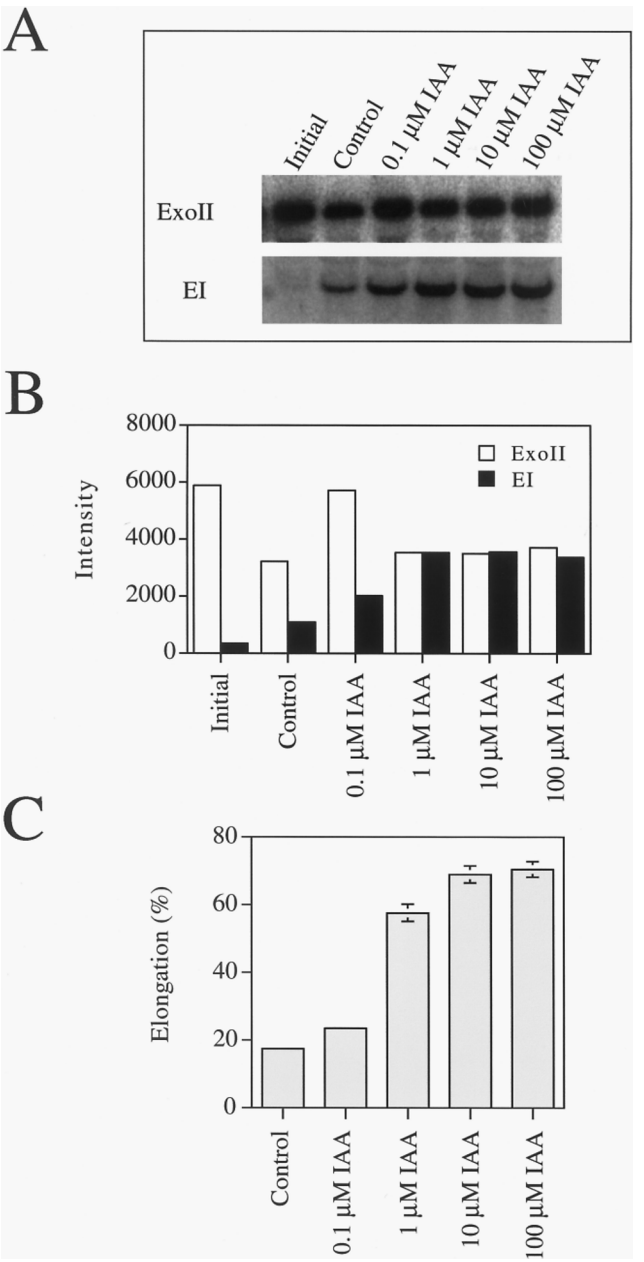

Fig. 3 Effect of different concentrations of IAA on the amount of ExoII and EI transcripts. Coleoptile segments (5 mm in length) excised from 5–10 mm region from the tip were incubated with 10^{-7}–10^{-4} M IAA in 10 mM citrate-phosphate-potassium buffer (pH 6.6) for 8 h in the dark. A, ExoII and EI transcripts were detected by Northern hybridization. B, the amount of transcript was qualified by a bioimaging analyzer. C, segment lengths were measured with a binocular microscope. Elongation growth was expressed as % from initial segment. Means and standard errors are given (n=10). Means without error bar show that the errors are less than 2%.

molecular weight distribution to low molecular weight region as did IAA-treatment within 2 h (Fig. 6B, C). The effects of FC and IAA on the decrease were more prominent at 4 h (Fig. 6E, F). Slight decrease in the control segments was also observed at 2 h (Fig. 6A), but the FC- or IAA-treated segments showed more decrease at 2 h than the control. The results indicate that FC stimulated degradation of hemicellulosic polysaccharide of barley coleoptiles as well as IAA.

Analysis of glucose component of hemicellulosic polysaccharides

Each fraction of polysaccharides eluted from a gel permeation column was analyzed by acetylation to detect the molecular weight shift of glucose component of hemicellulose B polysaccharides (Fig. 7). When the segments were incubated without IAA or FC, glucose was mainly eluted around void

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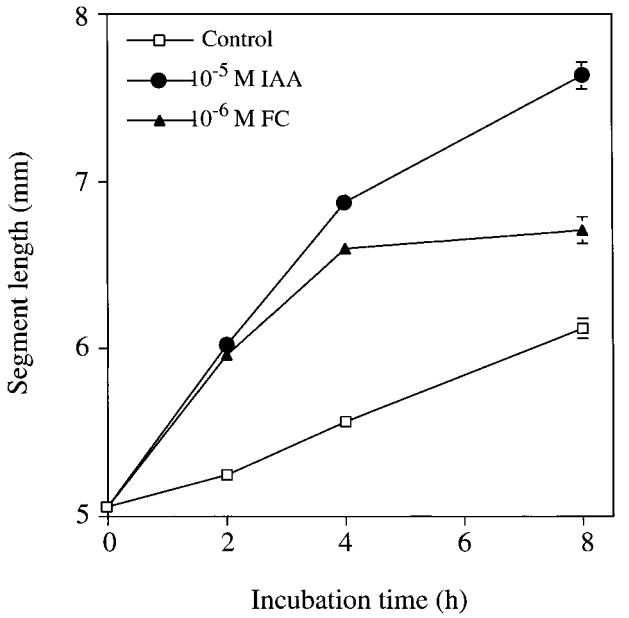

Fig. 4 Effects of IAA and FC on elongation growth of coleoptile segments of etiolated barley seedlings. Coleoptile segments (5 mm in length) were excised from 5–10 mm region from the tip with first leaves removed. The segments were treated with 10 μM IAA or 1 μM FC in 10 mM citrate-phosphate-potassium buffer (pH 6.6) in the dark up to 8 h. The segment lengths were measured with a binocular microscope. Means and standard errors are given (n=20). Means without error bar show that the errors are less than 0.1 mm.

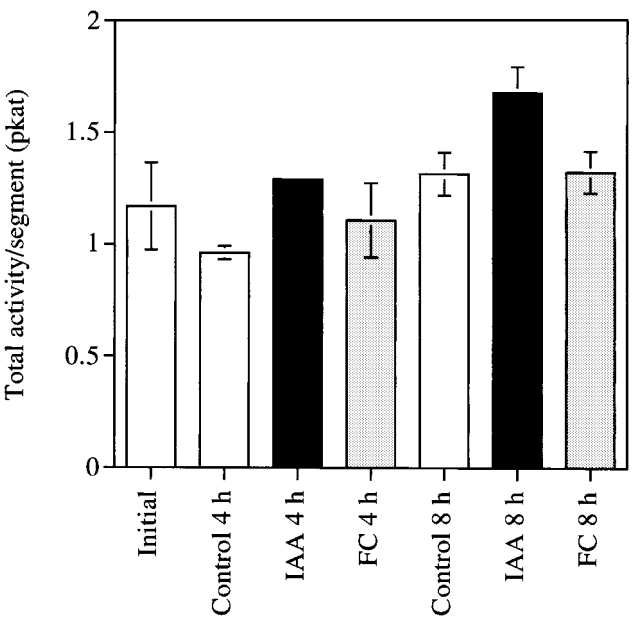

Fig. 5 Effects of IAA and FC on amount of wall-bound β-glucanase activity in coleoptile segments. Wall-bound glucanases were liberated as described in Materials and methods. Glucanase activity was measured using barley β-glucan as substrate. Means and standard errors are given (n=3).

volume with a minor peak eluted at Rt 20 min, indicating that the major 1,3:1,4-β-glucan was eluted around void volume. Treatment of IAA for 4 h shifted the glucose elution profile eluted around void volume to the lower molecular weight region as well as did FC. Weight-averaged molecular weight of the 1,3:1,4-β-glucan was 1,240 kDa for control, 710 kDa for IAA and 700 kDa for FC.

Discussion

Hemicellulosic polysaccharides in both monocotyledonous (Sakurai 1991) and dicotyledonous plants (Nishitani and Masuda 1981) undergo the decreases in amount and molecular weight in auxin-induced elongation growth. While hundreds genes related to cell wall metabolism have been cloned from higher plants, there was few report about the induction of the gene expression by auxin. Wu et al. (1996) reported that a putative endo-1,4-β-glucanase gene, EGL1 was auxin-inducible in pea epicotyl. They revealed that the amount of EGL1 mRNA was increased by treatment of synthetic auxin 2,4-D in 5 h. Catala et al. (1997) also found that gene expression of a putative endo-1,4-β-glucanase, Cel7 from tomato was stimulated by 2,4-D in 12 h. They reported that tomato Cel7 was an ortholog of pea EGL1. However, the functions and substrate specificities of transcripts of both pea EGL1 and tomato Cel7 remained to be determined. In the present study, gene expressions of biochemically characterized endo-1,3:1,4-β-glucanase EI (Woodward and Fincher 1982) and exo-β-glucanase ExoII (Hrmova et al. 1996, Kotake et al. 1997) were examined in barley coleoptiles in the presence of IAA. Induction of EI by IAA may, in part, explain the contribution of EI to the degradation of 1,3:1,4-β-glucan and to the elongation growth of barley coleoptiles. EI expression was detected only after 4 h of IAA treatment, whereas the elongation growth starts within 15 min. Therefore there might be another mechanism for the stimulation of glucanase activity in the early phase of the IAA-induced elongation growth of barley coleoptiles.

One can assume that the degradation of 1,3:1,4-β-glucan in vivo is rather controlled by the apoplastic pH regulated by IAA. Indeed, activity of exo-β-glucanase ExoII at pH 5.0 was approximately twice that at pH 6.5 (Kotake et al. 1997), and endo-1,3:1,4-β-glucanase EI has the maximum activity at pH 4.7 and lost 90% of the activity at pH 6.2 (Woodward and Fincher 1982). Molecular shift of 1,3:1,4-β-glucan caused by FC supports the above idea. However, FC did not promote EI expression, indicating that EI is not induced non-specifically in elongating coleoptiles and suggesting that EI is not induced by cell wall acidification caused by FC. The activation of wall metabolizing enzymes by proton extrusion triggered by auxin might occur in elongation growth of not only cereal but also dicotyledonous plants.

The contribution of EI to degradation of 1,3:1,4-β-glucan in intact growing coleoptiles might be small, because the level of EI mRNA was so low as not to be detected by Northern

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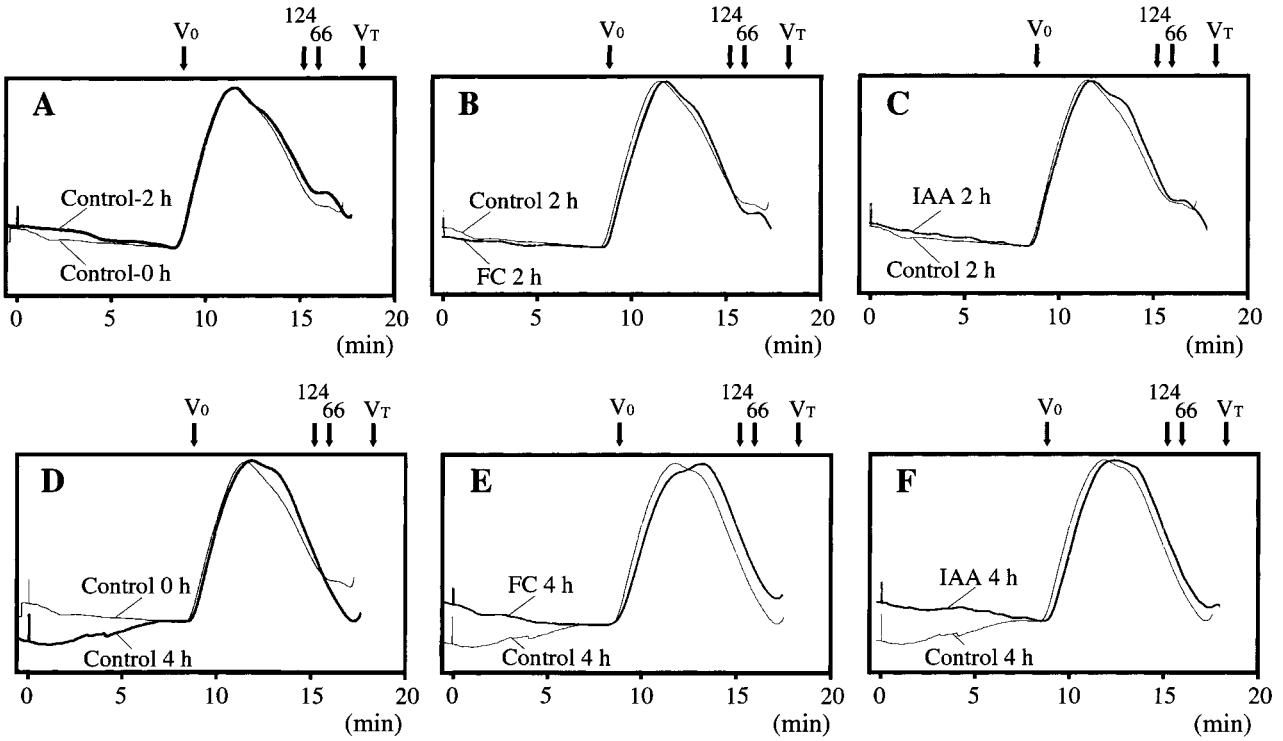

Fig. 6 Effects of IAA and FC on distribution of molecular weight of hemicellulose B in etiolated barley coleoptiles. Coleoptile segments from 5–10 mm regions were treated with 10^{-5} M IAA or 10^{-6} M FC in 10 mM citrate-phosphate-potassium buffer (pH 6.6) in the dark for 2 and 4 h. The extracted hemicellulose B was applied to a gel filtration column and detected with a refractive index detector. Dextrans of 124 and 66 kDa were used as molecular mass markers. A, initial (0 h) and control (2 h); B, FC and control at 2 h; C, IAA and control at 2 h; D, initial (0 h) and control (4 h); E, FC and control at 4 h; F, IAA and control at 4 h.

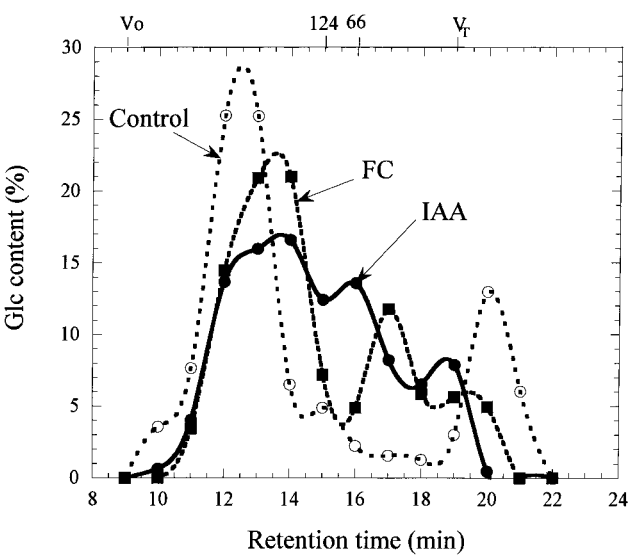

Fig. 7 Elution profile of glucose of hemicellulose B component. Hemicellulose B was extracted from coleoptile segments treated with or without IAA or FC for 4 h. Sample was eluted through a gel filtration column and fractionated polysaccharides were subjected to monosaccharide composition analysis. Dextrans of 124 and 66 kDa were used as molecular mass markers.

analysis. However, molecular weight of hemicellulosic polysaccharide extracted from a dwarf strain of barley that contains a low level of IAA, was higher than that from the isogenic normal strain (Sakurai and Kuraishi 1984), suggesting that endogenous IAA contributes to lowering of molecular weight of the β-glucan. Kinetic studies on EI (Woodward and Fincher 1982) and ExoII (Hrmova and Fincher 1998) suggest that the endo-type EI is more effective for 1,3:1,4-β-glucan degradation than exo-type ExoII, because EI not only acts on 1,3:1,4-β-glucan in endo-manner and contributes to the decrease in viscosity of cell walls more than ExoII, but also possesses 11 times higher kcat of EoxII (119 for EI and 11 for ExoII). Moreover, the action of EI on 1,3:1,4-β-glucan in endo-manner increases the number of non-reducing terminal glucose that is the substrate for ExoII. The stimulation of EI activity via both acidification of apoplast and induction of the gene expression by IAA probably causes an effective hydrolysis of 1,3:1,4-β-glucan in IAA-induced elongation growth of barley coleoptiles.

Present results suggest that the apoplast acidification by auxin causes the degradation of 1,3:1,4-β-glucan. As described above, both EI and ExoII can be activated by acidification of cell walls. We propose that the early phase of auxin-induced growth involves activation of glucanases due to low apoplastic

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pH rather than induction of β-glucanase gene. Since the activation of H^+-ATPase and following H^+ extrusion to cell walls are immediately caused by auxin, the stimulation of glucanase activities by the acidification can contribute to the early phase of auxin-induced growth. It is likely that the enzyme-linked growth is mediated by both the specific gene expression of a cell-wall degrading enzyme and the H^+ extrusion by H^+-ATPase. The effect of the acidification on the growth may continue in 4 h and thereafter the growth is sustained by the stimulation of gene expression of EI by IAA.

Another hypothesis of stimulation of glucanase activity has been proposed, i.e., a putative cell wall protein increases the exo- and endo-β-glucanase activity in the cell walls (Inouhe and Nevins 1997). Moreover expansin may be involved in the stimulation of glucanase activity, because it enhanced breakdown of hydrogen bonds between cellulose and other polysaccharides (Cosgrove 1999), resulting in an increase in opportunity for the above glucanases to digest 1,3:1,4-β-glucan. Contribution of EI and ExoII to elongation growth of barley coleoptiles should be evaluated in transgenic plants.

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