A Novel Type Carbohydrate-Binding Module Identified in α-Glucan, Water Dikinases Is Specific for Regulated Plastidial Starch Metabolism†

René Mikkelsen, Ken Suszkiewicz, and Andreas Blennow*

Center for Molecular Plant Physiology, Plant Biochemistry Laboratory, The Royal Veterinary and Agricultural University, 1876 Frederiksberg C, Copenhagen, Denmark

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ABSTRACT: The phosphorylation of the amylopectin fraction of starch catalyzed by the α-glucan, water dikinase (GWD, EC 2.7.9.4) plays a pivotal role in starch metabolism. Limited proteolysis of the potato tuber (*Solanum tuberosum*) GWD (StGWD, 155 kDa) by trypsin primarily produced stable fragments of 33 and 122 kDa, termed the SBD fragment and N11, respectively, as generated by trypsin cleavage at Arg-286. SBD and N11 were generated using recombinant DNA technology and purified to near homogeneity. Tandem repeat sequences, SBD-1 and SBD-2, of a region that is significantly similar in sequence to N-terminal regions of plastidial α-amylases are located in the N-terminus of StGWD. The SBD-1 motif is located within the sequence of the SBD fragment, and our results demonstrate that the fragment composes a new and novel carbohydrate-binding module (CBM), apparently specific for plastidial α-glucan degradation. By mutational analyses of conserved Trp residues located within the SBD-1 motif, W62 and W117, we show that these aromatic residues are vital for carbohydrate binding. N11 still possessed starch phosphorylating activity, but with a 2-fold higher specific activity compared to that of wild type (WT) StGWD using potato starch as the glucan substrate, whereas it had double the K_m value for the same substrate. Furthermore, investigation of the chains phosphorylated by WT StGWD and N11 shows that N11 exhibits a higher preference for phosphorylating shorter chains of the amylopectin molecule as compared to WT. From analyses of the glucan substrate specificity, we found up to 5-fold higher specific activity for N11 using amylose as the substrate.

Starch is the primary energy reserve in higher plants where it is found in both photosynthetic and nonphotosynthetic tissues. Starch found in the chloroplast of green leaves is termed transitory starch, whereas starch is deposited for long-term storage in the amyloplasts of storage organs such as seeds and tubers. A typical starch granule contains two distinct polysaccharides: amylose and amylopectin. While amylose is a linear chain of α-1,4-glucan with occasional α-1,6 branches, amylopectin is a much larger polyglucan with more frequent branch points (∼5%). The only naturally occurring covalent modification of starch is phosphoesterification of the amylopectin fraction at either C-3 (∼30%) or C-6 (∼70%) of the glucosyl unit (*1*, *2*). In potato storage starch, approximately 0.5% of the glucose residues is phosphorylated, i.e., one of 200−300 units, whereas cereal endosperm starches contain much less covalently linked phosphate (<0.01%) (*3*, *4*). In transitory leaf starch, approximately 0.1% of the glucose residues is phosphorylated (*5*).

The enzymatic mechanism responsible for starch phosphorylation has recently been elucidated, and it has been shown that a 155 kDa potato starch granule-bound protein, StGWD (GenBank accession number AAK11735), phosphorylates starch according to reaction I (*6*, *7*)

$$\text{ATP} + \alpha\text{-glucan} + H_2O \leftrightarrow \text{AMP} + \alpha\text{-glucan-P} + P_i \quad \text{(I)}$$

Hence, GWD[1] is an α-glucan, water dikinase (EC 2.7.9.4) catalyzing the transfer of the β-phosphate of ATP to either C-3 or C-6 of the glucosyl residue. During catalysis, a His residue at position 992 of potato GWD is autophosphorylated, generating a stable phosphohistidine intermediate containing the β-phosphate, which is subsequently transferred to either the C-3 or C-6 position of the glucosyl residue (*7*). Recently, it was demonstrated that the enzymatic activity of StGWD can be regulated by thioredoxin (*8*). Oxidation of StGWD inactivates the enzyme and induces the formation of a disulfide linkage between C1004 and C1008 which can be reduced by thioredoxin, thereby reactivating the enzyme. Furthermore, GWD displays a reversible binding to starch granules, partly existing in a soluble state in illuminated leaves and attached to the starch granule in darkened leaves

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* To whom correspondence should be addressed: Center for Molecular Plant Physiology (PlaCe), Plant Biochemistry Laboratory, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Copenhagen, Denmark. Telephone: +45 35283334. Fax: +45 35283333. E-mail: abl@kvl.dk.

[1] Abbreviations: AGE, affinity gel electrophoresis; Am, amylose; AMY3, plastidial α-amylase; Ap, amylopectin; CBM, carbohydrate-binding module; DP, degree of polymerization; GWD, α-glucan, water dikinase; HPAEC, high-performance anion exchange chromatography; HRP, horseradish peroxidase; PMSF, phenylmethanesulfonyl fluoride.

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(*9*). The reversible partitioning of StGWD to the starch granule appears to be modulated by the redox properties of StGWD (*8*) where the granule-bound form is in the oxidized state, whereas the reduced form is found in its soluble state.

Analysis of the functional domain organization of StGWD by limited proteolysis indicates a five-domain composition of 37, 24, 21, 36, and 38 kDa domains spanning the N- to C-termini (*10*). The catalytic histidine is located on the 36 kDa domain, whereas the 38 kDa C-terminal domain contains the ATP-binding site. The noncatalytic association of GWD with the glucan molecule has been thought to be confined to regions in the N-terminus of GWD (*5*). However, despite numerous well-defined and -characterized carbohydrate-binding modules (CBMs) (*11*−*13*) [currently 43 defined families; see http://afmb.cnrs-mrs.fr/CAZY/index.html (*11*)], neither is homologous to GWD. CBMs are generally found in glycoside hydrolases that degrade insoluble polysaccharides where they promote the noncatalytic association of the enzyme with the insoluble substrate.

Suppression of StGWD synthesis in potato using antisense technology results in a 90% reduction of starch bound phosphate (*14*, *15*). Mutations in a homologous gene in *Arabidopsis*, *sex1*, now termed AtGWD1, lead to a dramatic reduction or total suppression of the phosphate content of leaf starch (*5*). Interestingly, the transgenic potato plants as well as the *AtGWD1* mutant show a starch excess (sex) phenotype proposed to be a result of impaired starch degradation (*15*). This was corroborated by the fact that antisense GWD potato tubers stored at low temperatures exhibit a decreased cold sweetening (*15*). This strongly suggests that starch phosphorylation is required for normal starch degradation (*16*, *17*), but the precise relation between starch phosphorylation and starch degradation remains to be resolved.

In this article, we describe the engineering of truncated StGWDs by limited proteolysis with trypsin which produced the fragments SBD and N11, covering amino acid residues 1−286 and 287−1387 of StGWD (mature protein), respectively. We investigate the properties of the recombinantly produced SBD fragment and N11 and demonstrate that SBD possesses a novel CBM apparently specific for plastidial starch metabolism. The starch recognizing properties are mediated by at least two conserved Trp residues, W62 and W117. N11 exhibits an up to 5-fold higher glucan phosphorylating activity compared to WT StGWD when using amylose as the glucan substrate. Furthermore, we demonstrate that N11 shows altered chain length specificity versus WT.

MATERIALS AND METHODS

Expression and Purification of WT StGWD. The pGWD plasmid is an L-arabinose inducible pBAD/*Myc*-His C (Invitrogen) derived expression vector which contains the *StGWD* gene devoid of the transit peptide and the Myc-His tag (*7*). Expression and purification of WT StGWD were performed as described previously (*7*).

Limited Proteolysis of StGWD with Trypsin. Purified StGWD (0.4 mg/mL) was digested at 25 °C with trypsin (Roche) in 50 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, and 0.25 mM dithiothreitol (DTT). At the indicated time points, a 25 μL aliquot was removed and the digestion terminated by the addition of 2.5 μL of 20 mM phenylmethanesulfonyl fluoride (PMSF) in 2-propanol.

Protein Quantification, SDS−*PAGE, and Western Blotting*. Protein concentrations were measured by the method of Bradford (*18*), and with the BCA protein assay reagent (Pierce Chemical Co.) (*19*) using bovine serum albumin (BSA) as the standard. SDS−PAGE was performed using 8% homogeneous or 8 to 25% linear gradient polyacrylamide gels according to Laemmli (*20*) and protein visualized with Coomassie brilliant blue. Western blotting was carried out as described by Burnette (*21*). GWD was immunologically detected by rabbit anti-GWD IgG (1:1000 dilution) and horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG (1:5000 dilution, DAKO P0217) using a HRP substrate (Bio-Rad). N11 and SBD were detected by mouse anti-V5 IgG (1:5000 dilution, Invitrogen) and HRP-conjugated rabbit anti-mouse IgG (1:5000 dilution, DAKO P0161). Chemiluminescence was detected with a UVP AutoChemi System (UVP, Upland, CA).

Protein Sequencing. N-Terminal protein sequencing was performed at the Macromolecular Structure, Sequencing and Synthesis Facility at Michigan State University (East Lansing, MI).

Assay of GWD Activity. StGWD activity was measured as described previously using radiolabeled [β-^{33}P]ATP with small modifications. The assay was carried out in 2 mL Eppendorf tubes and contained, in a final volume of 100 μL, 25 mM Hepes-KOH (pH 7.0), 10 mM NH_4Cl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.2 mg/mL BSA, 20 μM [β-^{33}P]ATP (150 000 dpm, PerkinElmer Life Sciences), and 5 mg/mL gelatinized polyglucan. Reactions were initiated by adding the appropriate amount of GWD. The polyglucan was precipitated with 1.8 mL of 75% methanol and 1% KCl. Following centrifugation, the supernatant was discarded, and the pellet was resuspended in 200 μL of water, mixed briefly, and then precipitated like before. This procedure was repeated four times, and the final pellet was dissolved in 400 μL of water. Incorporated radioactivity was measured using a MicroBeta Trilux liquid scintillation counter (Wallac). One unit is defined as 1 μmol of phosphate incorporated into α-glucan per minute at 30 °C.

For production of linear glucan substrates for StGWD, 4, 8, or 16 mg of maltohexaose (Sigma) dissolved in 10 mL of 50 mM sodium citrate (pH 7.0), 200 mg of glucose 1-phosphate (G-1-P), and 5 mM AMP was incubated with 3 mg of phosphorylase *a* (Sigma) for 18 h at 37 °C. The reaction was stopped by boiling the mixture for 5 min, and the polyglucan was precipitated with 80% (v/v) ethanol. Following centrifugation, the polyglucan was washed twice with 30 mL of water and precipitated as described above. Finally, the polyglucan was resuspended in 30 mL of water and precipitated with 150 mL of acetone.

In addition, we used soluble potato starch and glycogen (type IX, bovine liver), both from Sigma, as glucan substrates.

High-Performance Anion Exchange Chromatography (HPAEC) of Oligosaccharides. The synthesized polyglucans were analyzed for chain length distribution by HPAEC using a Dionex DX 500 system (Dionex Corp., Sunnyvale, CA) equipped with an S-3500 autosampler and a CarboPac PA-100 column as described previously (*22*). The distribution of chains phosphorylated by GWD was analyzed following

radioactive labeling according to the method described above with a few modifications. We used 10 mg/mL polyglucan and 700 000 dpm [β-^{33}P]ATP to increase the amount of incorporated radioactivity. A total of 10 washes were performed to eliminate the background. Phosphorylated chains were treated with either isoamylase (Megazyme) to produce linear glucans or isoamylase and β-amylase (Megazyme) to produce linear phosphorylated chains in which the phosphate group is situated one or two glucose units from the nonreducing end. Treatment with β-amylase/isoamylase was carried out in 50 mM sodium citrate (pH 6.0). After enzymatic treatment, the phosphorylated chains were separated by HPAEC as described previously (*22*), and 1.6 mL fractions were collected. Radioactivity in the samples was determined with a MicroBeta Trilux liquid scintillation counter (Wallac).

The number of phosphate groups on each phosphorylated glucan chain was determined by HPAEC using the following conditions: flow rate of 1 mL/min and 0−145 min linear gradient from 0 to 800 mM NaOAc (pH 8.5). Fractions (1.6 mL) were collected, and radioactivity was determined as described above.

Construction of Plasmids. The pGWD plasmid was used as a PCR template with the following oligonucleotides: 5′-GAGGAATAATAAATGAGTTCTTTTGCCGTTGAA-3′ and 5′-CATCTGTGGTCTTGTCTGAACGAC-3′. For the PCR, we used the proofreading, single-3′-thymidine overhang producing DNA polymerase Easy A (Stratagene), which enabled the subsequent TA cloning into the pBAD-TOPO vector (Invitrogen). This produced plasmid pN11, encoding a 286-amino acid N-terminally deleted GWD with a V5 epitope and a polyhistidine (six-His) tag at the C-terminus. The construct was confirmed by restriction enzyme analysis and DNA sequencing of the entire coding region. The pSBD plasmid was constructed by using the pGWD plasmid as a template with the following oligonucleotides: 5′-GTACTTACCACTGATACC-3′ and 5′-TCTTTTCAGGTGCTTTTCCAC-3′. PCR was performed using Easy A (Stratagene) DNA polymerase, and the fragment was cloned into the pBAD-TOPO vector (Invitrogen). The pSBD plasmid encodes the 286 N-terminal amino acids of StGWD followed by a V5 epitope and a six-His tag. The desired Trp-to-Ala mutants of SBD were constructed using the QuickChange site-directed mutagenesis kit from Stratagene with the pSBD plasmid as a template using complementary oligonucleotides (new codons are bold): W52A, 5′-GATAAACTGTTTTTGCAC**GCG**GGGGCAGTAAAGTTCGGG-3′ and 5′-CCCGAACTTTACTGCCCC**CGC**GTGCAAAAACAGTTTATC-3′; W62A, 5′-GTTCGGGAAAGAAACA**GCG**TCTCTTCCTAATGATCGTCCAG and 5′-CTGGACGATCATTAGGAAGAGA**CGC**TGTTTCTTTCCCGAAC; and W117A, 5′-CGATGAAGCCCACGATAAA**GCG**ATAAAGAATATTGGTGG and 5′-CCACCAATATTCTTTAT**CGC**TTTATCGTGGGCTTCATCG.

Expression and Purification of N11 and SBD. Expression of N11 and SBD was carried out as described previously for WT (*7*) with 0.02% (w/v) L-arabinose used for induction of protein expression. Cells were resuspended and sonicated on ice in 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, and 5 μg/mL leupeptin. Following centrifugation, the sonicate concentration was set at 40 mM with imidazole, 500 mM with NaCl, and 20 mM with $NaPO_4$ (pH 7.4) and the sonicate thereafter loaded onto a 1 mL HisTrap HP (Amersham Biosciences) column equilibrated in buffer A [20 mM $NaPO_4$ (pH 7.4), 500 mM NaCl, 40 mM imidazole, 1 mM PMSF, 1 mM benzamidine, 5 μg/mL leupeptin, and 0.5 mM DTT]. Proteins were eluted with a linear gradient from 0 to 500 mM imidazole in buffer A. Further purification of N11 was required, and the protein solution was transferred into buffer B [50 mM Tris-HCl (pH 7.5), 3 mM EDTA, 2.5 mM DTT, 10% (v/v) glycerol, 0.5 mM PMSF, 1 mM benzamidine, and 5 μg/mL leupeptin] using a HiPrep 26/10 desalting column (Amersham Biosciences). The sample was applied to a MonoQ HR 5/5 (Amersham Biosciences) column equilibrated in buffer A, and proteins were eluted with a linear gradient from 0 to 1 M KCl in buffer B. N11 eluted at 0.11 M KCl, and fractions containing N11 were combined and concentrated using a 15 mL Millipore protein concentrator (10 kDa molecular mass cutoff). Aliquots of N11 were stored at −80 °C until they were used.

Starch Granule Binding Properties. Starch granule binding properties of SBDs were studied by incubating purified SBD (6.7 μg/mL) in 600 μL of 100 mM Hepes-KOH (pH 7.0) with 0.05% Triton X-100 containing 0, 0.1, 0.25, 0.5, 1.5, 2.5, 5.0, and 7.5% potato starch (Sigma). The suspension was incubated at 4 °C under continuous slow rotation for 45 min, and SBD bound to the granules was spun down at 4 °C for 3 min at 13000*g* and 450 μL of the supernatant removed. Following precipitation of protein by acetone, the relative amounts of unbound SBD were quantified following SDS−PAGE. Plots of the data obtained were fitted with an equation equivalent to the Hill equation.

$$B = \frac{B_{max}[S]^h}{K_d^{\,h} + [S]^h} \quad (1)$$

where B is the fraction of protein bound to the starch, [S] is the substrate concentration, K_d is the dissociation constant, and h is the Hill constant.

Soluble Starch Binding Properties. The capacity of SBDs to bind to soluble starch was analyzed by affinity gel electrophoresis (AGE) as follows. Native PAGE was performed at 4 °C and a constant current of 30 mA using gels composed of a separation gel of 7.5% acrylamide in 375 mM Tris-HCl (pH 8.8) and a stacking gel containing 3% acrylamide in 75 mM Tris-HCl (pH 6.8). The separation gel contained 0−3.0 mg/mL solubilized potato amylopectin (Sigma). The running buffer consisted of 50 mM Tris-HCl and 190 mM glycine (pH 8.8). Dissociation constants (K_d) obtained from AGE were calculated as described previously (*23*). For each lane, the relative mobility (R_m) of SBD in gels containing various amounts of substrate (S) and the relative mobility (R_0) of SBD in gels without substrate were determined, and K_d was calculated from $1/R_m$ versus [S] plots using the following expression:

$$\frac{1}{R_m} = \frac{1}{R_0}\left(1 + \frac{[S]}{K_d}\right) \quad (2)$$

RESULTS

Generation of Stable Proteolytic Fragments. Trypsin is a specific serine protease, which cleaves peptide bonds after

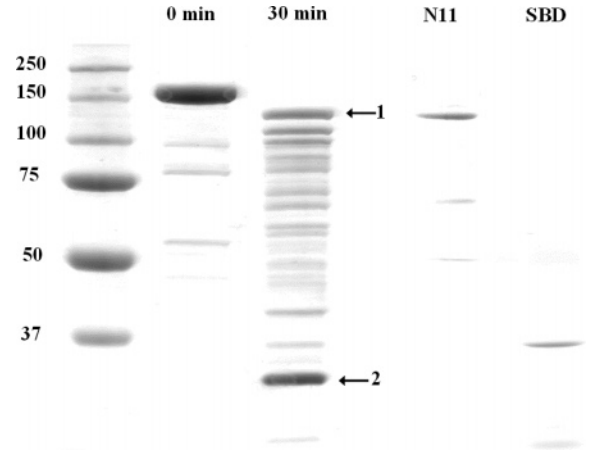

FIGURE 1: Coomassie blue-stained SDS−PAGE gel of StGWD tryptic fragments, purified N11 and SBD. StGWD was digested with trypsin at an enzyme:protease ratio of 50:1. After 30 min, the reaction was terminated by the addition of PMSF. The two major fragments generated by the trypsin treatment are indicated by the arrows. Five micrograms of total protein was loaded per lane (lanes 1 and 2); purified N11 (lane 3) and SBD (lane 4) were also loaded (1 μg of protein per lane).

arginine and lysine residues. It is generally assumed that peptide regions located between protein structural domains are less ordered and solvent exposed and, therefore, are more susceptible to proteolytic cleavage (*24*, *25*). Limited digestion of StGWD (155 kDa) by trypsin was monitored by SDS−PAGE and GWD activity. Initial experiments were performed with a 1:1000 molar ratio of trypsin to StGWD (data not shown). These experiments showed a slow digestion of StGWD over time generating 122 and 33 kDa fragments as the major degradation products with around half of the native StGWD left undigested after 90 min. Digestion using a 1:50 molar ratio of trypsin to StGWD also produced fragments of 122 and 33 kDa, fragments 1 and 2, respectively, as the two major digestion products with no apparent native StGWD left after 30 min (Figure 1). According to the size of these two fragments, they are likely to be generated from a single cleavage of StGWD (155 kDa). Activity analysis showed that 25% of the initial GWD activity remained after digestion for 30 min, most likely resulting from residual GWD activity of 122 kDa fragment 1. To obtain amino acid sequence information about fragments 1 and 2, the protein mixture obtained after trypsin digestion for 30 min was separated by SDS−PAGE and transferred to a PVDF membrane. The N-terminal sequence of fragment 1 was SSFAVERIQ, which shows that trypsin cleaves before serine 287. Sequencing of fragment 2 yielded a MVLTTDT sequence, which is identical to the N-terminal sequence of the mature StGWD protein. These results indicate that trypsin performs the first proteolytic cleavage between R286 and S287 generating fragments 1 and 2 with calculated molecular masses of 122.1 and 33.0 kDa, respectively. These results are consistent with previous results obtained by limited proteolysis with proteinase K which indicate that StGWD consists of an N-terminal domain of ~37 kDa (*10*). As proteolysis of GWD proceeds, the 122 kDa fragment is consumed and several smaller fragments are produced, whereas the 33 kDa fragment appears to be very stable, indicating that this fragment could represent a single structural domain.

Construction of N- and C-Terminally Deleted StGWDs, N11, and SBD. To further investigate the putative glucan, water dikinase activity of 122 kDa fragment 1 and putative starch binding properties of 33 kDa fragment 2, we used recombinant DNA techniques to generate an N-terminally truncated StGWD (fragment 1), termed N11, and a C-terminally truncated StGWD (fragment 2), termed SBD, by inserting start and stop codons at position 286, respectively. The truncated proteins, N11 and SBD (calculated molecular masses of 125.1 and 36.0 kDa, respectively, including the V5 epitope and six-His tag), were expressed in *Escherichia coli* and purified to near homogeneity (Figure 1). Purified N11 and SBD reacted with antibodies raised against WT StGWD and the V5 epitope, and N-terminal sequencing confirmed the predicted amino acid sequence (data not shown).

Conserved Tandem SBD Motif. In the N-terminus of StGWD tandem repeats, SBD-1 and SBD-2 (amino acid residues 50−126 and 381−461, respectively), of a region which is significantly similar in sequence to N-terminal regions of plastidial α-amylases, are located (Figure 2). These conserved tandem repeats have been identified in an N-terminal extension of several plastidial targeted α-amylases from the plant kingdom, i.e., *Arabidopsis*, rice, apple, and potato, where their exact function remains unknown (*26*). The tandem repeats have furthermore been identified in members of the GWD family from potato, *Arabidopsis*, rice, and apple. Interestingly, the GWD2 homologue from *Arabidopsis* contains only a single copy of this conserved sequence in its N-terminal region. Further investigation of this novel sequence reveals the complete conservation of several aromatic amino acid residues, including W52, W62, and W117 (StGWD numbering) (Figure 2). Conserved aromatic amino acid residues, especially tryptophans, are present in CBMs and carbohydrate-binding proteins (*27*−*29*), where they have been shown to play vital roles in the recognition of the polysaccharide molecule.

SBD Is a Novel Carbohydrate-Binding Module. To investigate whether SBD containing the SBD-1 motif comprises a novel CBM, the ability of the protein to bind to insoluble and soluble starch was tested using SBD purified as described above. Binding experiments using granular potato starch show a clear binding of SBD. The data were fitted to eq 1 (see Materials and Methods) and yielded a dissociation constant (K_d) of 7.2 mg/mL and a maximal fraction of bound protein (B_{max}) of 0.53 (Figure 3A), which is comparable to that observed for other CBMs (*30*, *31*). When evaluating the degree of cooperativity for granular starch binding, we calculated a Hill factor (h) of 1.02 (Table 1) which does not provide evidence for more than one site involved in binding of the polysaccharide molecule. Affinity gel electrophoresis (AGE) was employed to evaluate the ability of SBD to interact with soluble starch. The data were fitted to eq 2 (see Materials and Methods) (Figure 3B) and showed a rather weak interaction, [K_d = 1.2 mg/mL (Table 1)] with the solubilized polysaccharide compared to other CBMs (*30*, *31*).

To investigate the roles of the conserved aromatic amino acid residues within the SBD-1 motif, we constructed the following mutant SBDs: W52A, W62A, and W117A. Both W62A and W117A were purified to ~50% purity as determined by SDS−PAGE (data not shown), but the

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                 W52          W62                                                                  W117
                  ↓            ↓                                                                    ↓
StGWD     50 LHWGA--VKFGKETWSLP--NDRPDGTKVYKNKALRTPFVK-------SGSNSILRLEIRDTAIEAIEFLIYDEAHDKWIKNIGGNFH
StGWD    381 LHWAL---SKSPGEWMVPPSSILPPGSIILDK-AAETPFSAS-SSDGLTSKVQSLDIVIEDGNFVGMPFVLLSGEK--WIKNQGSDFY
AtGWD1    43 LHWGA--ILDNKENWVLP--SRSPDRTQNFKNSALRTPFVK-------SGGNSHLKLEIDDPAIHAIEFLIFDESRNKWYKNNGQNFH
AtGWD1   317 LHWAL---SQKGGEWLDPPSDILPPNSLPVRG-AVDTKLTIT-STD-LPSPVQTFELEIEGDSYKGMPFVLNAGER--WIKNNDSDFY
AtAMY3    71 LHWGVSYVGDTGSEWDQPPEDMRPPGSIAIKDYAIETPLKK-LS--EGDSFFEVAINLNLESSVAALNFVLKDEETGAWYQHKGRDFK
AtAMY3   249 VHWGV--CKNGTKKWEIP-SEPYPEETSLFKNKALRTRLQR------KDDGNGSFGLFSLDGKLEGLCFVLKLNEN-TWLNYRGEDFY
MdAMY3    74 LHWGVNYVDDVGSEWDQPPSEMRPAGSVSIKDYAIETPLKESLSPVGGDTSHEVKIDVTPNSAIAAINFVLKDEETGAWYQHRGRDFK
MdAMY3   309 VHWGV--CRDDAKRWEIP-AAPHPPETVVFKDKALRTRLQQ------REDGNGCSGLFTLEEGLAGFLFVFKLNET-MWLNCVGNDFY
             :**.          *  *     *  :      *  * :                              .: *::       * :  . :*
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FIGURE 2: Amino acid sequence alignment of the N-terminal repeat regions of GWDs and plastidial α-amylases (AMY3). Repeated N-terminal regions of StGWD (GenBank accession number AAK11735) and *Arabidopsis* GWD1, AtGWD1 (GenBank accession number AAG47821), were aligned with N-terminal regions of *Arabidopsis* AMY3, AtAMY3 (GenBank accession number AAK91414), and apple AMY3, MdAMY3 (GenBank accession number AAX33231), using ClustalX. An asterisk indicates all eight amino acid residues are conserved, a colon four to seven, and a dot four to six. The three conserved Trp residues [W52, W62, and W117 (StGWD numbering)] are shown in bold and denoted with arrows.

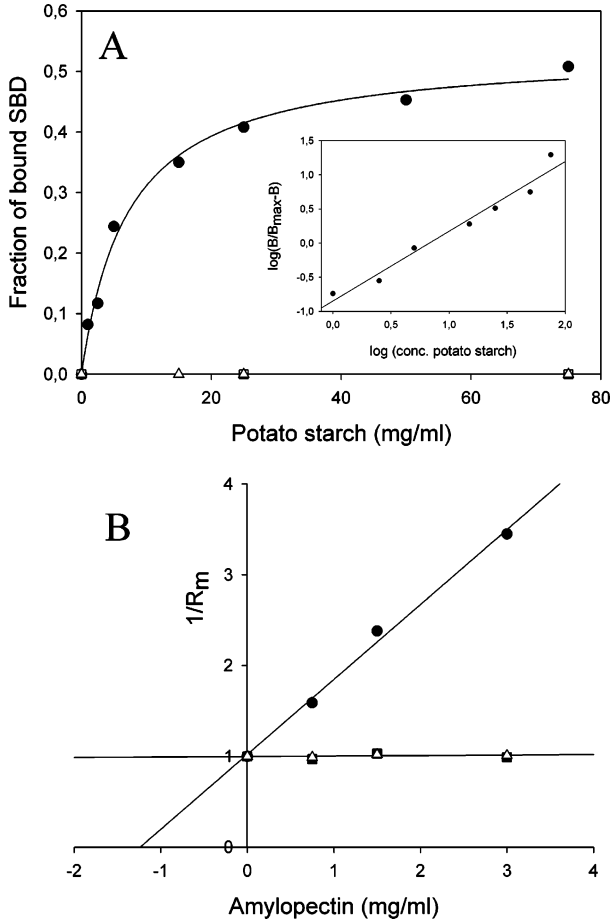

FIGURE 3: Binding of purified SBDs to granular potato starch (A) and soluble potato starch (B). (A) The binding of WT SBD (●), W62A (□), and W117A (△) to various concentrations of granular potato starch was performed as described in Materials and Methods. The Hill plot of WT SBD (●) is shown in the inset. (B) AGE was used to determine the affinities of WT SBD (●), W62A (■), and W117A (△) for soluble potato starch. R_m refers to the relative mobility.

purification of W52A was unsuccessful due to the poor expression and apparent instability of the mutant protein. From analysis with granular potato starch, we did not detect significant levels (K_d > 600 mg/mL) of starch binding for W62A and W117A (Figure 3A). The affinity for solubilized starch was measured by AGE and showed extremely weak glucan binding of W62A and W117A (Figure 3B) with dissociation constants of >100 mg/mL (Table 1), demonstrating that W62 and W117 are vital for binding of the glucan substrate.

Table 1: Binding of SBD to Potato Starch Granules and Solubilized Potato Starch

	granular potato starch			solubilized starch[a]
protein	B_{max}	K_d (mg/mL)	*h*	K_d (mg/mL)
WT	0.53 ± 0.03	7.2 ± 0.9	1.02	1.2 ± 0.1
W62A	<0.01	>600	–	>100
W117A	<0.01	>600	–	>100

[a] As measured by affinity gel electrophoresis.

Table 2: Kinetic Parameters of WT StGWD and N11 GWD[a]

	WT	N11
specific activity (milliunits/mg)	5.8 ± 0.2	12.7 ± 0.7
k_{cat} (s^{-1})	0.015 ± 0.001	0.026 ± 0.001
starch		
K_m (mg/mL)	1.3 ± 0.2	2.7 ± 0.3
k_{cat}/K_m (s^{-1} mg^{-1} mL)	0.012	0.0096
ATP		
K_m (μM)	3.1 ± 0.4	4.5 ± 1.4
k_{cat}/K_m (s^{-1} M^{-1})	4900	5700

[a] Kinetic parameters were measured using soluble potato starch as the glucan substrate. One unit is defined as 1 μmol of phosphate incorporated into α-glucan per minute at 30 °C.

Increased Activity of N11. Activities of WT StGWD and N11 were characterized using soluble potato starch as described previously (*7*). Analyses showed a 2-fold increase in specific activity for N11 compared to that of the WT enzyme (Table 2). However, calculation of k_{cat} yielded a 70% higher specific activity for N11 than for the WT enzyme (Table 2). The starch binding properties of the N-terminus of WT StGWD led us to further investigate the kinetic parameters of N11. With respect to the glucan substrate, N11 exhibited an increase in its K_m value to double that determined for WT (Table 2), resulting in a virtually unaltered catalytic efficiency. From investigation of the kinetic parameters for ATP, we observed essentially similar K_m values for WT and N11, indicating that the lacking N-terminus does not play a role in binding of ATP.

Enhanced Phosphorylation of Amylose by N11. Structural data for native starch have shown that amylopectin and not amylose is phosphorylated. We have previously demonstrated in vitro that WT StGWD exhibits a very low activity for phosphorylation of potato amylose (Am), which most likely reflects a specific substrate requirement of StGWD. The substrate specificities of WT and N11 were tested using commercially available glucan substrates as well as a series of enzymatically generated synthetic amyloses of various mean chain lengths as determined by HPAEC analyses (data

Table 3: Specific Activities of WT StGWD and N11 GWD

substrate	mean chain length (DP)	specific activity (milliunits/mg)[a] WT	N11	ratio of activity (N11:WT)
glycogen	–	<0.005	<0.005	–
potato Ap	–	3.26	6.19	1.9
Am 18	18	<0.005	<0.005	–
Am 24	24	<0.005	<0.005	–
Am 53	53	0.20	1.01	5.1
Am 85	85	0.41	1.18	2.9
potato Am	670	0.07	0.14	2.0

[a] One unit is defined as 1 μmol of phosphate incorporated into α-glucan per minute at 30 °C.

not shown). Glycogen was a very poor substrate for both WT and N11, showing extremely low levels of activity (Table 3). The plant analogue, amylopectin (Ap), proved to be a much better substrate, with N11 displaying almost twice the specific activity as WT. When testing very short chain amyloses with mean chain lengths of degree of polymerization (DP) of 18 and 24, we observed extremely low levels of activity. An increase in mean chain length to DP 53 resulted in a boost in activity with N11 showing more than 5-fold higher activity as compared to WT, indicating that N11 has a stronger preference for phosphorylation of relatively short linear glucan chains than WT. A further increase in the mean chain length to 85 yielded a slightly higher specific activity for both WT and N11, where N11 still exhibited an almost 3-fold higher specific activity than WT. Analysis of purified potato amylose with a mean chain length of DP 670 (*32*) as the substrate resulted in a decrease in activity to 0.07 and 0.14 milliunit/mg for WT and N11, respectively. These results demonstrate that the N-terminal CBM of StGWD plays a very important role in determining glucan substrate specificity and requirements and that these properties can be altered significantly by modification of the N-terminus of GWD.

WT StGWD and N11 Phosphorylate α*-Glucans of Different Lengths*. The results given above, which demonstrate a difference in substrate specificity between WT StGWD and N11, led us to investigate the specific nature of the glucan chains phosphorylated by WT and N11. For this analysis, we used starch isolated from the *waxy* maize genotype as the glucan substrate due to its extremely low levels of starch-bound phosphate and amylose, which enabled us to investigate the phosphate groups introduced by WT StGWD and N11 in a branched glucan system with no background levels of phosphate. WT and N11 were incubated with *waxy* maize starch and [β-^{33}P]ATP as described in Materials and Methods. The enzymatic units of WT and N11 used in this experiment were adjusted to ensure equal incorporation of phosphate. Previously, it has been shown that StGWD phosphorylates both C-3 and C-6 of glucosyl moieties (*6*). To study whether the same phosphorylation pattern was observed for WT and N11, the radioactively labeled glucan was subjected to acid hydrolysis and the resulting monomers were separated by HPAEC (*33*). This analysis showed no difference in the C-3:C-6 phosphorylation ratio for WT and N11 (data not shown).

Figure 4 shows the distribution of linear labeled chains as analyzed by HPAEC after isoamylase-catalyzed debranching of the *waxy* maize amylopectin and indicates that N11

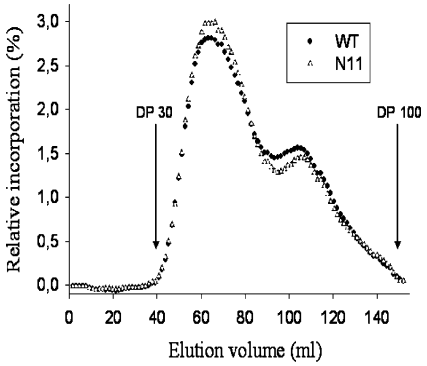

FIGURE 4: Distribution of linear glucan chains phosphorylated by WT StGWD and N11. *Waxy* maize, which does not contain any starch-bound phosphate and amylose, was radiolabeled using [β-^{33}P]-ATP and equivalent units of WT StGWD and N11. Following enzymatic debranching of the amylopectin by isoamylase treatment, the chains were separated by HPAEC, and 1.6 mL fractions were collected. Radioactivity was measured, and the relative level of incorporation of radiolabel observed for WT and N11 is shown. The elution volumes of DP 30 and DP 100 are indicated. Values are averages of three independent experiments.

has a stronger preference for phosphorylating shorter chains than WT. On the basis of previous data (*22*), the phosphorylated chains shown in Figure 4 range between DP 30 and 100. Whereas the separation of neutral α-glucans by HPAEC proceeds strictly according to chain length, the chromatographic behavior of the phosphorylated glucans is more complex because of additional influence by the position and number of phosphate groups in each α-glucan chain. To exclude the possibility that the altered chain profile is a result of differences in the two enzymes in multiple phosphorylation of single chains, we analyzed the number of phosphate groups in each phosphorylated glucan chain using HPEAC analysis at pH 8.5, which ensures ionization of only the phosphate groups during chromatographic separation. Separation of glucans phosphorylated by WT as well as N11 resulted in only one major peak indicating that the labeled glucan chains are monosubstituted (Figure 5). To further analyze the position of the introduced phosphate groups in relation to the branch point, we treated the phosphorylated glucans with isoamylase and β-amylase before separation by HPAEC. β-Amylase hydrolyzes maltose units from the nonreducing end of glucans but cannot bypass a phosphate group. The resulting product will therefore be shortened linear phosphorylated chains in which the phosphate group is situated one or two glucose units from the nonreducing end (*34*). As seen in Figure 6A, the β-amylase-treated chains elute as expected earlier than the full-length chains (Figure 4). Chains phosphorylated by N11 show a clear shift toward shorter chains and a different overall phosphorylation profile compared to that of WT. The relative peak area difference between N11 and WT is shown in Figure 6B. These results further indicate that for N11 the specific site of phosphorylation is closer to the branch point than that of WT.

Quaternary Structure of N11. Previous experiments have demonstrated that WT StGWD exists as a homodimer in its native and active state (*7*). Gel filtration chromatography was employed to investigate the apparent molecular mass of N11, and Figure 7 shows the elution profile of WT StGWD and N11. The molecular mass of N11 was estimated to be 270 kDa, demonstrating that the active form of N11 also exists

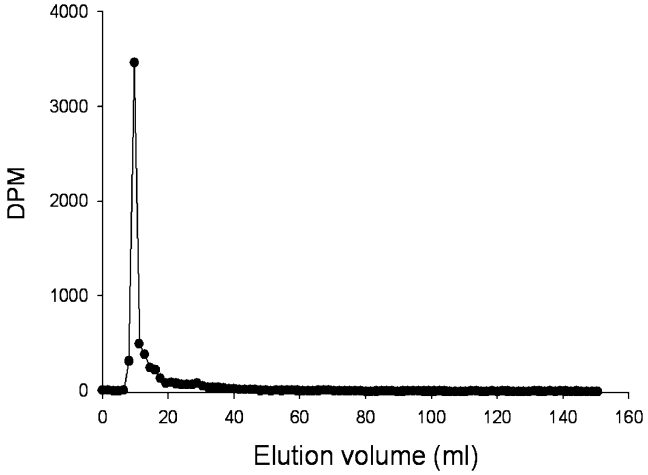

FIGURE 5: Degree of substitution of each phosphorylated glucan chain. ^{33}P-radiolabeled phosphate groups were introduced into *waxy* maize starch by WT StGWD and N11 using [β-^{33}P]ATP. Linear chains were produced by isoamylase treatment and analyzed by HPAEC at pH 8.5, which ensures ionization of only the phosphate group. Both WT and N11 produced a single peak (here shown for WT StGWD), demonstrating that each chain phosphorylated by WT and N11 is monosubstituted.

as a homodimer, thus indicating that the lacking N-terminal CBM is not involved in major interactions leading to the dimeric state.

DISCUSSION

A collected array of evidence suggests that α-glucan, water dikinase plays a pivotal role in the regulation of starch degradation. During starch biosynthesis and, as recently indicated, also during starch mobilization (*17*), GWD is responsible for phosphorylating starch. During synthesis, GWD phosphorylates C-3 as well as C-6 of α-glucan chains and displays a reversible binding toward the starch granule. Very little is known of the structural features of GWD responsible for these observations.

Here we report on the identification and characterization of a novel CBM found in GWDs and plastidial α-amylases. Furthermore, deletion of the N-terminal CBM of StGWD results in an enzyme, N11, with altered starch phosphorylating properties compared to those of WT StGWD. Our results show that trypsin performs its first cleavage of StGWD between R286 and S287, generating two stable fragments of 122 and 33 kDa (Figure 1), indicating that these could represent stable folding domains of StGWD. This agrees well with previous results suggesting that a structural domain of ~37 kDa is found in the far N-terminus of StGWD (*10*). On the basis of these results, SBD and N11, which encode and lack the first 286 amino acid residues of WT StGWD, respectively, were constructed and expressed as recombinant polypeptides.

Analysis of the StGWD amino acid sequence shows the existence of repeat sequences, SBD-1 and SBD-2 (amino acid residues 50−126 and 381−461, respectively), located in the N-terminus with significant similarity to regions in an N-terminal extension specific for the plastidial α-amylases (Figure 2). Interestingly, this extension is not required for catalytic activity in the plastidial α-amylases (*26*). To investigate the function of these repeat motifs further, we successfully expressed and purified SBD containing the

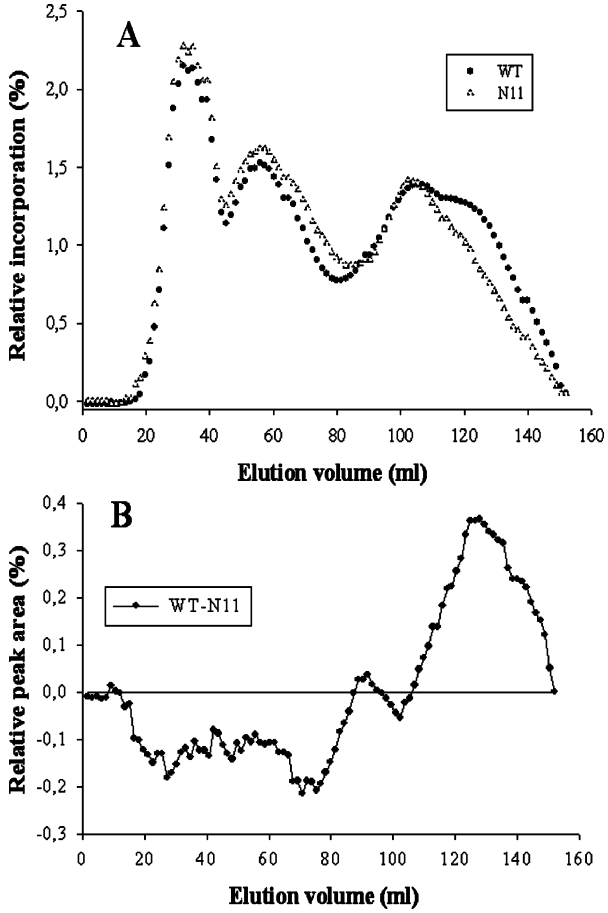

FIGURE 6: Distribution of linear β-amylase-treated glucan chains phosphorylated by WT StGWD and N11. As described previously, *waxy* maize starch was radiolabeled using [β-^{33}P]ATP and equivalent units of WT StGWD and N11. Following isoamylase and β-amylase treatment, which produces linear shortened chains where the phosphate group is situated one or two glucose units from the nonreducing end, the chains were separated by HPAEC, and 1.6 mL fractions were collected. (A) Relative incorporation of radiolabel observed for WT and N11. (B) Difference in phosphorylated chain distribution observed for WT and N11. The difference was the relative peak areas from N11 subtracted from the relative peak areas from WT. Values are averages of three independent experiments.

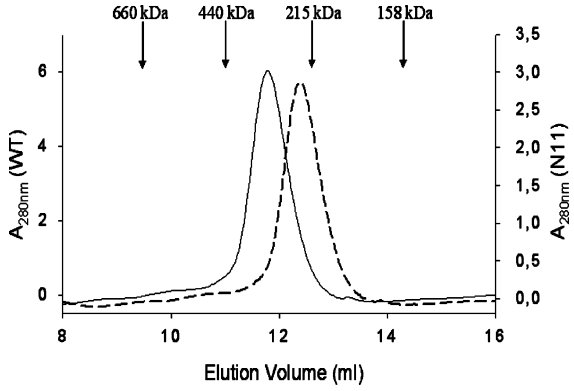

FIGURE 7: Quaternary structure of WT StGWD and N11. The purified enzyme was eluted from a Superdex 200 HR 10/30 column (Amersham Biosciences) in a buffer, as described in Materials and Methods. Elution volumes of the molecular mass standards are indicated with arrows. Data for WT and N11 are shown with solid and dashed lines, respectively.

SBD-1 sequence from *E. coli* and demonstrated starch binding properties of this domain (Figure 3). Binding

experiments with granular potato starch show a clear binding of SBD (K_d = 7.2 mg/mL). In addition to granular starch binding capacity, a clear interaction of SBD with soluble starch (K_d = 1.2 mg/mL) was observed in AGE experiments (Figure 3B). Thus, the results given above demonstrate that SBD contains a novel CBM and binding of the polysaccharide molecule most likely involves residues located within the SBD-1 sequence. Aromatic residues of well-characterized CBMs often play key roles in substrate binding (*12*, *29*), and substitution of either conserved W52 or conserved W117 within the SBD-1 sequence to an alanine residue of SBD (Figure 2) resulted in almost complete loss of starch binding capacity (Figure 3), identifying these tryptophan residues as being active in glucan recognition. The results support the suggestion that SBD contains only one binding site, as also indicated by the Hill factor of 1.0, and employs a binding mechanism similar to that of other CBMs where tryptophan residues play key roles in substrate binding via stacking interactions (*27*, *28*).

Analysis of the kinetic parameters of WT StGWD and N11 representing the remaining StGWD sequence shows that N11 exhibited a 2-fold higher specific activity, and removal of the N-terminal CBM resulted in a K_m value for the glucan substrate double that of WT (Table 2), yielding similar catalytic efficiencies.

When investigating the affinity for the nucleotide substrate, we determined essentially similar K_m values for WT StGWD and N11, demonstrating that the lacking N-terminal CBM is not involved in binding of the ATP molecule (Table 2). These findings are consistent with previous reports which suggest that ATP binding is confined to a domain located in the C-terminus of StGWD (*10*). However, the K_m value for ATP determined in this report (3.1 μM) is 13-fold higher than that reported previously (*6*). The reason for this discrepancy is not known.

Interestingly, our experiments show that N11 has a stronger preference for phosphorylating shorter glucan chains of the branched amylopectin molecule and possibly phosphorylates closer to the α-1,6 branch point than WT StGWD (Figure 6). These results demonstrate that the N-terminal CBM of StGWD plays an important role in determining the specific phosphorylation site and pattern possibly by directing the enzyme to specific glucan chains. Extremely low levels of glucan phosphorylating activity were observed when glycogen was tested as a substrate, which is highly branched and consists of relatively short side chains compared to amylopectin (Table 3). From an investigation of the preference between the two glucan substrates, amylopectin and amylose, we found a shift toward relatively short chain amyloses for N11 compared to WT (Table 3) with N11 showing a 5-fold higher specific activity than WT using Am 53 as the glucan substrate, again demonstrating that the N-terminal CBM plays a major role in determining the substrate preference. It is tempting to speculate that the two N-terminal CBMs each reflect the ability of StGWD to phosphorylate both C-3 and C-6 of the glucosyl residues. However, N11 proved to be capable of phosphorylating both C-3 and C-6, and we found no change in the C-3:C-6 phosphorylation ratio, indicating that this is an intrinsic property of StGWD, which possibly could be confined to the second CBM or to the C-terminal part catalyzing phosphoryl transfer.

It would be of great interest to investigate the starch binding properties of the 24 kDa domain of StGWD containing the SBD-2 motif and compare these to the properties combined, as determined for SBD described in this paper. The two modules within, SBD-1 and SBD-2, are likely to have arisen from a gene duplication event, which indeed would allow for evolution toward a more specialized function. In addition, it is possible that SBD-1 and SBD-2 may act in synergy to bind to their substrate and enable the recognition of target molecules with very distinct chain lengths, branching patterns, etc. This could help explain the altered properties observed for N11. Actually, it is plausible that the two CBMs as found in WT StGWD exhibit bivalent binding properties resulting in quite high glucan affinity. Such affinity, generating very strong glucan−enzyme complexes, can be nonproductive in the classical sense that diffusion of the enzyme is strongly inhibited in the glucan matrix by the nonsubstrate or phosphorylated product glucans preventing diffusion to chains with higher reactivity. It should be noted that in starch very few glucose units (>1%) are phosphorylated so "hyperphosphorylation" of starch is counterproductive for the plant. Slow diffusion of GWD helps such activities remain suppressed.

Previous results indicate that GWD attached to the starch granule in darkened leaves is found in an oxidized state (*8*), and despite oxidized StGWD being incapable of starch phosphorylation in vitro, new experiments have shown that it is capable of autophosphorylation (unpublished results). Furthermore, we have shown that autophosphorylation of reduced StGWD is accompanied by large conformational changes probably involving domain rearrangements (*10*). It can be suggested that this rearrangement enables the CBMs located in the N-terminus of GWD to interact with the glucan molecule. Thus, in darkened leaves, autophosphorylation of the oxidized form of StGWD could result in exposure of the N-terminal CBMs to allow for the noncatalytic association of StGWD with the starch granule.

The presence of a homologous motif in plastidial α-amylases (Figure 2) suggests a general role for this motif in plastidial starch metabolism where it seems to play a role in the diurnal modulation of starch recognition.

From a biotechnological viewpoint, the generation of enzymes with increased activity and altered specificity is of special interest brought about by the increased hydration capacity of starch generated by the phosphate groups (*14*, *35*, *36*). Increasing the phosphate content in crop starch is therefore highly desirable for preventing or minimizing expensive and environmentally unfriendly industrial chemical processes. In this work, the principle of generating enzymes with increased activity and changed substrate specificity has been demonstrated using an enzyme that is important for crop quality. Specifically, we generated an enzyme with higher specificity for phosphorylating shorter α-glucan chains that is relevant for producing freeze−thaw-stable hydrocolloids (*37*). Using recombinant DNA technology, it would be of interest to construct additional deletion mutants of StGWD, as well as fusion of N11 with well-characterized starch-binding domains. We believe this would allow for further investigation of the determinants of substrate specificity and chain phosphorylation patterns. These experiments are currently in progress in our laboratory. This approach may enable rational design of substrate specificity and chain

phosphorylation patterns of GWD, which may lead to *in planta* production of desirable polysaccharides with novel and improved properties for specific industrial uses.

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