Short Communication

Changes in Levels of mRNAs for Cell Wall-Related Enzymes in Growing Cotton Fiber Cells 1

Yoshinori Shimizu2, Satoshi Aotsuka3, Osamu Hasegawa3, Toshinari Kawada4, Tomoyasu Sakuno4, Fukumi Sakai2 and Takahisa Hayashi2,5

1 Wood Research Institute, Kyoto University, Gokasho, Uji, Kyoto, 611 Japan
2 Tokyo Research Center, Nishinbo Industries, Inc., Adachi-ku, Tokyo, 123 Japan
3 Faculty of Agriculture, Tottori University, Koyama-cho, Tottori, 680 Japan

mRNAs for cell wall-related enzymes in developing cotton fiber cells were measured by reverse transcription-PCR analysis. Both endo-1,4-β-glucanase and expansin mRNA levels were high during cell elongation but decreased when cell elongation ceased, and xyloglucan decreased. The endo-1,3-β-glucanase mRNA level was very low in the elongating cells but increased gradually at the onset of secondary wall synthesis, accompanying the massive deposition of cellulose. Endoxyloglucan transferase and sucrose synthase mRNA levels were constant during all stages of growth.

Key words: Cotton fibers — Endo-1,3-β-glucanase — Endo-1,4-β-glucanase — Endoxyloglucan transferase — Expansin — Sucrose synthase.

Synchronous cell growth occurs in cotton fibers during ovule development; the epidermal cells (10 to 20 μm long) of the seed elongate about 2.5 to 3 cm during the primary wall synthesis and a high rate of cellulose synthesis starts at the onset of secondary wall synthesis. The transition of the primary to the secondary wall synthesis occurs between 18 and 21 days post anthesis. The change of cell development may involve changes in the level of expression of genes coding for proteins (cell wall-related enzymes) required for cell wall loosening and cellulose synthesis. In fact, there are marked changes in the pattern of translatable mRNAs associated with the transition from primary to secondary wall synthesis (Delmer et al. 1985). Therefore, regulation between cell elongation and cellulose deposition during development may occur at many levels with the increase and decrease in protein (enzyme) levels.

Random sequencing of 1,000 clones from the cDNA library of the fiber cells revealed 173 clones with sequences found in the GeneBank database. The clones identified contain several cDNAs for cell wall-related enzymes, endo-1,4-β-glucanase, expansin, endoxyloglucan transferase, endo-1,3-β-glucanase and sucrose synthase (Hasegawa et al. 1994). These enzymes have a major role in cell wall loosening during cell elongation and are closely associated with the massive synthesis of cellulose. The endo-1,4-β-glucanase probably belongs to family E, to which the poplar endo-1,4-β-glucanase belongs (Nakamura et al. 1995), and its activity may be responsible for solubilization of xyloglucan in the primary wall, which may be associated with the auxin-induced cell elongation in plants (Hayashi 1989). Expansin has the highly conserved region in amino acid sequence (Shcherban et al. 1995). It may function to loosen the wall via an unusual nonhydrolytic mechanism that disrupts hydrogen bondings not only between cellulose microfibrils potentially but also between xyloglucan and cellulose (McQueen-Mason and Cosgrove 1994). The endoxyloglucan transferase belongs to the EXT group of xyloglucan-related proteins on the dendrogram based on the UPGMA method (Nishitani 1995). Endoxyloglucan transferase may participate in the reconstruction of xyloglucan-cellulose networks in the walls of growing plant cells. The endo-1,3-β-glucanase exhibits 58% identity at the DNA level to barley endo-1,3-β-glucanase exon. 1,3-β-Glucan in cotton fibers occurs at the maximum level just before the massive synthesis of secondary wall cellulose (Jaquet et al. 1982). Meier et al. (1981) showed the transformation of the radioactivity of 1,3-β-glucan to cellulose, suggesting the transglucosylation of 1,3-β-glucan to 1,4-β-glucan by endo- and exo-1,3-β-glucanases in the walls of cotton fibers. Sucrose synthase might serve as an UDP-glucose synthase like cellulose and callose synthases to supply glycosyl residues from sucrose in the plasma membrane (Amor et al. 1995).

Herein, we measured the levels of mRNAs for endo-

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2 To whom all correspondence should be addressed: e-mail, taka@kuwri.kyoto-u.ac.jp
3 The nucleotide sequences reported in this paper have been submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession numbers: D88412 for sucrose synthase, D88413 for endoxyloglucan transferase, D88414 for actin, D88415 for expansin, D88416 for endo-1,3-β-glucanase and D88417 for endo-1,4-β-glucanase.
4 Faculty of Agriculture, Tottori University, Koyama-cho, Tottori, 680 Japan
mRNAs in cotton fibers

1,4-β-glucanase, expansin, endo-1,3-β-glucanase, endoxylanoglan transferase and sucrose synthase in the growing cotton fiber cells by using reverse transcription-PCR analysis (Hentzen et al. 1996) with mRNA for actin as a control.

Materials and methods—Cotton (Gossypium hirsutum L.) was grown in a growth chamber. Ovules were harvested into liquid nitrogen at different developmental stages. Xyloglucan was measured in the 24% KOH extracts of ovules by the method of Kooiman (1960) and cellulose by the method of Updegraff (1969). Total RNA was isolated from cotton fibers according to the method of Hall et al. (1978) and poly(A)+RNA was isolated from total RNA with an oligo-dT cellulose.

A cDNA library was constructed in the plasmid vector Bluescript by the Lambda Uni-Zap vector (Stratagene, La Jolla, CA, U.S.A.) using cDNA with poly(A)+RNA of cotton fibers harvested at 18 to 24 day as the template. One thousand clones were randomly selected from the cDNA library, and the plasmid DNA inserts were sequenced using an Applied Biosystems model 373A DNA sequencer (Hasegawa et al. 1994).

For reverse transcription-PCR analysis, first-strand cDNA was synthesized using 5 µg of total RNA at 42°C for 2 h using oligo(dT) (n=20) and SuperScript (SuperScript Preamplification System from Gibco BRL). PCR was performed with final volumes of 25 µl containing 0.6 unit of AmpliTaq (Perkin-Elmer), 50 mM dNTPs, 0.4 mM MgCl2 and 10 pM gene-specific primers with first-strand cDNA. The gene-specific primers were designed to unique regions (124 to 343 bp) of each cDNA as summarized in Table 1. Primer specificity was tested by amplifying 1 ng each of the cDNA inserted in pBK-CMV with a set of primers for 20 cycles. Since the amplification of DNA was not observed in the PCR reaction containing 5 µg of total RNA instead of cDNA, there was no contaminating genomic DNA in the total RNA preparation. The PCR reaction was initially denatured at 94°C for 5 min and in the subsequent cycles at 94°C for 30 s, annealing cycles were 30 s long at adjusted temperature (Table 1), and elongation cycles were 30 s long at 72°C. PCR products were size-separated by electrophoresis in a 1.5% agarose gel and blotted to nylon membranes (Hybond-N from Amersham). Membranes were hybridized in 5 × SSC, 1.0% blocking reagent, 0.1% lauroylsarcosine and 0.02% SDS at 65°C to digoxigenin-dUTP-labeled pro-

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Table 1. PCR amplification of cDNAs

<table>
<thead>
<tr>
<th>Template</th>
<th>Primer</th>
<th>PCR</th>
<th>Length of amplified DNA (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endo-1,4-β-glucanase</td>
<td>forward: 5'-TTCGAGAAGTACCCTCAGAAGGC-3' reverse: 5'-GGTGAACCTGGCTGGATATG-3'</td>
<td>55, 25, 27, 30, 35</td>
<td>330</td>
</tr>
<tr>
<td>Expansin</td>
<td>forward: 5'-AGTCTGACATACCCATGAGCG-3' reverse: 5'-CCCAATTGCGGACATAGCTGC-3'</td>
<td>57, 14, 15, 17, 20</td>
<td>329</td>
</tr>
<tr>
<td>Endoxylanoglan transferase</td>
<td>forward: 5'-GATATTTCATGGTTTGATGTACCCTTTCT-3' reverse: 5'-GATATTGGTTGGAAAGCTGTAATGGC-3'</td>
<td>55, 14, 15, 16, 17, 20</td>
<td>343</td>
</tr>
<tr>
<td>Endo-1,3-β-glucanase</td>
<td>forward: 5'-ATATGGGTATTTAAAATCCAGCAGATG-3' reverse: 5'-AGGGACGATGTTGGTTGAAACCGT-3'</td>
<td>55, 15, 17, 20</td>
<td>305</td>
</tr>
<tr>
<td>Sucrose synthase</td>
<td>forward: 5'-ATATGCGGTTTAAAATCCAGCAGATG-3' reverse: 5'-AGGGACGATGTTGGTTGAAACCGT-3'</td>
<td>50, 28, 30, 35</td>
<td>272</td>
</tr>
<tr>
<td>Actin</td>
<td>forward: 5'-GATATGGACTTCAAGCAGCGAAGG-3' reverse: 5'-GGAGAATGAAACCGCATTGGCAG-3'</td>
<td>61, 14, 15, 16, 17, 20, 25, 27, 28, 30, 35</td>
<td>124</td>
</tr>
</tbody>
</table>
bes (DIG DNA Labeling kit from Boehringer Mannheim) which were synthesized from the first-strand cDNAs by gene-specific primers. Following hybridization, the membranes were washed in 2× SSC for 5 min at room temperature and then two times in 0.1× SSC, 0.5% SDS at 68°C for 15 min. The washed membranes were developed with an immunostaining system using a DIG DNA Detection kit (Boehringer Mannheim).

**Results and discussion**—We measured the levels of mRNAs for cell wall-related enzymes in the growing cotton fiber cells by reverse transcription-PCR analysis. The amounts of first strand cDNA in the PCR reaction mixture were adjusted by the levels of amplified DNA for actin. The levels were estimated from the linear portion of the amplifying curve. Each PCR reaction contained a certain amount of first strand cDNA equivalent to 16 to 75 ng of total RNA, derived from 2 to 75 fiber cells. This suggests that the reverse transcription-PCR analysis of mRNA is a highly sensitive assay for small amounts of differentiated cells (tissues) as well as low copy number RNA species (Chelly et al. 1988).

We have also measured the amounts of xyloglucan and cellulose in relation to cell length in growing cotton fiber cells. As shown in Fig. 1, the increase in amount of xyloglucan is due to the primary wall synthesis (until 18 days post-anthesis) and the massive deposition of cellulose (after 21 days post-anthesis) is due to the secondary wall synthesis. The transition of the primary to the secondary wall synthesis occurs between 18 and 21 days post-anthesis. The rate of cell elongation was correlated with the increase in xyloglucan (Fig. 1). When the rate of cell elongation decreased, the amount of xyloglucan was decreased gradually and, finally, became one fifth.

Reverse transcription-PCR analysis of mRNAs for cell wall-related enzymes during growth is summarized in Fig. 2 under the conditions as described in Table 1. Signals for endo-1,4-β-glucanase appeared at 25 cycles of DNA amplification at the stages of cell elongation (9 to 15 days post-anthesis) and were clearly visible at 27 and 30 cycles during the primary wall synthesis (Fig. 3A). There were still distinguishably different intensities of signals between the primary and secondary wall syntheses even at 30 cycles but constant signal intensities were observed at 35 cycles at all stages of cell growth. Signals for expansin were strong even at 15 cycles during the primary wall synthesis (Fig. 3B), but no difference was observed at 20 cycles. Signals for endoxylanase transferase appeared to be just visualized constant at 15 cycles during both the primary and secondary

![Fig. 2 Reverse transcription-PCR Southern blot analysis of mRNAs for cell wall-related enzymes during growth.](image)

![Fig. 3 Relative amounts of mRNAs for cell wall-related enzymes during growth by reverse transcription-PCR analysis at various cycles. Endo-1,4-β-glucanase (A): 25 (●), 27 (○) and 30 (△) cycles; expansin (B): 15 (●), 17 (○) and 20 (△); endoxylanase transferase (C): 15 (●), 17 (○) and 20 (△); endo-1,3-β-glucanase (D): 15 (○), 17 (●) and 20 (△); sucrose synthase (E): 28 (○), 30 (●) and 35 (△) cycles. Quantities of reverse transcription-PCR Southern blots were measured using an Imaging Plate Scanner JX-250 (Sharp, Tokyo, Japan) with a NIH image.](image)
wall syntheses and showed constant signal intensities at any cycle during growth (Fig. 3C). These findings suggest that the levels of mRNAs for both endo-1,4-β-glucanase and expansin are high during cell elongation (6 to 15 days post-anthesis) and decrease gradually when cell elongation ceased. The two mRNAs also decreased with the decrease in xylanase in the cell wall of cotton fibers. Signals for endo-1,3-β-glucanase appeared at 15 cycles at the secondary wall synthesis. The level of mRNA for endo-1,3-β-glucanase was very low in the elongating cells but increased gradually at the onset of secondary wall synthesis (Fig. 3D), accompanying the massive deposition of cellulose (Fig. 1). This is in agreement with an earlier proposal (Meier et al. 1981) that a high level of endo-1,3-β-glucanase activity is required during massive deposition of cellulose in cotton fibers, although direct evidence that 1,3-β-glucan is an intermediate in biosynthesis of cellulose is lacking. Signals for sucrose synthase appeared at 30 cycles at all stages of cell growth (Fig. 3E). Since the level was not increased during the secondary wall synthesis, the sucrose synthase may not be a membrane-bound form which is associated with cellulose synthase (Amor et al. 1995).

Among the mRNAs for cell wall-related enzymes, those for expansin and endo-xylanase transferase were present at high levels. In addition, endo-xylanase transferase mRNA level was constant at all stages of growth. This suggests that the gene for endo-xylanase transferase is always expressed to maintain cell wall architecture, where its translate could function for cross-linking of the wall layers during the process of cell growth. On the other hand, the endo-1,4-β-glucanase mRNA level changed at relatively low copy numbers during cell growth. It is uncertain whether the expression level for endo-1,4-β-glucanase is relatively low or its mRNA is unstable in cotton fiber cells during cell growth.

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References


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