J Wood Sci (2002) 48:425–428

NOTE

Yuri Ihara · Takumi Takeda · Fukumi Sakai
Takahisa Hayashi

Transferase activity of GhCesA2 (putative cotton cellulose 4-β-glucosyltransferase) expressed in *Pichia pastoris*

Received: August 22, 2001 / Accepted: November 28, 2001

Abstract *GhCesA2* is a cotton (*Gossypium hirsutum*) homologue of the bacterial cellulose synthase gene that encodes cellulose 4-β-glucosyltransferase. The central catalytic region of GhCesA2 was expressed as a soluble protein in the methylotrophic yeast *Pichia pastoris*. The molecular size of the recombinant protein was 100 kDa, which decreased to 85 kDa after treatment with endoglycosidase H. The recombinant GhCesA2 catalyzed transfer of glucose from UDP-glucose into unknown products in the presence of an extract of cotton hypocotyls, but the products were not β-1,4-glucan.

Key words GhCesA2 · Gene expression · *Pichia pastoris*

Introduction

Based on electron microscopy observations, cellulose is probably synthesized at a rosette-like structure on the plasma membrane that may be a large complex of cellulose synthase.[1] Cellulose synthase genes were first identified from *Acetobacter xylinum*[2] as an operon encoding four subunit proteins, one of which (bcsA) was a 4-β-glucosyltransferase.[3] The plant homologues of *bcsA* were isolated from cotton (*Gossypium hirsutum*) fiber cells and named cotton *celA1* and *celA2*[4] (*pcsA2*[5]), which were renamed *GhCesA1* and *GhCesA2* recently.[6] These GhCesA gene products are membrane-bound proteins and contain transmembrane helices at the N- and C-termini. The central cytosol region of the GhCesA contains conserved residues for β-glycosyltransferases[7] (D, D, D, QxxRW) and is capable of binding UDP-glucose, the predicted substrate for cellulose synthesis.[4] When treated with herbicide CGA 325′615, which inhibits plant cell wall synthesis, cotton fiber cells accumulated noncrystalline β-1,4-glucan associated with GhCesA protein, suggesting that GhCesA is involved in the synthesis of β-1,4-glucan.[8] In addition, mutation of the *AtCesA* gene (*Arabidopsis thaliana* homologue of *GhCesA*) caused cell wall abnormalities in vivo, such as radial swelling (*rsw1*[9]) and irregular xylem (*irx3*[10] and *irx1*[11]) in *Arabidopsis* mutants. The mutation also caused a decreased crystalline cellulose content in *rsw1*, *irx*,[12] and *prc1* (*procuste1*).[13] Despite all indirect evidence supporting the role for CesA protein in cellulose biosynthesis, there is no distinct evidence to show that CesA catalyzes the transfer of glucose from UDP-glucose to β-1,4-glucan. In this study, *GhCesA2* was expressed in the methylotrophic yeast *Pichia pastoris*, and the transferase activity of the recombinant GhCesA2 was tested.

Materials and methods

Construction of recombinant plasmid vector

The DNA fragment encoding α-factor was amplified with *Eco*R1 site at the 5′ end by the polymerase chain reaction (PCR) using the pPIC9 vector (Invitrogen) as a template. The amplified fragment was digested with *Eco*R1 and *Sna*B1 and then inserted into the same sites of the pPICZ C vector (Invitrogen). The resultant plasmid vector was linearized with digestion of *Sna*B1 and added ddTTP at the 3′ ends with terminal deoxynucleotidyl transferase. A 1.6-kb DNA fragment of *GhCesA2* was amplified by PCR using the plasmid *GhCesA2*/pGEM as a template and then inserted into the above T-vector derived from pPICZ C. The PCR reaction was carried out using primers (forward: 5′-GGTTCCCTATTACTCGCG-3′; reverse: 5′-GCAAGCC TCTCGAGCCA-3′) with initial denaturation at 95°C for 30 s followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and elongation at 68°C for 100 s.

Y. Ihara · T. Takeda · F. Sakai · T. Hayashi (✉)
Wood Research Institute, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan
Tel. +81-774-38-3618; Fax +81-774-38-3600
e-mail: taka@kuwri.kyoto-u.ac.jp

Expression of GhCesA2

Pichia pastoris GS115 cells were transformed with the recombinant plasmid vector by electroporation. Transformed cells were screened on YPDS plates (1% yeast extract, 2% peptone, 2% glucose, 1M sorbitol, 2% agar) containing Zeocin (Invitrogen) 500μg/ml. Single colonies were cultured in 10ml of BMGY medium (100mM potassium phosphate pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol, and 4×10^{-3}% histidine) at 30°C for 3 days. Cells were centrifuged and resuspended in 3ml of BMMY medium (100mM potassium phosphate pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol, and 4×10^{-3}% histidine) and incubated at 30°C for 3 days with addition of 0.5% methanol for each 24h. The cells were centrifuged at 3000rpm for 10min and lysed by sonication for 20s in 10mM sodium phosphate buffer (pH 7.4) containing 1mM EDTA and 5% glycerol. The cell lysate was used as an enzyme preparation after centrifugation.

Western blotting

The transformed cells and cell lysate were suspended in sample buffer: (50mM Tris HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), mercaptoethanol 60ml/ml, glycerol 0.1ml/ml, and bromophenol blue 0.1mg/ml). This then was heated for 3min at 100°C, after which the samples were applied to protein gel electrophoresis using 4% (stacking gel) and 10% (running gel) polyacrylamide mini-gel in the presence of 0.1% SDS. Following electrophoresis, the proteins were silver-stained or transferred to a PVDF membrane (NEN Life Science Products) using a semidry transblot apparatus (Bio Craft). The membrane was blocked overnight with phosphate-buffered saline (PBS) containing 0.5% Triton X-100 (PBST) and 5% nonfat milk at 4°C and then treated with mouse anti-myc monoclonal antibody (Bio Mol) diluted 1:1000 in PBST for 1h at room temperature. The membrane was washed with PBST and incubated with horseradish peroxidase-conjugated anti-mouse antibody diluted 1:1000 in PBST for 30min at room temperature. The antibody-bound proteins were visualized by a Western blotting detection kit, ECL (Amersham Life Science).

Preparation of lipids and soluble and membrane materials from cotton hypocotyls

The cotton lipids were extracted as follows: young hypocotyls were ground, mixed with 7 volumes of chloroform/methanol (1:1), and vortexed well. After incubation at −30°C for 48h, the mixture was filtrated through a glass filter and evaporated twice. The residue was solubilized with 100% methanol and used as the lipid material. The soluble and membrane materials were isolated as follows: young hypocotyls were ground in 10mM HEPES/KOH buffer (pH 7.4) and centrifuged at 48000g for 60min. The supernatant was used as the soluble material, and the precipitate was solubilized with the same buffer containing 0.5% CHAPS. The solution was centrifuged at 48000g, and the supernatant was used as the membrane material.

Characterization of reaction products

The reaction mixture (100μl) containing an enzyme preparation, 1mM UDP-[^{14}C]glucose (0.25mCi/mmol), 2mM $MnSO_4$, 10mM $MgCl_2$, 1mM cellopentaose, 5mM cellobiose, and cotton cell extract were incubated at 25°C for 20min. The reactions were routinely terminated by passage through a Dowex 1 × 8 (Cl form) column (0.5 × 1.0cm). The column was washed two times with 10ml of water, and the eluate was obtained as the soluble fraction. The reactions were terminated by addition of 1ml of 70% ethanol containing 0.5mM EDTA. The mixture was left for at least 30min and filtered over Whatman GF/C glass fiber filters. The filters were washed three times in 70% ethanol and counted with a liquid scintillation counter as the 70% ethanol-insoluble fractions.

Results and discussion

We had cloned the full length of *GhCesA2* as a homologue of bacterial cellulose synthase gene from the cDNAs of cotton fiber cells, previously.[5] GhCesA2 is a putative cellulose 4-β-glucosyltransferase that may contain three transmembrane helices at the N-terminus and six at the C-terminus. The central cytosol region of GhCesA2 contains a putative catalytic site. To obtain the soluble form of the protein, the partial sequences (Trp^{267} to Tyr^{812}) between the third and fourth transmembrane helices were expressed in the methylotrophic yeast *Pichia pastoris*. On the transformation of *P. pastoris GS115*, we used YPDS plates containing a high concentration (500μg/ml) of Zeocin to select the transformants where multiple gene insertion had occurred. Sixteen colonies were obtained in the presence of Zeocin, and one that grew faster than the others was employed for production of the central region of GhCesA2.

Figure 1 shows the results from Western blotting of GhCesA2 expressed in *P. pastoris*. Although the α-factor was fused at the N-terminal region to produce the recombinant protein as a secretory form in the medium, the protein was not detected in the medium. After mild sonication of the cells for a short period (10s × 2), however, the gene product appeared in the cell lysate. This could be explained by the fact that the protein accumulated in the periplasm between the plasma membrane and cell wall was extracted by mild sonication. The molecular size of the recombinant protein was 100kDa, which was far larger than the size calculated from cDNA sequences (65kDa). When the extracted proteins were treated with endoglycosidase H, the immune signal shifted from 100kDa to 85kDa (Fig. 2). This result showed that the recombinant protein had been *N*-glycosylated because endoglycosidase H cleaves the 1,4-β-chitobiosyl linkage between the high mannosyl moiety and the aspartic acid residue of protein. The central region of

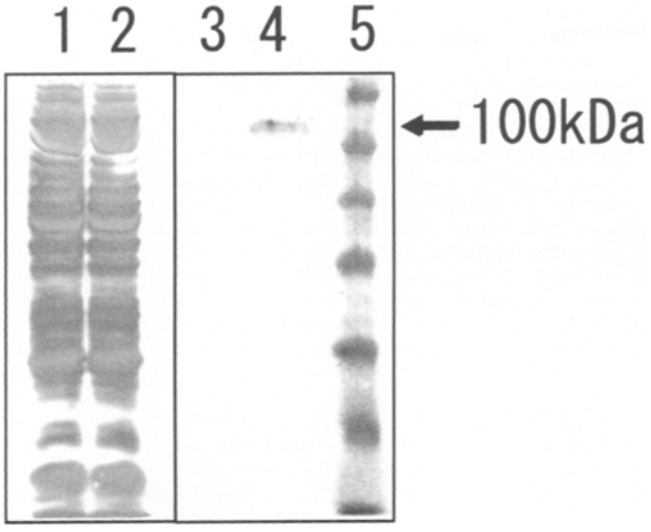

Fig. 1. Expression of GhCesA2 in *Pichia pastoris*. The proteins were visualized by silver staining (lanes *1* and *2*) and by Western blotting with anti-c-myc antibody (lanes *3* and *4*). Lane *1*, wild-type cell; lane *2*, transformant; lane *3*, cultured medium of transformant; lane *4*, cell lysate; lane *5*, molecular markers (MBP-β-galactosidase 175 kDa; MBP-paramyosin 83 kDa; glutamic dehydrogenase 62 kDa; aldolase 47.5 kDa; triosephosphate isomerase 32.5 kDa; β-lactoglobulin A 25 kDa; lysozyme 16.5 kDa)

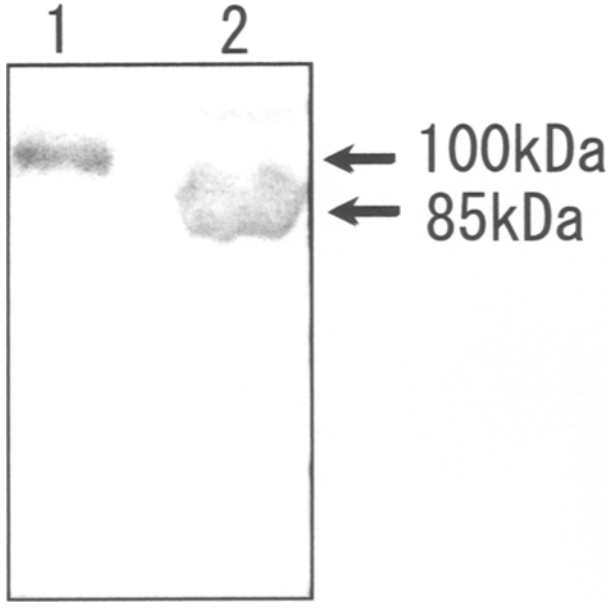

Fig. 2. Treatment of GhCesA2 with endoglycosidase H. Lane *1*, cell lysate of transformant; lane *2*, cell lysate after treatment with 500 units of endoglycosidase H

GhCesA2 contains three putative *N*-glycosylation sites, such as Asn^{508}, Asn^{703}, and Asn^{777}. Because the size of the deglycosylated protein was still larger than the expected size, another type of glycosylation (*O*-linked)[14] or modification of the recombinant protein might occur.[15]

GhCesA2 is believed to be cellulose synthase, so incorporation of glucose from UDP-glucose into β-1,4-glucan was measured with the recombinant protein. Because cellulose synthase is believed to form a large complex in the plasma membrane,[1] transfer of glucose may require other components such as proteins and lipids. Thus, the crude extracts from cotton young cells fractionated into cytosol (soluble), lipid, and membrane extracts were added to the reaction mixture. The reaction product was fractionated into and soluble 70% ethanol-insoluble fractions, and the radioactivity was measured (Table 1). The two fractions indicated incorporation into low-molecular-weight product (soluble) and polymer product (70% ethanol-insoluble), respectively.

Table 1. Incorporation of glucose from UDP-glucose

Fraction	Incorporation (nmol Glc/min/mg protein)	
	Wild-type	Transformant
Soluble fraction		
No additive	35.6 ± 6.4	36.0 ± 3.9
+Lipid[a]	29.7 ± 3.5	47.9 ± 3.4
+Membrane[a]	32.9 ± 0.1	38.5 ± 1.6
+Soluble[a]	36.1 ± 1.6	34.0 ± 2.0
+Lipid + membrane[a]	32.2 ± 4.0	34.9 ± 3.1
+Lipid + soluble[a]	30.8 ± 1.4	34.6 ± 2.0
70% Ethanol-insoluble fraction		
No additive	1014.4 ± 205.1	294.1 ± 47.7
+Lipid[a]	1153.4 ± 84.1	101.0 ± 12.7
+Membrane[a]	50.0 ± 13.3	80.5 ± 1.4
+Soluble[a]	360.5 ± 16.1	177.6 ± 4.5
+Lipid + membrane[a]	279.5 ± 12.4	548.5 ± 21.4
+Lipid + soluble[a]	390.6 ± 1.8	1035.1 ± 46.5

Each measurement was repeated three times and the means ± SD are shown

[a] Lipid, membrane, and soluble materials were prepared from cotton young hypocotyls

In the absence of cotton extract, more incorporation into the 70% ethanol-insoluble fraction was observed with the wild-type cells than with the transformant. This was probably because at the presence of the recombinant protein reduced the relative content of endogenous yeast glycosyltransferases, or recombinant protein inhibited the activities of yeast protein. With the wild-type cells in the 70% ethanol-insoluble fraction, the amount of incorporation was decreased to 5%–40% in the presence of cotton extract except lipid. This might be due to the inhibition by cotton extract except lipid. With the transformant, addition of cotton lipid lowered the incorporation of glucose into the 70% ethanol-insoluble fraction, which might be related to the higher incorporation into the soluble fraction (+lipid in Table 1). In the presence of two cotton fractions, the amount of incorporation with the transformant increased two- to threefold over that seen without additive in the 70% ethanol-insoluble fraction (+lipid+membrane and +lipid+soluble in Table 1). This shows that cotton lipid accelerates GhCesA2 to catalyze the transfer of glucose into the 70% ethanol-insoluble fraction (polymer product) in the presence of cotton proteins.

Thus, the recombinant protein could synthesize low-molecular-weight product and polymer product in the presence of cotton extract. However, hydrolysis of all products

by cellulase (*Trichoderma* sp.) yielded glucose but not cellooligosaccharides (data not shown). The higher incorporation of glucose with the recombinant GhCesA2 was not due to the synthesis of β-1,4-glucan. Nevertheless, it is possible that the product was an intermediate for cellulose synthesis or a substance hardly hydrolyzed with cellulase. Detailed analysis of the products is in progress.

Acknowledgment The authors thank Ms. Teruko Konishi for the gift of pPICZ C T-vector.

References

1. Mueller SC, Brown RM Jr (1980) The control of cellulose microfibril deposition in the cell wall of higher plants. J Cell Biol 84:315–326
2. Wong HC, Fear AL, Calhoon RD, Eichinger GH, Mayer R, Amikam D, Benziman M, Gelfand DH, Meade JH, Emerick AW, Bruner R, Ben-Basat BA, Tal R (1990) Genetic organization of the cellulose synthase operon in *Acetobacter xylinum*. Proc Natl Acad Sci USA 87:8130–8134
3. Saxena IM, Lin FC, Brown RM Jr (1990) Cloning and sequencing of the cellulose synthase catalytic subunit gene of *Acetobacter xylinum*. Plant Mol Biol 15:673–683
4. Pear JP, Kawagoe Y, Schreckengost WE, Delmer DP, Stalker DM (1996) Higher plants contain homologs of the bacterial *CelA* genes encoding the catalytic subunit of cellulose synthase. Proc Natl Acad Sci USA 93:12637–12642
5. Ihara Y, Sakai F, Hayashi T (1997) Cloning and sequencing of cotton homologs of *bcsA* gene encoding cellulose 4-β-glucosyltransferase. Wood Res 84:1–6
6. Delmer DP (1999) Cellulose biosynthesis: exciting times for a difficult field of study. Annu Rev Plant Physiol Plant Mol Biol 50:245–276
7. Saxena IM, Brown RM Jr (1997) Identification of cellulose synthase(s) in higher plants: sequence analysis of processive β-glycosyltransferases with the common motif "D, D, D35Q(R,Q)XRW." Cellulose 4:33–49
8. Peng L, Xiang F, Roberts E, Kawagoe Y, Greve LC, Kreuz K, Delmer DP (2001) The experimental herbicide CGA 325′615 inhibits synthesis of crystalline cellulose and causes accumulation of non-crystalline β-1,4-glucan associated with CesA protein. Plant Physiol 126:981–992
9. Arioli T, Peng L, Betzner AS, Burn J, Wittke W, Herth W, Camilleri C, Hofte H, Plazinski J, Birch R, Cork A, Glover J, Redmond J, Williamson RE (1998) Molecular analysis of cellulose biosynthesis in *Arabidopsis*. Science 279:717–720
10. Taylor NG, Scheible WR, Cutler S, Somerville CR, Turner SR (1999) The irregular xylem3 locus of *Arabidopsis* encodes a cellulose synthase required for secondary cell wall synthesis. Plant Cell 11:769–779
11. Taylor NG, Laurie S, Turner SR (2000) Multiple cellulose synthase catalytic subunits are required for cellulose synthesis in *Arabidopsis*. Plant Cell 12:2529–2539
12. Turner SR, Somerville CR (1997) Collapsed xylem phenotype of *Arabidopsis* identifies mutants deficient cellulose deposition in the secondary cell wall. Plant Cell 9:689–701
13. Fagard M, Desnos T, Desprez T, Goubet F, Refregier G, Mouille G, McCann M, Rayon C, Vernhettes S, Hofte H (2000) *PROCUSTE1* encodes a cellulose synthase required for normal cell elongation specifically in roots and dark-grown hypocotyls of *Arabidopsis*. Plant Cell 12:2409–2423
14. Bewley MC, Tam BM, Grewal J, He S, Shewry S, Murphy MEP, Mason AB, Woodworth RC, Baker EN, MacGillivray RTA (1999) X-ray crystallography and mass spectroscopy reveal that the N-lobe of human transferring expressed in *Pichia pastoris* is folded correctly but is glycosylated on Serin-32. Biochemistry 38:2535–2541
15. Gemmill TR, Trimble RB (1999) Overview of *N*- and *O*-linked oligosaccharide structures found in various yeast species. Biochim Biophys Acta 1426:227–237