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Enhancement of cellulose production by expression of sucrose synthase in *Acetobacter xylinum*

(cellulose synthesis/sucrose metabolism)

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ABSTRACT Higher plants efficiently conserve energy ATP in cellulose biosynthesis by expression of sucrose synthase, in which the high free energy between glucose and fructose in sucrose can be conserved and used for the synthesis of UDP-glucose. A mixture of sucrose synthase and bacterial cellulose synthase proceeded to form UDP-glucose from sucrose plus UDP and to synthesize 1,4-β-glucan from the sugar nucleotide. The mutant sucrose synthase, which mimics phosphorylated sucrose synthase, enhanced the reaction efficiency (V_{max}/K_m) on 1,4-β-glucan synthesis, in which the incorporation of glucose from sucrose was increased at low concentrations of UDP. Because UDP formed after glucosyl transfer can be directly recycled with sucrose synthase, UDP-glucose formed appears to show high turnover with cellulose synthase in the coupled reaction. The expression of sucrose synthase in *Acetobacter xylinum* not only changed sucrose metabolism but also enhanced cellulose production, in which UDP-glucose was efficiently formed from sucrose. Although the level of UDP-glucose in the transformant with mutant sucrose synthase cDNA was only 1.6-fold higher than that in plasmid-free cells, the level of UDP was markedly decreased in the transformant. The results show that sucrose synthase serves to channel carbon directly from sucrose to cellulose and recycles UDP, which prevents UDP build-up in cellulose biosynthesis.

Cellulose, the most abundant biopolymer on earth, occurs in plants and certain algae, fungi, and bacteria. Bacteria have a biosynthetic system different from the system in higher plants, in which sucrose and sucrose synthase participate in cellulose biosynthesis (1). Higher plants have two systems for the formation of UDP-glucose, one with sucrose synthase (EC 2.4.1.13) and the other with UDP-glucose pyrophosphorylase (EC 2.7.7.9), although bacteria contain only the latter. Sucrose synthase catalyzes the freely reversible reaction: UDP-glucose + fructose ⇌ sucrose + UDP. The amount of the enzyme is much higher in nonphotosynthetic tissues (2, 3), a major sink in plants where sucrose is the source of carbon that is translocated and cleaved by the enzyme to produce UDP-glucose for synthesis of cellulose. Therefore, the reaction of the enzyme proceeds to the cleavage of sucrose for the synthesis of UDP-glucose in the plant tissues (4). In developing cotton fibers, the sucrose synthase is localized in arrays in parallel with the helical pattern of cellulose deposition, participating in the biosynthesis of cellulose (1).

Mung bean (*Vigna radiata*, Wilczek) sucrose synthase is a tetramer composed of identical subunits of 95 kDa (5). A recombinant protein from *Escherichia coli* encoded by the mung bean gene conserves the activity to synthesize sucrose from UDP-glucose plus fructose but hardly catalyzes its reversible reaction (6). The recombinant enzyme was phosphorylated *in vitro* at Ser^{11} by a Ca^{2+}-dependent protein kinase from soybean root nodules (7), suggesting that Ser^{11} is the regulatory target residue in mung bean sucrose synthase (8). The apparent affinity for sucrose was increased in this phosphorylated enzyme and also in an engineered mutant in which Ser^{11} (S11E) was replaced by glutamic acid. This shows that the glutamic acid residue at position 11 mimics the phosphorylated Ser^{11} residue to bind and cleave sucrose for the synthesis of UDP-glucose. Therefore, the mutant enzyme would be expected to carry out enhanced formation of UDP-glucose from sucrose without any regulation such as phosphorylation.

Sucrose is potentially the most suitable carbon source for the production of cellulose in bacterial fermentation, not only because energy can be conserved in the formation of UDP-glucose with sucrose synthase, but also because this carbon source is economically attractive throughout the world. There are potential industrial applications for bacteria that produce cellulose during fermentation with sucrose as a carbon source (9). In *Acetobacter xylinum*, which has no sucrose synthase, there are at least four enzyme steps in the pathway from sucrose to UDP-glucose. In addition, *A. xylinum* strains show various pathways of UDP-glucose formation. For example, the level of UDP-glucose pyrophosphorylase activity in *A. xylinum* in strain ATCC23768 differs from that in ATCC23769, although cellulose production is similar (10). Herein, we demonstrate the direct synthesis of UDP-glucose from the carbon source sucrose during cellulose synthesis both *in vitro* and *in vivo*. We then find the major function of sucrose synthase, which is the prevention of UDP build-up in the cellulose biosynthesis of higher plants.

The 5′ upstream region (about 3.1 kb) of cellulose synthase operon (*bcs* operon) has been isolated by cloning from *A. xylinum* BPR 2001 (11). The expression level of the upstream region was determined by using sucrose synthase cDNA as a reporter gene in the shuttle vector pSA19. The expression occurred with the 1.1-kb upstream sequence from the ATG of *bcs* operon in *A. xylinum* G7 (12) and *Acetobacter aceti* 10–852 (13), although the upstream sequence did not cause any expression as a promoter in *E. coli* TB1 (New England Biolabs). This shows that the upstream region functions as a specific promoter for the *Acetobacter* genus (11). However, much more sucrose synthase was produced with the *lac* promoter than with the upstream region of *bcs* operon. Nevertheless, the production of recombinant protein with the ribosome-binding site of *lac* promoter was reduced to about half in *A. xylinum*. Therefore, for the expression of sucrose synthase, we used the *lac* promoter and the ribosome-binding site of *bcs* operon.

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MATERIALS AND METHODS

Bacterial Strains and Culture Conditions. *E. coli* BL21(DE3) was used as the host to produce wild-type and mutant recombinant sucrose synthases from mung bean by using the pET-21d expression vector under the control of a T7 promoter (Novagen). Transformants of strain BL21(DE3) containing various plasmids were cultivated in LB medium at pH 7.0 and 37°C in a shaker flask; ampicillin (50 μg/ml) was included when the bacteria carried plasmids conferring drug resistance. The cells of *A. xylinum* subsp. *sucrofermentans* BPR 2001 were grown at 30°C for 24 h in a shaking flask in a liquid medium (300 ml) containing 2% glucose, 0.5% yeast extract, 0.5% bactopeptone, 0.3% K_2HPO_4 (pH 6.0), and 0.1% cellulase preparation (14). *A. xylinum* subsp. *nonacetoxidans* LD-2 was cultured at 28°C for 72 h statically in a roux flask containing sucrose/corn steep liquor medium consisting of 4% sucrose and 4% corn steep liquor (15). This strain incorporates sucrose, which may be hydrolyzed by the activity of intracellular invertase. Its cell suspension was inoculated into the production culture to a concentration of 10% (vol/vol). A standard culture was used in 75 ml of the sucrose/corn steep liquor medium in a 300-ml flask and a small-scale culture with 2 ml of the medium containing [^{14}C-*glucose*]sucrose (26 μCi/mmol;1 Ci = 37 GBq; New England Nuclear) as a carbon source. The fermentation culture was done at 28°C in a baffled flask with rotary shaking at 180 rpm. Ampicillin (100 μg/ml) was included when the bacteria carried plasmids conferring drug resistance.

Plasmid Construction. The oligonucleotides used for cloning a sucrose synthase cDNA into the expression vector pET-21d (Novagen) are the following two forward primers: one is (nucleotides 41–60: GCTACCGATCGTTTGACCCG) for wild-type sucrose synthase, which is homologous to the coding strand, and the other is (nucleotides 41–83: GCTACCGATCGTTTGACCCGTGTTCACGAACTCCGTGAGAGGC) for mutant [Glu^{11}]sucrose synthase (the bases changed to achieve the Glu^{11} mutation are underlined), and reverse primer (nucleotides 2464–2489: TCTCGGTCGACAAGCCGGTTCCTCCTCCATTTCTTCATCC) was complementary to the coding strand, which had a *Sal*I recognition site. The oligonucleotides were used for amplification with PCR by using plasmid pM-SS-5 (5, 16) as a template, which contained sucrose synthase cDNA. The PCR product was digested with *Sal*I and cloned into pET-21d that had been digested with *Xho*I. After ligation, the products were used to transform *E. coli* BL21(DE3)-competent cells, and a minipreparation was used to isolate the recombinant plasmids pED-01 for wild-type sucrose synthase and pED-01-S11E for mutant sucrose synthase. The constructed plasmids contained full-length cDNAs encoding 805 amino acids.

To achieve the expression of sucrose synthase activity in *A. xylinum*, we introduced the wild-type and mutant sucrose synthase cDNAs into the shuttle vector pSA19 (17) as described above. The vectors were designed as pSA-SD and pSA-SD-S11E, respectively. The 5′ end of each cDNA was directed to lac promoter in the two plasmids. The plasmids contained full-length cDNAs for wild-type and mutant sucrose synthases with *lac* promoter. The alignments of the resulting plasmids were confirmed by sequencing the entire cDNA insert by using the primer-labeled dideoxynucleotide chain-termination method (18).

Isolation of Recombinant Sucrose Synthase. *E. coli* BL21(DE3) cells harboring each plasmid were grown in LB medium at 37°C in a shaker flask and the expression of the recombinant protein was induced by the isopropyl β-D-thiogalactoside at a final concentration of 0.3 mM. The bacterial cells harvested by centrifugation were suspended with 30 mM Tris·HCl (pH 7.5) containing 0.1 mM EDTA and 0.1 mM DTT and then disrupted by sonication. After centrifugation, the supernatant was adjusted to 65% saturation with ammonium sulfate and the precipitating proteins were collected and dissolved in the same buffer. The suspension was centrifuged and the resultant supernatant was applied to a Sepharose CL-6B column (2.5 × 30 cm). The active fractions (V_o) were pooled and further applied to a Superdex 200 column (2.6 × 60 cm) that had been equilibrated with the same buffer containing 0.1 M NaCl. The peak fractions were combined, dialyzed against the Tris buffer, and applied to a Mono Q column (0.5 × 5 cm) that had been equilibrated with the same buffer. The active fractions containing sucrose synthase activity were collected and pooled. The final preparation of each recombinant sucrose synthase appeared to be homogeneous at 95 kDa by SDS/PAGE. The specific activities of the purified wild-type and mutant enzymes were 1.46 and 1.35 units/mg of protein, respectively, for the formation of sucrose. One unit of sucrose synthase activity was defined as 1 μmol of sucrose formed per min at 30°C.

Assay for Sucrose Synthase. For determination of the incorporation of glucose from sucrose into UDP-glucose, the reaction mixture contained 40 mM [^{14}C]sucrose (2.5 mCi/mmol, Amersham), 10 mM UDP, 50 mM Tris·HCl (pH 7.5), and the enzyme preparation (usually 20–30 μg of protein) in a total volume of 20 μl. For determination of the incorporation of glucose from UDP-glucose into sucrose, the reaction mixture contained 3 mM UDP-[^{14}C]glucose (420 μCi/mmol, Amersham), 50 mM fructose, 50 mM Tris·HCl (pH 7.5), and the enzyme preparation in a total volume of 20 μl. The reaction was allowed to proceed for 30 min to form UDP-glucose and for 5 min to form sucrose, and the reaction mixture was immediately spotted on a 2-cm wide Whatman 3MM filter paper strip, followed by electrophoresis in 50 mM sodium tetraborate (pH 9.6) at 250 V for 3 h. After electrophoresis, the paper strips were dried and subjected to autoradiography. The area corresponding to UDP-glucose and sucrose on each strip was excised and neutralized, and its radioactivity was measured in 5 ml of a toluene scintillator with a Beckman LS-1800 liquid scintillation counter.

Preparation of Cellulose Synthase and Cyclic Diguanylic Acid from *A. xylinum*. The cultured cells of *A. xylinum* subsp. *sucrofermentans* BPR 2001 were washed three times in TME buffer containing 50 mM Tris·HCl (pH 7.0), 10 mM $MgCl_2$, and 1 mM EDTA by centrifugation and suspended in the buffer containing 20% PEG 4000. They were disrupted in Parr Bomb with nitrogen gas at 1,800 psi (1 psi = 6.89 kPa). The suspension was centrifuged at 12,000 × *g* for 15 min. The pellet was suspended in TME buffer and centrifuged at 27,000 × *g* for 20 min. The resulting pellet was suspended in TME buffer containing 10% glycerol and 1 mM DTT and used for the membrane preparation of cellulose synthase. After cell disruption, the supernatant obtained was used for the preparation of cyclic diguanylic acid (19). The cyclic diguanylic acid was obtained after incubation (at 32°C for 1 h) of the supernatant enzyme preparation with 10 mM GTP, 1 mM $CaCl_2$, 3.2 mM creatine phosphate, and 20 units of creatine kinase in a total volume of 1 ml.

Assay for Cellulose Synthase. For determination of cellulose synthesis from sucrose, incubation mixtures contained the membrane preparation of cellulose synthase (20 μg), 75 mM Tris·HCl (pH 8.6), 15 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EDTA, cyclic diguanylic acid (20 μg/ml), 0.6 mM [^{14}C]sucrose (620 mCi/mmol, Amersham), 10 mM UDP, and purified recombinant sucrose synthase preparation (0.3 μg) in a total volume of 100 μl (20). For the synthesis of cellulose from UDP-glucose, 0.4 mM UDP-[^{14}C]glucose (290 mCi/mmol) was added to the reaction mixture instead of [^{14}C]sucrose, 10 mM UDP, and recombinant sucrose synthase preparation. The reaction was allowed to proceed for 10 min at 30°C and then terminated by the addition of 1 ml of 1 M NaOH containing 5 mg of carrier cellulose. An alkali-insoluble product (cellulose) was obtained

by incubation with 1 M NaOH at 100°C for 20 min. The insoluble residue was washed six times with water and once with 0.1 M acetic acid by centrifugation, and radioactivity was measured in 5 ml of Aquasol as described above.

Determination of Nucleotides and Sucrose. For determination of UDP-glucose, UDP and sucrose, the bacteria were cultured in the presence of 0.1% cellulase preparation. Bacterial cells were washed twice with water and a brief centrifugation, and cells were extracted with 0.2 M perchloric acid at 2°C for 3 h. The extract was collected by centrifugation and neutralized with 2 M KOH, and the insoluble cell debris and $KClO_4$ formed were removed by centrifugation. The supernatant was concentrated by adsorption on charcoal and material was eluted with a mixture of ammonia/ethanol/water, 4:50:46 (vol/vol). The eluate was concentrated to a quarter volume and freeze-dried. The amount of UDP-glucose was determined by the procedure of Tochikura *et al.* (21). The reaction mixture contained 1.0 μmol of NAD, 200 μmol of glycine buffer (pH 8.6), 5 mU UDP-glucose dehydrogenase (Sigma), and the sample to be assayed in a total volume of 0.3 ml. The mixture was incubated at 25°C for 30 min and absorbance at 340 nm was measured. The amount of UDP was analyzed by HPLC on a Gen-Pak column (3.9 × 150 mm). Analysis was carried out at room temperature in 20 mM NH_4Cl containing 10% acetonitrile. A UV monitor with detection at both 260 and 280 nm was used. For determination of sucrose, the extract with 0.2 M perchloric acid was incubated with 2 units of invertase (Wako Biochemicals, Osaka, Japan) at 37°C for 5 min, followed by oxidation with 1 unit of mutarotase/glucose oxidase (Wako Biochemicals) at 37°C for 10 min. The oxidized glucose was monitored at 505 nm.

For determination of radioactive glucose of UDP-glucose, the bacterial cell extract with 0.2 M perchloric acid was applied to a Dowex AG1x8 ($HCOO^-$ type) column (0.5 × 1.5 cm), and an eluate of the column with 4 M HCOOH containing 0.8 M $HCOONH_4$, after washing with 4 M HCOOH containing 0.4 M $HCOONH_4$, was collected as a UDP-glucose fraction (22). The eluate (UDP-glucose) was freeze-dried to remove HCOOH and $HCOONH_4$ and hydrolyzed with 0.01 M trifluoroacetic acid (pH 2.0) at 100°C for 15 min. The sample was subjected to paper chromatography with 1-propanol/1-butanol/water, 3:2:1 (vol/vol), and radioactivity in the area corresponding to glucose was measured in a toluene-based scintillation mixture.

Determination of Cellulose. The amount of cellulose produced in the culture was measured as the dry weight of the insoluble fraction obtained after washing of the cells/cellulose mixture with 0.1 M NaOH at 80°C for 20 min (23).

For determination of radioactive cellulose in the small scale culture, the insoluble residue after extraction with 0.2 M perchloric acid was heated with 0.1 M NaOH at 80°C for 20 min to dissolve the bacterial cell walls and other carbohydrates. The resulting insoluble cellulose was washed four times with water by centrifugation and solubilized with ice-cold 72% sulfuric acid for 1 h. In a portion of the mixture, radioactivity was measured with 5 ml of Aquasol, and a portion of the mixture was also analyzed by the phenol/sulfuric acid method (24).

RESULTS

Biosynthesis of 1,4-β-Glucan from Sucrose with Recombinant Sucrose Synthase. Mung bean sucrose synthase was subjected to the site-directed mutagenesis, and the recombinant protein was expressed in *E. coli* (6, 8). Wild-type (Ser^{11}) and mutant (Glu^{11}) sucrose synthases were purified to give a single band at 95 kDa on SDS/PAGE, although their activities were eluted at 350 kDa as a homotetrametric structure (5) on a Superdex 200 column. Replacement of Ser^{11} with Glu^{11} increased the affinity for sucrose to produce UDP-glucose, although both enzymes had a similar affinity for UDP-glucose

Table 1. Kinetic constants of recombinant sucrose synthases and cellulose synthase for UDP-glucose and sucrose

	K_m, mM		V_{max}, nmol per min per μg	
Enzyme	UDP-glucose	Sucrose	UDP-glucose	Sucrose
SuSy (wild type)	0.40*	161*	1.67*	0.24*
SuSy (mutant)	0.45*	23*	1.00*	0.11*
Cellulose synthase + SuSy (mutant)	0.20	ND	0.03	ND
+10 mM UDP	—	0.33	—	0.06
+1 mM UDP	—	0.42	—	0.12
+0.1 mM UDP	—	0.83	—	0.28

SuSy, sucrose synthase.
*Conditions were the same as those of the standard assay, except for the concentrations of UDP-glucose or sucrose (8).

(Table 1). We expected Glu^{11} to mimic the phosphorylated Ser^{11}, because the acidic residue causes the enzyme to bind and cleave sucrose for the synthesis of UDP-glucose (8).

On incubation of [^{14}C]sucrose with membrane preparations derived from extracts of *A. xylinum*, [^{14}C]cellulose was formed in the presence of purified recombinant sucrose synthase and UDP. Reactions were linear for 20 min (Fig. 1*A*) and were also linear with the concentration of mutant sucrose synthase in the assay up to about 1 μg (Fig. 1*B*). The incorporation from sucrose was increased at low concentrations of UDP at initial rates of 1,4-β-glucan synthesis for 20 min and even after 20 min. The inactivation for UDP-glucose after 20 min may be due to the instability of the substrate rather than that of 1,4-β-glucan synthase. The β-glucan production with mutant sucrose synthase exceeded that with the wild-type enzyme by a factor of 15 (Fig. 1), although the V_{max}/K_m ratio of the mutant enzyme for sucrose was only 3-fold higher than that of the wild-type enzyme (Table 1). K_m and V_{max} values for sucrose in the coupled reaction leading to 1,4-β-glucan synthesis were increased with the decreasing amount of UDP (Table 1). High concentrations of UDP caused a low K_m value for sucrose (0.33 mM), similar to that of cellulose synthase for UDP-glucose (0.2 mM). However, V_{max} value for sucrose was increased in the presence of low concentrations of UDP. This is in agreement with the finding (Fig. 1*A*) that the amount of glucose incorporation from sucrose was increased with the decreased amount of UDP at initial rates of 1,4-β-glucan synthesis. These results show that low concentrations of UDP decrease the affinity of sucrose synthase but increase the velocity of cellulose synthesis.

Effect of Sucrose Synthase Expressed in *A. xylinum*. The expression of mutant sucrose synthase cDNA has been dem-

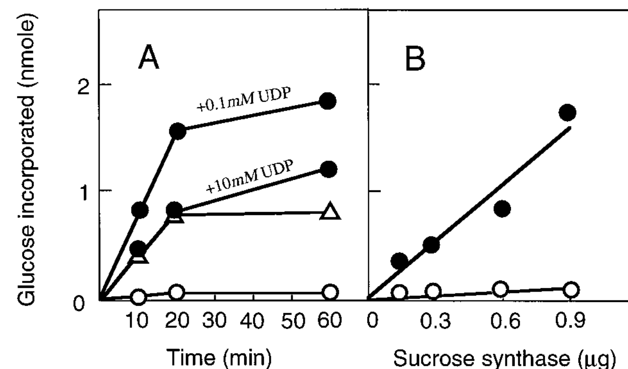

FIG. 1. Effect of time and recombinant sucrose synthase on cellulose formation from sucrose by *A. xylinum* membrane preparations. ●, Mutant sucrose synthase; ○, wild-type sucrose synthase; △, cellulose formation from UDP-glucose without sucrose synthase.

Table 2. Levels of sucrose synthase activity and its substrates/products in *A. xylinum* transformants

Plasmid	Forming activity, nmol per mg per min		Content, μmol/ml*		
	UDP-glucose	Sucrose	UDP	Sucrose	UDP-glucose
None	0	0	0.20	25	0.25
pSA-SD (wild type)	60	37	0.14	20	0.31
pSA-SD-S11E (mutant)	140	42	0.02	18	0.41

*Concentrations of UDP-glucose, UDP, and sucrose are shown per packed cell volume (ml).

onstrated in transformants of *A. xylinum* subsp. nonacetoxidans with mutant sucrose synthase and wild-type cDNAs inserted into the shuttle vector pSA19 to construct pSA-SD and pSA-SD-S11E, respectively. The transformants showed sucrose synthase activity that catalyzes the formation of UDP-glucose from sucrose plus UDP and the formation of sucrose from UDP-glucose plus fructose, but the plasmid-free cells did not (Table 2). The level of UDP-glucose in the transformant with mutant sucrose synthase cDNA was 1.6-fold higher than that in plasmid-free cells, and the level of UDP was markedly decreased in the transformant at the exponential stage in a large scale culture (Table 2). When three kinds of *A. xylinum* were grown in a 300-ml baffled flask, the transformant with mutant sucrose synthase cDNA produced cellulose at about 2- to 3-fold higher levels than plasmid-free cells (Fig. 2). Thus, the expression of mutant sucrose synthase in *A. xylinum* caused the increased level of UDP-glucose and the decreased level of UDP, resulting in massive synthesis of cellulose.

Functional Analysis of Sucrose Synthase Activity in *A. xylinum*. To determine whether sucrose synthase activity is functionally expressed in cells, we used asymmetrically labeled [*glucose*-^{14}C]sucrose as a carbon source in the culture (2 ml) of transformants of *A. xylinum* subsp. nonacetoxidans. The transformant with mutant sucrose synthase cDNA markedly produced UDP-[^{14}C]glucose from the asymmetrically labeled sucrose, but plasmid-free cells hardly incorporated the radioactivity into UDP-glucose (Fig. 3*A*). This shows that sucrose is incorporated into the bacterial cells, where UDP-glucose is directly formed from the glucose moiety of sucrose in the transformant but is mainly derived from the nonradioactive fructose moiety of sucrose in the plasmid-free cell. The total production of cellulose was higher for transformants with higher radioactive cellulose and lower for plasmid-free cells with lower radioactivity (Fig. 3*B*). The cellulose formed in the

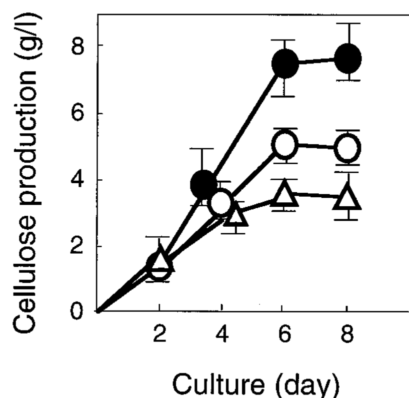

FIG. 2. Production of cellulose in transformants of *A. xylinum*. ●, Transformant with mutant sucrose synthase cDNA; ○, transformant with wild-type sucrose synthase cDNA; △, plasmid-free cells. Because the solid and open symbols overlapped for 2 days, only the open symbols were shown.

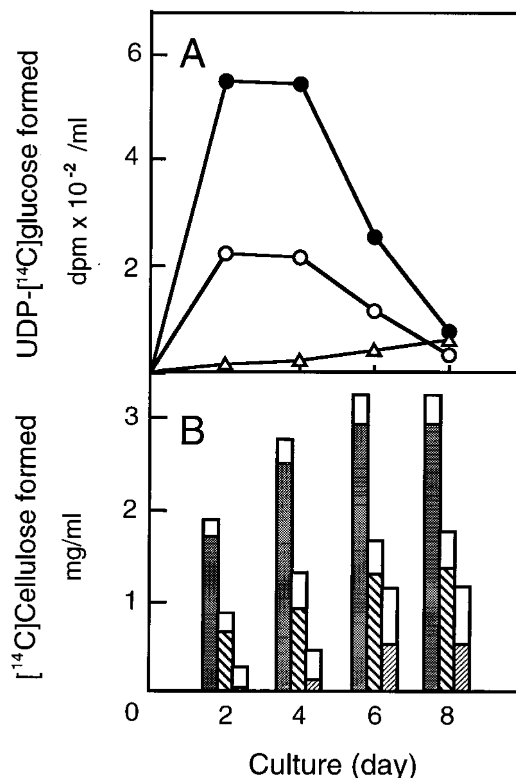

FIG. 3. Formation of radioactive UDP-glucose and cellulose from [*glucose*-^{14}C]sucrose in transformants of *A. xylinum.* (*A*) UDP-[^{14}C]glucose formed from [*glucose*-^{14}C]sucrose. ●, Transformant with mutant sucrose synthase cDNA; ○, the transformant with wild-type sucrose synthase cDNA; △, plasmid-free cells. (*B*) [^{14}C]Cellulose formed from [*glucose*-^{14}C]sucrose, in which the amount of cellulose is shown as the height of bar due to total radioactive plus nonradioactive cellulose in each fraction, the solid part of which is due to the radioactive cellulose equivalent to specific radioactivity of [^{14}C]glucose moiety: ■, mutant sucrose synthase cDNA; ▧, wild-type sucrose synthase cDNA; ▨, plasmid-free cells.

transformant maintained the high specific radioactivity of glucosyl residues equivalent to the glucose moiety of [*glucose*-^{14}C]sucrose. In conclusion, the plant-specific gene sucrose synthase cDNA not only changed sucrose metabolism but also enhanced the production of cellulose in bacteria.

DISCUSSION

We have demonstrated that a plant-specific gene, a cDNA for sucrose synthase, not only changed sucrose metabolism but also enhanced the production of cellulose in *A. xylinum*, where UDP-glucose was efficiently formed from sucrose and the massive synthesis of cellulose was promoted (Figs. 2 and 3). The rate of cellulose formation in the transformants was promoted during cell growth. Because the cellulose formed in the transformant with mutant sucrose synthase maintained the high specific radioactivity of glucosyl residues equivalent to the labeled glucose moiety of [*glucose*-^{14}C]sucrose (Fig. 3*B*), the sucrose synthase served to channel carbon directly from sucrose to cellulose in bacteria. The enhanced cellulose production was probably due to the low level of UDP in cells because low concentrations of UDP increased in the incorporation of glucose into 1,4-β-glucan (Fig. 1 and Table 1). It appears that UDP formed after glucosyl transfer is directly recycled in a short time by sucrose synthase. Because UDP inhibits cellulose synthase in *A. xylinum* (K_i = 0.14 mM) (25), sucrose synthase apparently functions to prevent UDP build-up during cellulose (1, 4-β-glucan) synthesis. This is the most important role of sucrose synthase on cellulose biosyn-

thesis in higher plants, where its biosynthetic mechanism is superior to bacterial systems. In this study, we confirmed the function of sucrose synthase not only on the activation of 1,4-β-glucan synthase at low concentrations of UDP but also on the enhanced production of cellulose at low levels of UDP in the transformant with mutant sucrose synthase.

The high free energy between glucose and fructose in sucrose can be conserved and used for the synthesis of UDP-glucose and finally for cellulose in transformants (Fig. 3), although two ATPs are required for the biosynthesis of UDP-glucose from sucrose. It was surprising that UDP-glucose in plasmid-free cells might be derived from the fructose moiety of sucrose. Essentially, four enzyme steps are required for the complete pathway from fructose to UDP-glucose—i.e., the phosphorylation of fructose by fructokinase, the isomerization of fructose 6-phosphate to glucose 6-phosphate by phosphoglucomutase, the isomerization of glucose 6-phosphate to glucose 1-phosphate by phosphoglucomutase, and the synthesis of UDP-glucose by UDP-glucose pyrophosphorylase. The validity of the above series of reactions can be verified by the *in vivo* flow of carbon from [*fructose*-^{14}C]sucrose through UDP-glucose.

We also demonstrated that cellulose synthase was activated with mutant sucrose synthase in 1,4-β-glucan formation from sucrose (Fig. 1). The 1,4-β-glucan production with mutant sucrose synthase exceeded that with wild-type enzyme by a factor of 15 (Fig. 1), although the V_{max}/K_m ratio of mutant enzyme for sucrose was only 3-fold higher than that of the wild-type enzyme (Table 1). The mutant sucrose synthase might act in concert with cellulose synthase because sucrose synthase serves to channel carbon directly from sucrose to cellulose as shown in cotton fiber cells (1). The acidic side chain (Glu) of sucrose synthase at position 11 probably activates the enzyme not only to form UDP-glucose from sucrose but also to cooperate with membrane-bound cellulose synthase. Therefore, phosphorylation of sucrose synthase may change a switch-on to form UDP-glucose in the cooperation with membrane-bound cellulose synthase in plant tissues. It should also be noted that our finding is inconsistent with an earlier observation by Winter *et al.* (26) that phosphorylation of sucrose synthase in maize causes a soluble form to be dissociated with cellulose synthase.

Our findings also show that the production of cellulose can be increased by the enhanced production of UDP-glucose and the decreased amount of UDP. The production of cellulose could be even more enhanced by the expression manner with mutant sucrose synthase in *A. xylinum* rather than by the use of *lac* promoter (11), although the transformant with mutant sucrose synthase produces cellulose at 2- to 3-fold higher levels than plasmid-free cells (Table 2 and Fig. 2). The expression of mutant sucrose synthase in woody plants would also be expected to increase the deposition of cellulose. The constitutive production of UDP-glucose from sucrose may ensue without the regulation of the enzyme activity by phosphorylation. Because cellulose has a strong tendency to self-associate into fibrils, which are not easily hydrolyzed either chemically or biologically and accumulate in the walls of woody plants, cellulose is a good biological sink for CO_2 on the earth (27).

1. Amor, Y., Haigler, C. H., Johnson, S., Wainscott, M. & Delmer, D. P. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 9353–9357.
2. Xu, D. P., Sung, S. J. S., Loboda, T., Kormanik, P. P. & Black, C. C. (1089) *Plant Physiol.* **90,** 635–642.
3. Geigenberger, P., Langenberger, S., Wilke, I., Heineke, D., Heldt, H. W. & Stitt, M. (1993) *Planta* **190,** 446–453.
4. Nolte, K. D., Hendrix, D. L., Radin, J. W. & Koch, K. E. (1995) *Plant Physiol.* **109,** 1285–1293.
5. Delmer, D. P. (1972) *J. Biol. Chem.* **247,** 3822–3828.
6. Nakai, T., Tonouchi, N., Tsuchida, T., Mori, H., Sakai, F. & Hayashi, T. (1997) *Biosci. Biotechnol. Biochem.* **61,** 1500–1503.
7. Zhang, X.-Q. & Chollet, R. (1997) *FEBS Lett.* **410,** 126–130.
8. Nakai, T., Konishi, T., Zhang, X. Q., Chollet, R., Tonouchi, N., Tsuchida, T., Yoshinaga, F., Mori, H., Sakai, F. & Hayashi, T. (1998) *Plant Cell Physiol.* **39,** 1337–1341.
9. Yoshinaga, F., Tonouchi, N. & Watanabe, K. (1997) *Biosci. Biotechnol. Biochem.* **61,** 219–224.
10. Ross, P., Mayer, R. & Benziman, M. (1991) *Microbiol. Rev.* **55,** 35–58.
11. Nakai, T., Moriya, A., Tonouchi, N., Tsuchida, T., Yoshinaga, F., Horinouchi, S., Sone, Y., Mori, H., Sakai, F. & Hayashi, T. (1998) *Gene* **213,** 219–224.
12. Takemura, H., Tsuchida, T., Yoshinaga, F., Matsushita, K. & Adachi, O. (1994) *Biosci. Biotechnol. Biochem.* **58,** 2082–2083.
13. Okumura, H., Uozumi, T. & Beppu, T. (1985) *Agric. Biol. Chem.* **49,** 1011–1017.
14. Toyosaki, H., Kojima, Y., Tsuchida, T., Hishino, K. I., Yamada, Y. & Yoshinaga, F. (1995) *J. Gen. Appl. Microbiol.* **41,** 307–314.
15. Seto, A., Kojima, Y., Tonouchi, N., Tsuchida, T. & Yoshinaga, F. (1997) *Biosci. Biotechnol. Biochem.* **61,** 735–736.
16. Arai, M., Mori, H. & Imaseki, H. (1992) *Plant Cell Physiol.* **33,** 503–506.
17. Tonouchi, N., Tsuchida, T., Yoshinaga, F., Beppu, T. & Horinouchi, S. (1994) *Biosci. Biotechnol. Biochem.* **58,** 1899–1901.
18. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
19. Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D., Weinberger-Ohana, P., Mayer, R., Braun, S., de Vroom, E., van der Marel, G. A., van Boom, J. H. & Benziman, M. (1987) *Nature (London)* **325,** 279–281.
20. Lin, F. C., Brown, M. R. M., Jr., Cooper, J. B. & Delmer, D. P. (1985) *Science* **230,** 822–825.
21. Tochikura, T., Kawai, H., Tobe, S., Kawaguchi, K., Osugi, M. & Ogata, K. (1968) *J. Ferment. Technol.* **46,** 957–969.
22. Hayashi, T. & Matsuda, K. (1981) *Agric. Biol. Chem.* **45,** 2907–2908.
23. Toyosaki, H., Naritomi, T., Seto, A., Matsuoka, M., Tsuchida, T. & Yoshibaga, F. (1995) *Biosci. Biotechnol. Biochem.* **59,** 1498–1502.
24. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) *Anal. Chem.* **28,** 350–356.
25. Benziman, M., Aloni, Y. & Delmer, D. P. (1983) *J. Appl. Polym. Sci.* **37,** 131–143.
26. Winter, H., Huber, J. L. & Huber, S. C. (1997) *FEBS Lett.* **420,** 151–155.
27. Hayashi, T., Ihara, Y., Nakai, T., Takeda, T. & Tominaga, R. (1998) in *Advances in Chemical Conversions for Mitigating Carbon Dioxide*, eds. Inui, T., Anpo, M., Izui, K., Yanagida, S. & Yamaguchi, T. (Elsevier, Tokyo), pp. 243–248.