

Communication

Interdomain Disulfide Bridge in the Rice Granule Bound Starch Synthase I Catalytic Domain as Elucidated by X-Ray Structure Analysis

Mitsuru MOMMA[†] and Zui FUJIMOTO[†]

Biomolecular Research Unit, National Institute of Agrobiological Sciences,
2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan

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The catalytic domain of rice (*Oryza sativa japonica*) granule bound starch synthase I (OsGBSSI-CD) was overexpressed and the three-dimensional structures of the ligand-free and ADP-bound forms were determined. The structures were similar to those reported for bacterial and archaeal glycogen synthases, which belong to glycosyltransferase family 5. They had Rossmann fold N- and C-domains connected by canonical two-hinge peptides, and an interdomain disulfide bond that appears to be conserved in the *Poaceae* plant family. The presence of three covalent linkages might explain why both OsGBSSI-CD structures adopted only the closed domain arrangement.

Key words: ADP-binding; crystal structure; granule bound starch synthase; *Oryza sativa*

Starch and glycogen are megadalton-sized molecules of α -1,4- and α -1,6-linked D-glucose (hereafter glucose) polymers. They are the major energy source in most living organisms, including plants and animals. Starch is the most widespread and most abundant storage carbohydrate found in plants. Humans depend on starch for nutrition and as a raw material in various industries, including paper making and bio-ethanol production. Metabolic pathways conducting glucose to starch/glycogen synthesis commonly involve several enzymatic steps.^{1,2} Starch synthases (SSs) as well as glycogen synthases (GSs) catalyze the α -1,4-linked glucose chain elongation process by adding glucose from nucleotide diphosphate glucose (UDP-glucose/ADP-glucose) to the non-reducing end of the growing chain. These enzymes share two EC numbers, EC 2.4.1.11 and EC 2.4.1.21, based on the glucose donor. The GSs in EC 2.4.1.11 use UDP-glucose and the GSs and SSs in EC 2.4.1.21 use ADP-glucose. Some SSs can use either UDP- or ADP-glucose, and these are occasionally classified into EC 2.4.1.242. SSs are classified into at least six classes in higher plants: granule-bound SS (GBSS; two isoforms exist: GBSSI and GBSSII) and soluble SS (I, II, III, IV, and V). Waxy protein GBSSI, encoded by the *wx* locus, is an enzyme essential for amylose synthesis, whereas soluble SSs are responsible for regulating the quality and quantity of starch molecules, including amylopectin chain-length distribution.^{1,2} On the other hand, SSs and

GSs are classified into glycosyltransferase families (GTs) 3 and 5 in the CAZy database (<http://www.cazy.org/>) according to sequence similarities,^{3,4} and they are members of the GT-B superfamily which includes GT-3 and GT-5. SSs are classified into GT-5, whereas GSs are classified into either GT-3 or GT-5.^{3,5} Fungal, yeast, and animal GSs are classified into GT-3 enzymes, because they catalyze chain elongation using UDP-glucose as glucose donor. Structural information on *Saccharomyces cerevisiae* GS, a member of GT-3, was recently reported.^{6,7} The structure revealed that yeast GS proteins possess a unique insertion that is engaged in subunit oligomerization. The subunit interfaces of yeast GS proteins are responsible for phosphorylation and glucose-6-phosphate binding, which regulates enzyme action. In contrast to GT-3 GSs, bacterial GSs, archaeal GSs, and plant SSs are classified into the GT-5 family, and are non-regulated enzymes. Three-dimensional structures of GT-5 family members have been reported for the bacterial GSs from *Agrobacterium tumefaciens* (AtGS)⁸ and *Escherichia coli* (EcGS)^{9,10} and an archaeal GS from *Pyrococcus abyssi* (PaGS).^{11,12} Monomer subunits of AtGS and EcGS adopt a typical GT-B fold composed of distinct N and C-domains each comprising a Rossmann fold, with an active cleft formed between the domains. The AtGS structures of ligand-free and ADP-complexed forms are both in the relaxed open state. Recent intensive studies of the structure of EcGS complexed with ADP and maltooligosaccharides have unveiled the dynamic closure movement of the two Rossmann fold domains to form a functional closed active center associated with the conserved N-terminal KTGGL motif and the C-terminal ADP-binding pocket. The catalytic domains of SSs and bacterial GSs show significant amino acid sequence identity (30 to 36%), suggesting that they have a similar overall fold and catalytic mechanism. Despite progress in GSs structural studies, there is currently no structural information on plant SSs. Rice GBSSI belongs to GT-5 and conducts amylose synthesis.^{1,2,13} Here we report the three-dimensional structure of the rice (*Oryza sativa japonica* cultivar-group) GBSSI catalytic domain (OsGBSSI-CD) as the first example of the plant SS structure.

A putative OsGBSSI-CD DNA fragment (Met83-Pro609), deduced from amino acid sequence alignment

[†] To whom correspondence should be addressed. Mitsuru MOMMA, Fax: +81-29-838-7877; E-mail: momma@affrc.go.jp; Zui FUJIMOTO, Fax: +81-29-838-7877; E-mail: zui@affrc.go.jp

Abbreviations: AtGS, *Agrobacterium tumefaciens* glycogen synthase; EcGS, *Escherichia coli* glycogen synthase; GBSS, granule bound starch synthase; GS, glycogen synthase; GT, glycosyltransferase family; OsGBSSI-CD, rice granule bound starch synthase I catalytic domain; PaGS, *Pyrococcus abyssi* glycogen synthase; PDB, Protein Data Bank; rms, root-mean-square; SS, starch synthase

with bacterial GSs, was amplified by the PCR method using as template the cDNA (GenBank accession no. AK070431) from the rice cDNA clone library (KOME, <http://cdna01.dna.affrc.go.jp>).¹⁴⁾ PCR was performed at 30 cycles with Phusion DNA polymerase (Thermo Fisher Scientific, Waltham, MA) and the following primers: 5' CAT ATG AAC GTC GTG TTC GTC GGC 3' and 5' AAC TCT AGA TCA AGG AGC AGC CAC GTT C 3' (gene specific sequences underlined). The resulting PCR products were digested with *Xba*I and cloned into pET45b (Novagen-Merk, Darmstadt, Germany) using the *Pml*I and *Avr*II sites, and OsGBSSI-CD was overexpressed in *E. coli* BL21-Gold (DE3) (Stratagene, La Jolla, CA). After the cells grew at 37 °C for 3 h, isopropyl β -thiogalactopyranoside was added to the culture to a final concentration of 0.5 mM and growth continued at 25 °C overnight. The harvested cells were washed, suspended in 20 mL of potassium buffer saline, and disrupted using ultrasonicator Insonator 201M (Kubota, Tokyo), and then centrifuged at 35,000 *g* for 1 h at 4 °C. The supernatant was diluted with the same volume of 40 mM phosphate buffer pH 7.2, containing 50 mM imidazole and 1 M sodium chloride. The diluted solution was loaded onto a HisTrap Chelating FF column 5 mL (GE Healthcare, Buckinghamshire, UK) equilibrated with the binding buffer (20 mM phosphate buffer pH 7.2, 0.5 M sodium chloride, and 25 mM imidazole). After the column was washed with at least 5 volumes of the binding buffer, OsGBSSI-CD was eluted by step-wise raising of the imidazole concentration to 0.1, 0.2, and 0.5 M. The fractions containing OsGBSSI-CD were further purified by gel-filtration column chromatography with a HiLoad 16/60 Superdex 200 pg column connected to an ÄKTA Explorer10 (GE Healthcare) equilibrated with 20 mM Tris-HCl pH 8.0 and 0.2 M sodium chloride. The fractions primarily showing a single band on SDS-PAGE were pooled and concentrated to 6 mg/mL for crystallization trials. The molecular weight was estimated to be 59 kDa by SDS-PAGE and 60 kDa by gel filtration with a Superose 12 HR 10/30 column (GE Healthcare). The molecular weight of OsGBSSI-CD was calculated to be 59.3 kDa with 527 residues. Thus the purified OsGBSSI-CD was present in a monomeric form in solution. The protein concentration was determined photometrically.¹⁵⁾

OsGBSSI-CD was crystallized by the sitting-drop vapor-diffusion method in 96-well Intelli Plates (Art Robbins Instruments, Sunnyvale, CA) at 20 °C. The reservoirs contained 50 μ L solutions, and each drop consisted of 0.3 μ L of the protein solution and the same volume of the reservoir solution. The precipitant conditions consisted of 1.5 M lithium sulfate, and 0.1 M HEPES-sodium buffer (pH 7.5). Crystals of 0.15 $\times$ 0.15 $\times$ 0.15 mm were obtained after 4 weeks of incubation. Crystals with ADP were prepared by soaking 5 mM ADP for 10 min in the crystallization solution. A crystal was harvested and soaked in the mother liquid containing 20% glycerol as a cryo-protectant and flash-cooled in a nitrogen stream at -180 °C. Diffraction data sets were collected at beamlines AR-NW12¹⁶⁾ and BL5 of the Photon Factory (High Energy Accelerator Research Organization, Tsukuba, Japan) using Quantum CCD detectors (Area Detector Systems Corporation, Poway,

Table 1. Data Collection and Refinement Statistics
Values in parentheses are for the highest resolution shell.

PDB code	apo form 3VUE	ADP complex form 3VUF
Data collection		
Beamline	PF-BL12	PF-BL5
Wavelength (Å)	1.000	1.000
Space group	<i>P</i> 432	<i>P</i> 432
Unit cell (Å)	<i>a</i> = <i>b</i> = <i>c</i> = 152.9	<i>a</i> = <i>b</i> = <i>c</i> = 153.4
Resolution (Å)	50-2.7 (2.8-2.7)	50-3.0 (3.1-3.0)
Total no. reflections	232,915	180,430
Unique reflections	17,202 (1,681)	12,909 (1,254)
<i>R</i> _{merge}	0.046 (0.370)	0.067 (0.669)
Completeness (%)	99.3 (99.9)	99.8 (99.9)
Redundancy	13.5 (13.9)	14.0 (12.7)
Average <i>I</i> / σ (<i>I</i>)	27.5 (7.0)	24.5 (4.4)
Refinement		
Resolution (Å)	33.4-2.7	35.2-3.0
<i>R</i> / <i>R</i> _{free}	0.218/0.267	0.221/0.271
No. of reflections	16,018	12,161
No. of atoms	3,893	3,864
rms deviations		
in bond length (Å)	0.0015	0.0016
in bond angles (°)	1.602	1.619

CA). The data sets were integrated and scaled using the programs Denzo and Scalepack in the HKL-2000 program suite.¹⁷⁾ The OsGBSSI-CD structure was solved by the automated molecular replacement method with the MrBUMP program in the CCP4 program packages,^{18,19)} using the EcGS structure, with Protein Data Bank (PDB) ID 3GUH and with a sequence identity of 36%,⁹⁾ as a search model. Starting from the initial solution, the model was manually built iteratively using Coot, and was refined using REFMAC5 and phenix.refine.²⁰⁻²²⁾ The statistics for data collection and refinement are summarized in Table 1. Graphic figures were generated using the program PyMOL (DeLano Scientific, Palo Alto, CA). The atomic coordinates of the OsGBSSI-CD structure and its complex with ADP have been deposited in the PDB, through PDB Japan, Osaka University, under IDs 3VUE and 3VUF.

The OsGBSSI-CD crystals belonged to space group *P*432 and diffracted to a resolution of 2.7 Å in apo form and to 3.0 Å in ADP complex form (Fig. 1A). The asymmetric unit contained one OsGBSSI-CD peptide chain with a solvent content of 51.5%.²³⁾ The N-terminal amino acids (MAHHHHH) derived from the expression and purification tag, the C-terminal region (586-609), and the loop formed by residues 174-191 did not show a clear electron density, indicating that these regions were disordered. OsGBSSI-CD folded into two Rossmann fold domains, as has been observed for GTs and glycogen phosphorylases of the GT-B superfamily. The structure showed a deep cleft between the two domains where the catalytic center is located. The N-terminal domain consisted of residues 83-355 along with the C-terminal helix (residues 569-585), and the C-terminal domain consisted of residues 356-568. The structures of the domains showed similar " β - α - β sandwich" structures with parallel β -sheets surrounded by α -helices.

Compared with the bacterial GSs, most of the secondary structures were well conserved (Fig. 1B). Although these structures were in good topological agreement and show good superposition, five insertions

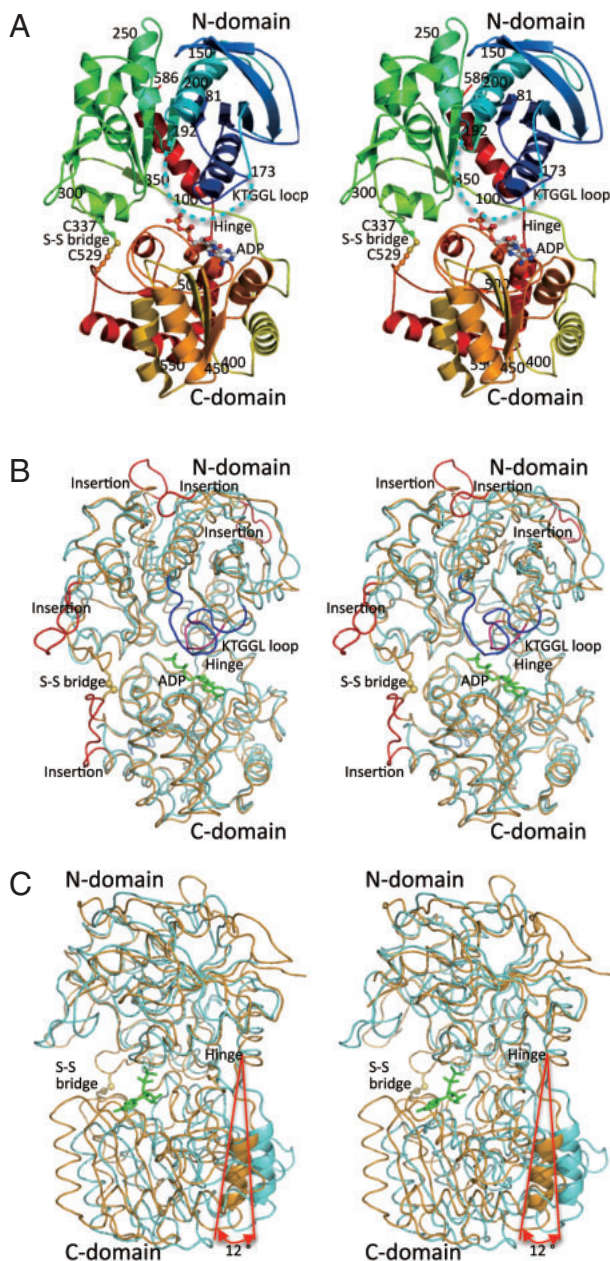


Fig. 1. Structure of OsGBSSI-CD.

A, Stereo view of the ribbon model of ADP-complexed OsGBSSI-CD shown in rainbow-ordered colors. Disulfide bridge and the bound ADP molecules are shown in ball-and-stick models. Amino acids are numbered every 50 residues. Broken blue line shows the disordered flexible loop region (174–191). B, Stereo view of a superposed model of the ADP-complexed OsGBSSI-CD structure (yellow) and the closed EcGS structure (light blue, PDB ID 2QZS). The ADP molecule is represented by a green stick model, the disulfide bridge by a yellow ball-and-stick model, the KTGL loop in magenta, and the five insertion sequences in red. The flexible loop region of EcGS, whose corresponding region in OsGBSSI-CD was disordered, is in blue. C, Stereo view of a superposed model of the OsGBSSI-CD structure (yellow) and the open EcGS structure (light blue, PDB ID 3D1J). The models were superimposed based on their N-domain structures, and domain movement was estimated for the highlighted helix before the C-terminal hinge.

and one deletion were observed, most of which displayed elongates or shorten loop structures located outside the core structure. The C-domain structure was more conserved than the N-domain. The bacterial GSs, EcGS and AtGS, had two structural states of the deep and wide clefts: (i) the enzymatic inactive open state and

(ii) the active closed state.^{8–10} Although ADP can bind to the open state of AtGS, the closed structure is perhaps an important structure for enzyme action. The closed state of EcGS has been solved in complex with various ligands including ADP bound in the catalytic cleft between the N-terminal and the C-terminal domains. The glucosyl donor (ADP-glucose) and acceptor glucan or mimics of them appear to be essential to construct the enzymatically competent closed cleft conformation. In contrast to these AtGS and EcGS structures, the solved OsGBSSI-CD structure displayed similar folds in ligand-free and ADP-bound forms (Fig. 1B). The root-mean-square (rms) difference between the ligand-free and ADP-bound structures was 0.375 Å, suggesting that OsGBSSI-CD is restricted to one state regardless of whether it binds a ligand in the catalytic cleft. The rms difference between OsGBSSI-CD and the closed EcGS structure (PDB ID 2QZS) was 1.65 Å. This value is lower than the value of 2.14 Å between OsGBSSI-CD and the open EcGS structure (PDB ID 3D1J). Much higher rms differences were calculated (2.21–2.72 Å) against open forms of AtGS (PDB IDs 1RZU and 1RZV) and PaGS (PDB IDs 3FRO and 3L01). These results indicate that the solved OsGBSSI-CD structure was in the closed state. The superimposed models in Fig. 1C indicated that the closed domain movement of OsGBSSI-CD was approximately 12° around the hinge peptide against the open EcGS structure.

The N and C-terminal domains were connected with narrow hinge peptides formed by two sequence segments (352–357 and 566–570) located at the center of the domains (Fig. 1), as observed for bacterial and archaeal GSs. In addition to these two linkages, OsGBSSI-CD contained a disulfide bond between Cys337 and Cys529, as the third covalently connected interaction, which has not been observed in other GT-5 GSs' structures. The position of the disulfide bond was on the side opposite to the two narrow hinges. Cys337 was located at the point of the extended loop from the N-domain. Residues 338–347 after Cys337 adopted no secondary structure in OsGBSSI-CD, but the corresponding residues of EcGS or AtGS folded into α -helices, and the loop around Cys337 of OsGBSSI-CD appeared to be extended as compared to those of EcGS and AtGS. Cys529 was located at the point of the inserted loop from the C-domain. In bacterial and archaeal GSs, these cysteine residues are not conserved. There are some interdomain loops in bacterial and archaeal GSs, but they make hydrogen-bond and hydrophobic interactions to connect the two domains. In plant GBSSIs, the second cysteine, Cys529 in rice GBSSI, is conserved and sequence insertions around this cysteine were similarly observed (Fig. 2). In contrast, the first cysteine, Cys337 in rice GBSSI, is conserved in the *Poaceae* family, which includes rice, wheat, rye, barley, corn, and sorghum, but is replaced by valine in non-*Poaceae* plants (e.g., potato, sweet potato, soybean, kidney bean, pea, cassava, and *Arabidopsis thaliana*). Therefore, the disulfide bridges are present only in poaceous GBSSIs, although extended loops from both domains appear to be conserved in plant GBSSIs. This disulfide bridge might restrict domain movement between the open and closed structures seen in bacterial and archaeal GSs. A preliminary mutational study

Rice	327-EELISGIARGCELDNIMRLTG-	519-GFHMGRSLVDCNVVEPSDVKK-
Wheat	332-EELISGEARGCELDNIMRLTG-	525-GFHMGRSLVDCNVVEPADVKK-
Rye	331-EELISGEARGCELDNIMRLTG-	514-GFHMGRSLVDCNVVEPADVKK-
Barley	320-EELISGEARGCELDNIMRLTG-	513-GFHMGRSLVDCNVVEPADVKK-
Sorghum	326-EELISGIARGCELDNIMRLTG-	518-GFHMGRSLVDCNVVEPADVKK-
Corn	319-EELISGIARGCELDNIMRLTG-	514-GFHMGRSLVDCNVVEPADVKK-
<i>A. thaliana</i>	328-QELISGVDGVELHKLRLMKT-	520-GFHIGRFNVKCEVVDVDDVIA-
Potato	325-QELISAVDKGVELDSVLRKTC-	517-GFHMGAFFNVCEVVDVDDVIA-
Sweet potato	326-KELVSCVSKGVELDNHRLDCG-	518-GFHMGAFFNVCEVVDVDDVIA-
Soybean	326-QELVSGEERGVELNNIRSCG-	518-GFHMGAFFNVCEVVDVDDVIA-
Kidney Bean	324-KELVSGEDRGVELDNIRKTC-	516-GFHMGAFFNVCEVVDVDDVIA-
Pea	321-KELISGEDRGVELDNIRSTG-	513-GFHAGFPFVCEVVDVDDVIA-
Cassava	326-QEIVSGVERGVELDNIRKTC-	518-GFQMGALHVECDKIDSADVAA-

Fig. 2. Amino Acid Sequence Alignment of Rice GBSSI with Other Plant GBSSIs around the Two Cysteines Forming the Disulfide Bond in OsGBSSI-CD.

The two cysteines and the corresponding residues are in bold font. Sequence accession numbers are as follows: wheat, CAA40509; rye, ACM41699; barley, CAA30755; sorghum, Q43134; corn, ABW95927; *A. thaliana*, AEE31537; soybean, ACB97679; kidney bean, BAA82346; pea, Q43092; and cassava, Q43784.

replacing the first cysteine with valine (C337V) caused an overexpressed protein to be produced in insoluble form (data not shown), suggesting that the disulfide bond plays an important role in stabilizing the three-dimensional structure by maintaining the closed inter-domain arrangement. It is interesting to note that the disulfide bond is observed in poaceous GBSSIs, including major cereal plants that accumulate abundant starch in their seeds. The expected restricted domain movement caused by this disulfide bridge might aid in efficient starch biosynthesis.

Plant SSs, including rice GBSSI, as well as bacterial and archaeal GT-5 GSs, can use nucleotide diphosphate glucose (UDP-glucose/ADP-glucose) as glucosyl donor substrates. The ADP-complexed structure shows an electron density map of the bound ADP (Fig. 3A) and an ADP-binding structure arrangement (Fig. 3B, C). The ADP-binding pocket consisted of the C-terminal domain wall and the conserved N-terminal KTGGGL loop motif located in the inter-domain region. This was similar to that of the other GT-5 enzymes. Most amino acid residues were conserved, except for two residues, Phe463 and Gln493, which were different from Tyr355 and from Tyr385 in EcGS respectively (Fig. 3D). On the other hand, the acceptor (starch or maltooligosaccharide) binding site has been found to lie in the cleft between the N and C-domains by structural analysis of the EcGS-maltohexaose complex.¹⁰⁾ The amino acids involved in the maltooligosaccharide recognition at the catalytic site were conserved in the OsGBSSI-CD structure (Fig. 3D), indicating that rice GBSSI binds maltooligosaccharides in a manner similar to EcGS, while the N-domain loop formed by residues 174–191 was disordered and its electron density was not observed, indicating that this region shows flexibility (Fig. 1A, B). In EcGS, Tyr95 and His96 from this loop participate in maltooligosaccharide binding. Since this loop covers the catalytic cleft, it might regulate the accessibility of the catalytic site to the ligands under the condition of the open-close domain movement, which is restricted by the disulfide link in rice GBSSI. On the contrary, the structure of the distal region from the catalytic center was not conserved. Starch bound in the catalytic site appears to stretch to the end of the cleft, where the disulfide link and the insertion loops are present in rice GBSSI. This raises the question whether these unique structures are related to starch recognition in plants. Further structural and biochemical studies

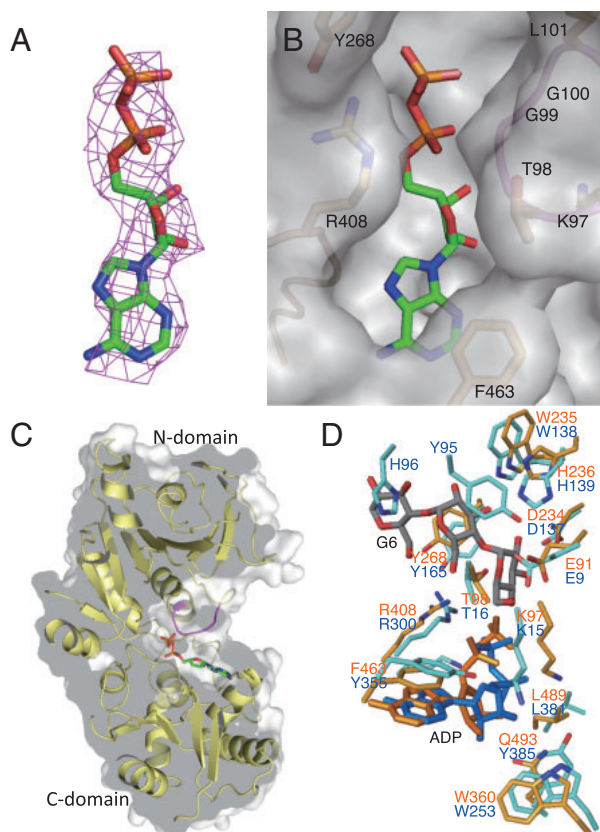


Fig. 3. Structure of ADP-Complexed OsGBSSI-CD.

A, Electron density map of the bound ADP molecule contoured at 0.7 σ . B, Catalytic pocket surface structure showing the arrangement of ADP-binding and surrounding residues. The N-terminal conserved KTGGGL loop is colored magenta. C, Sliced surface model of the overall structure of ADP-complexed OsGBSSI-CD. D, Superimposition of OsGBSSI-CD structure (yellow) and the maltooligosaccharide-bound EcGS structure (light blue, PDB ID 3CX4) around the catalytic site. The bound maltooligosaccharide and ADP in EcGS are shown by gray and blue stick models respectively, and ADP in OsGBSSI-CD is shown in orange.

should be done to understand the details of the mechanism of starch biosynthesis in plants.

Here we report the expression, and purification of OsGBSSI-CD and crystal structure determination of it in the ligand-free and ADP-bound forms, providing the first example of a plant SS structure. The OsGBSSI-CD structure was composed of the typical twin Rossmann fold structure in the GT-5, GT-B superfamily. ADP was bound in the catalytic pocket between the N-terminal and the C-terminal domain. The ligand-free and ADP-bound structures were both in the closed state, and OsGBSSI-CD appeared to have a rigid structure. The twin domain movement might be restricted by the disulfide bond and the two narrow inter-domain hinges. The disulfide bond is unique in the *Poaceae* family GBSSIs, and is expected to prove to be functionally important in facilitating efficient starch biosynthesis, because the first cysteine is not conserved in non-*Poaceae* family GBSSIs.

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