Lysine-81 and Threonine-82 on Maize β-Glucosidase Isozyme Glu1 Are the Key Amino Acids Involved in β-Glucosidase Aggregating Factor Binding[†]

Hyun Young Yu,[‡] Farooqahmed S. Kittur,[‡] David R. Bevan,[§] and Asim Esen*[,‡]

Departments of Biological Sciences and Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0406

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ABSTRACT: In certain maize genotypes (nulls), β-glucosidase specifically interacts with a chimeric lectin called β-glucosidase aggregating factor (BGAF), resulting in high molecular weight complexes. Previously, we showed that three regions (S1-T29, E50-N127, and F466-A512) on the maize β-glucosidase isozyme Glu1 are involved in interaction and aggregation with BGAF. Recently, we found that the peptide span I72−T82 within E50−N127 is essential and sufficient for BGAF binding, whereas the S1−T29 and F466−A512 regions are required for formation of large complexes. To define the contribution of individual amino acids in the above three regions to BGAF binding, we constructed mutant β-glucosidases based on sequence differences between maize β-glucosidase and sorghum β-glucosidase (dhurrinase 2, Dhr2), which does not bind BGAF. Binding was evaluated by gel-shift assay and affinity by frontal affinity chromatography (FAC). In the gel-shift assay, Glu1 mutants K81E and T82Y failed to bind BGAF, and their FAC profiles were essentially similar to that of Dhr2, indicating that these two amino acids within the I72−T82 region are important for BGAF binding. Substitution of N481 with E (as in Dhr2) lowered affinity for BGAF, whereas none of the mutations in the S1−T29 region showed any effect on BGAF binding. To further confirm the importance of K81 and T82 for BGAF binding, we produced a number of Dhr2 mutants, and the results showed that all four amino acids (I72, N75, K81, and T82) that differ between Glu1 and Dhr2 in the peptide span I72−T82 are required to impart BGAF-binding ability to Dhr2.

Family 1 β-glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) are of common occurrence in all three domains (Archaea, Eubacteria, and Eukarya) of living organisms. They catalyze the hydrolysis of glycosidic bonds in aryl and alkyl β-D-glucosides and cellobiose or short glucooligosaccharides (*1*). The physiological functions of plant β-glucosidases are reasonably well established. The key function of these enzymes is defense against pathogens and herbivores (*2*−*4*). In maize, β-glucosidases play a key role in defense against pests by hydrolyzing natural β-glucosides. The main natural β-glucoside in maize is 2-*O*-β-D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOAGlc),[1] which is found mostly in young seedling parts. When DIMBOAGlc is hydrolyzed by the enzyme, a toxic aglycon, DIMBOA, is released, which is the major defense chemical in maize against aphids and the European corn borer (*5, 6*). Although the main function of β-glucosidases in plants is defense, they also have a diverse array of other physiological roles such as lignification (*7*−*9*), floral development (*10*), and activation and degradation of phytohormones (*11*−*13*). To match this functional diversity, a typical plant (e.g., *Arabidopsis*, rice, poplar, grape) produces more than 40 β-glucosidase isoforms encoded by a multigene family whose members are subject to temporal and spatial regulation. β-Glucosidases also have significance for biotechnological applications, e.g., aroma and flavor improvement in tea (*14*) and wine (*15*) and cellulose degradation (*16*) for biofuel production.

In some maize genotypes, β-glucosidases interact with a chimeric lectin called β-glucosidase aggregating factor (BGAF) and form high molecular weight complexes that are mostly insoluble aggregates (*17*). BGAF is comprised of an N-terminal dirigent (disease response) domain and a C-terminal jacalin-related lectin (JRL) domain. The JRL domain of BGAF is involved in binding to β-glucosidase, but the JRL domain alone fails to cause aggregation because it is a monomer, whereas BGAF is a dimer. To produce aggregating complexes with β-glucosidases, the dirigent domain is required, and thus the dirigent domain is indirectly involved in β-glucosidase interaction and aggregation. The JRL domain also has a sugar binding site, but the sugar and β-glucosidase binding sites are separate (*18*). The physiological role of BGAF is not known at this time, but recently,

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* Corresponding author. E-mail: aevatan@vt.edu. Tel: 540-231-5894. Fax: 540-231-9307.

[‡] Department of Biological Sciences, Virginia Polytechnic Institute and State University.

[§] Department of Biochemistry, Virginia Polytechnic Institute and State University.

[1] Abbreviations: PBS, phosphate-buffered saline; Glu1, maize β-glucosidase isozyme Glu1; Dhr1, sorghum β-glucosidase isozyme Dhr1; Dhr2, sorghum β-glucosidase isozyme Dhr2; BGAF, β-glucosidase aggregating factor; JRL, Jacalin-related lectin; FAC, frontal affinity chromatography; PCR, polymerase chain reaction; 4-MUGlc, 4-methylumbelliferyl β-D-glucoside; *p*NPGlc, *p*-nitrophenyl β-D-glucoside; IPTG, isopropyl β-D-1-thiogalactopyranoside; DIMBOAGlc, 2-*O*-β-D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one.

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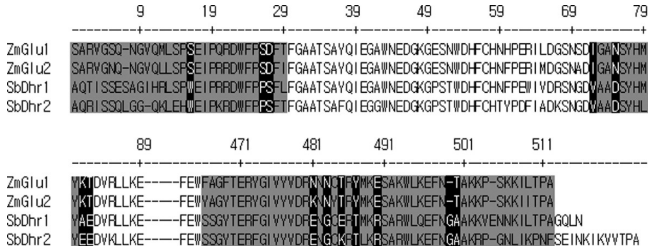

FIGURE 1: Sequence alignment of the maize β-glucosidase isozymes Glu1 and Glu2 and their sorghum homologues Dhr1 and Dhr2. The three regions (S1−T29, I72−T82, and F466−A512) of Glu1 involved directly and indirectly in BGAF binding are shown in gray background, and the amino acids that map to the predicted BGAF-binding site or affect binding indirectly in these regions are shown in black background.

the Hessian fly resistance (*Hfr-1*) gene product, a BGAF homologue from wheat, was shown to prevent Hessian fly larvae from feeding on resistant plants (*19*).

The maize β-glucosidase isozymes Glu1 and Glu2 have high sequence identity (~70%) with the sorghum β-glucosidase isozymes dhurrinase 1 and 2 (Dhr1 and Dhr2) (*20*). However, they differ with respect to substrate specificity and BGAF binding. Maize β-glucosidases hydrolyze a broad spectrum of natural and artificial substrates, including DIMBOAGlc, *p*-nitrophenyl β-D-glucoside (*p*NPGlc), 4-methylumbelliferyl β-D-glucoside (4-MUGlc), and others, but do not hydrolyze dhurrin, a natural substrate of sorghum β-glucosidases. In contrast, sorghum β-glucosidases exhibit narrow substrate specificity in that they hydrolyze their natural substrate dhurrin. Dhr2, but not Dhr1, shows weak activity on certain artificial substrates, including *p*NPGlc and 4-MUGlc. Although dhurrin is not hydrolyzed by Glu1 and Glu2, and DIMBOAGlc is not hydrolyzed by Dhr1 and Dhr2, dhurrin is a potent competitive inhibitor of Glu1 and Glu2 as is DIMBOAGlc of Dhr1 and Dhr2 (*20*). With respect to BGAF binding, maize β-glucosidases can bind BGAF with high affinity, but sorghum β-glucosidases do not (*21*). This difference provides an ideal model system to address questions related to BGAF-binding specificity and mapping the BGAF-binding site on maize β-glucosidases.

Previously, the BGAF-binding site on maize β-glucosidase was broadly mapped by Blanchard et al. (*21*). The results indicated that an N-terminal region (E50−N127) and a C-terminal region (F466−A512) of Glu1 play a key role in BGAF binding. Recently, we found that the N-terminal region I72−T82 on Glu1 is sufficient for BGAF binding, whereas the C-terminal region (F466−A512) plays a minor role in binding BGAF (in review). The BGAF-binding site is away from the active site, and the interaction does not affect enzyme activity (*21, 22*), suggesting that BGAF binding does not block the active site or cause significant conformational change in the enzyme. Although we knew the general BGAF-binding regions on Glu1, the contribution of individual amino acids within these regions responsible for BGAF binding was not defined (Figure 1).

We hypothesized that amino acid substitutions in peptide spans (S1−T29, I72−T82, and F466−A512) are responsible for differences in the BGAF-binding ability of maize and sorghum β-glucosidases. If true, replacement of variant amino acids within these regions in Glu1 with the corresponding residues of Dhr1 or Dhr2 should abolish BGAF interaction, whereas their replacement in Dhr1 or Dhr2 with the corresponding amino acids of Glu1 may confer BGAF-binding ability to Dhr1 and Dhr2. To identify and determine the contribution of individual amino acids on Glu1 to BGAF binding, we performed site-directed mutagenesis (SDM) experiments and evaluated the interaction of mutant enzymes with BGAF by gel-shift assay and frontal affinity chromatography (FAC). Our gel-shift assay and FAC data showed that K81 and T82 are the most important amino acids in Glu1 for BGAF binding because their replacement with the corresponding amino acids from Dhr1 or Dhr2 resulted in complete abolishment of binding or sharply decreased affinity for BGAF. Furthermore, introducing K81 and T82 along with I72 and N75 of Glu1 in Dhr2 conferred upon it the ability to bind BGAF.

EXPERIMENTAL PROCEDURES

Materials. pfu DNA polymerase and plasmid vector pBluescript I SK^+ were purchased from Stratagene (La Jolla, CA). All restriction endonucleases were from New England Biolabs Inc. (Ipswich, MA). T4 DNA ligase was purchased from Invitrogen (Carlsbad, CA). The expression vectors pET21a and pET28a were purchased from Novagen (Madison, WI). The protein purification column, lactosyl agarose affinity matrix, and nickel−Sepharose 6 Fast Flow were purchased from EY Laboratories (San Mateo, CA) and GE Healthcare Biosciences Corp. (Piscataway, NJ), respectively. Artificial substrates, 4-methylumbelliferyl β-D-glucoside (4-MUGlc), and *p*-nitrophenyl β-D-glucoside (*p*NPGlc), and isopropyl β-D-1-thiogalactopyranoside (IPTG) were from Research Products International Corp. (Mount Prospect, IL). Bradford assay reagent was purchased from Bio-Rad (Hercules, CA).

Cloning, Expression, and Purification of Mutant β-Glucosidase. Cloning and expression methodology used for wild-type, chimeric, and mutant β-glucosidases was as described by Cicek et al. (*23*). We constructed Dhr2 mutants instead of Dhr1 because Dhr2 hydrolyzes two artificial substrates, *p*NPGlc and 4-MUGlc (used in FAC and gel-shift experiments, respectively), whereas Dhr1 does not. Mutant β-glucosidase cDNAs (Figure 2) were constructed by overlap extension PCR using wild-type Glu1 or Dhr2 cDNA as template. The cDNAs of wild-type Glu1, Dhr2, and mutant β-glucosidases were blunt end ligated to the *Sma*I digested pBluescript SK^+. The cloned cDNAs were sequenced at the VBI (Virginia Bioinformatics Institute, Virginia Tech) Core Facility, and inserts were isolated by digesting the above constructs with *Nhe*I and *Hin*dIII or *Xho*I, gel-purified, and then ligated to the pET28a expression vector, which had been digested with the same set of restriction endonucleases. The expression construct was transformed into *Escherichia coli* BL21 CodonPlus competent cells. Wild-type Glu1, Dhr2, and mutant β-glucosidase expressions were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.6 mM final concentration) at room temperature for overnight. Cells were collected by centrifugation, suspended in extraction buffer (100 mM Tris-HCl, pH 8, containing 50 mM NaCl and 0.05% SDS), and lysed using a French press. Wild-type and mutant β-glucosidases (all with His tag at the N-terminus) were purified by Ni^{2+}-chelation chromatography. Fractions containing high concentration of protein were combined and dialyzed against two changes of PBS, pH 7.0, overnight. To

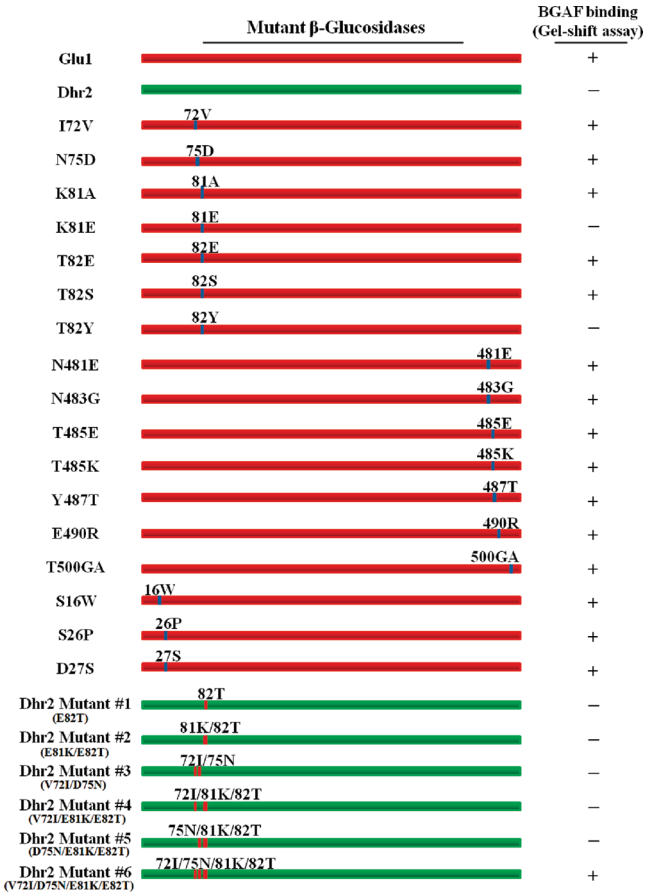

FIGURE 2: Line diagrams of wild-type and mutant β-glucosidases showing the positions of key amino acids that are altered by site-directed mutagenesis to study their role in the Glu1−BGAF interaction. The results of gel-shift assay [(+) for binding and (−) for not binding] are summarized under BGAF binding (gel-shift assay).

ascertain proper folding of the mutant enzymes, the enzyme activities of all mutants were assayed and found to be similar to those of the wild-type enzymes (data not shown).

Cloning and Expression of BGAF cDNA in E. coli and Purification of Recombinant BGAF (rBGAF). Cloning, expression, and purification of recombinant BGAF in *E. coli* were as described by Kittur et al. (*18*). Recombinant BGAF was used for all experiments described in the present work.

Protein Concentration Determination. Protein concentration was determined with the Bradford assay (*24*) according to manufacturer's protocol (Bio-Rad, Hercules, CA). Protein concentrations were also measured spectrophotometrically, using $\varepsilon_{280nm}^{1\%cm} = 18.7$, 17.1, and 14.9 for Glu1, Dhr2, and BGAF, respectively. (The extinction coefficients were computed by web-based tool: http://ca.expasy.org/tools/protparam.html.) Because of underestimation of BGAF concentration by the Bradford method, the protein concentration values obtained by Bradford assay were multiplied by a factor of 2.6 to correct for BGAF concentration.

β-Glucosidase Gel-Shift Assay. Gel-shift assay for β-glucosidase−BGAF interaction was carried out as described by Blanchard et al. (*21*) and Kittur et al. (*18*). Briefly, wild-type Glu1 and its N-terminal (I72−T82) region mutants (I72V, N75D, K81A, T82E, K81E, T82S, and T82Y, each 150 nM) were mixed with 7800 nM BGAF in 100 μL total volume in PBS, pH 7.0, and incubated at room temperature for 2 h. In the case of wild-type Dhr2 and Dhr2 mutants [Dhr2 mutant 1 (E82T), Dhr2 mutant 2 (E81K/E82T), Dhr2 mutant 3 (V72I/D75N), Dhr2 mutant 4 (V72I/E81K/E82T), Dhr2 mutant 5 (D75N/E81K/E82T), and Dhr2 mutant 6 (V72I/D75N/E81K/E82T); see Figure 2], each mutant enzyme (500 nM) was incubated with 39000 nM BGAF. The extreme N-terminal (S1−T29) and C-terminal (F466−A512) region mutants [N481E, N483G, T485E, T485K, Y487T, E490R, T500GA (this construct was made by substituting T500 of Glu1 with GA of Dhr1/Dhr2), S16W, S26P, and D27S, each 150 nM] were mixed with 390 nM BGAF. After 2 h incubation, 25 μL of the mixture was added to 5 μL of native gel sample buffer and electrophoresed through an 8% native gel at 15 mA for 2 h. The native gel was first equilibrated with two changes of 50 mM citrate−100 mM phosphate buffer, pH 5.8, and then incubated with 1 mM solution of the fluorogenic substrate 4-MUGlc at room temperature for 10 min, and β-glucosidase activity zones were visualized under UV light (*21*). Wild-type Glu1 and Dhr2 were used as positive and negative controls, respectively.

Frontal Affinity Chromatography (FAC) and Determination of Dissociation Constant (K_d). Frontal affinity chromatography for determining dissociation constants (K_d) was as described by Ohyama et al. (*25*), with slight modification. Briefly, 6 mL of lactosyl agarose gel was packed in a 10 cm long glass column, and the column was washed with PBS, pH 7.0, for equilibration. Purified BGAF was applied to the column [BGAF is a chimeric lectin and binds to the lactosyl agarose column (*18*)] and washed with the same buffer to remove unbound BGAF. BGAF-bound lactosyl agarose was transferred to a vial and kept at 4 °C until use. For FAC, 200 μL of BGAF-immobilized lactosyl agarose gel was packed in a column (4.5 × 70 mm; bed volume, 1 mL). Wild-type or mutant β-glucosidases (30 mL of 250 nM solution) in PBS, pH 7.0, were applied to the column at a flow rate of 0.25 mL/min at room temperature. Elution of the front was monitored by measuring β-glucosidase activity in the eluate as described by Cicek et al. (*22*). Wild-type Dhr2 (nonbinder) was used for determination of the void volume (V_0).

Determination of Dissociation Constant (K_d) Using FAC Data. The equation for determination of K_d has been described by Ohyama et al. (*25*).

$$V = \frac{B_t}{K_d + [A]_0} + V_0$$

The analyte is denoted as A and the concentration of the analyte applied to the column is referred to as $[A]_0$ (i.e., Glu1, Dhr2, and mutant β-glucosidases). Total concentration of ligand in the column is denoted as B_t (i.e., BGAF). The elution volume (for binder) and the void volume (for nonbinder) of the front refer to V and V_0, respectively, and K_d is the dissociation constant. If K_d, B_t, and V_0 are constant, V will change by $[A]_0$, and the equation can be modified as follows, so that the plot of $1/[A]_0(V - V_0)$ versus $1/[A]_0$ will be linear.

$$\frac{1}{[A]_0(V - V_0)} = \frac{K_d}{B_t}\left(\frac{1}{[A]_0}\right) + \frac{1}{B_t}$$

The values of K_d and B_t can be determined from calculation and once B_t is known, K_d for other enzymes

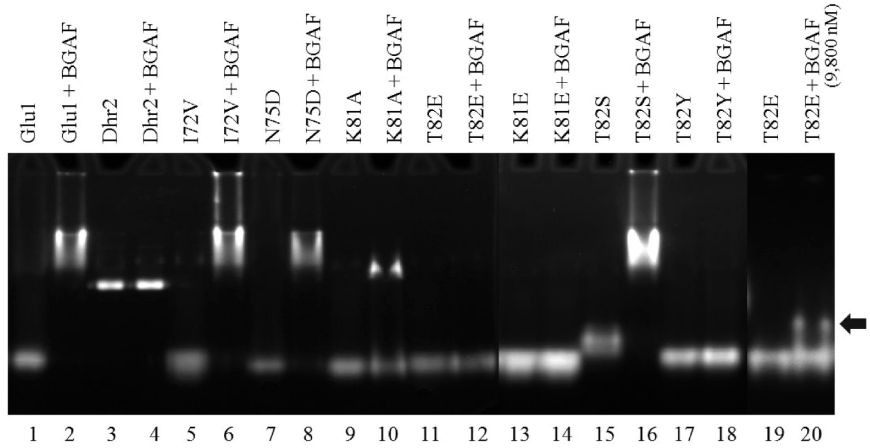

FIGURE 3: Gel-shift assay to detect the interaction of wild-type and mutant Glu1 (mutated in the N-terminal I72−T82 region) with BGAF. The smeared or discrete zones of activity with reduced electrophoretic mobility in lanes containing enzymes + BGAF indicate binding of enzymes to BGAF. Note that the Glu1 mutants K81E, T82E, and T82Y failed to bind BGAF, showing that K81 and T82 are essential for the binding of Glu1 to BGAF at a BGAF concentration of 7800 nM. In the case of T82E, at high molar excess of BGAF (9800 nM) over T82E, a small amount of T82E−BGAF complex is detected [lane 20, upper band (arrowhead)].

is calculated by placing the V value for other enzymes into the equation:

$$K_d = \frac{B_t}{V - V_0} - [A]_0$$

RESULTS AND DISCUSSION

Cloning, Expression, and Purification of Mutant β-Glucosidase. Previously, we showed that an N-terminal region (E50−N127), together with a C-terminal region (F466−A512), of Glu1 is involved in binding to BGAF (*21*). However, recently we found that the I72−T82 peptide span in the N-terminal region (E50−N127) of Glu1 is sufficient for BGAF binding, and the C-terminal region (F466−A512) plays a minor role in interaction with BGAF (in review). In addition, Blanchard et al. showed that chimera 5 (C-5), constructed by replacing S1−T29 of Glu1 with the corresponding region from Dhr1, showed two distinct bands instead of a smear (*21*), so we expected that the extreme N-terminal region (S1−T29) of Glu1 is also involved in BGAF interaction. Therefore, on the basis of the sequence comparison between maize and sorghum β-glucosidases (Figure 1), we generated mutant Glu1 and Dhr2 enzymes by SDM to determine which specific amino acids within the above regions are involved in BGAF binding, as well as their relative contribution to the interaction. We added a His tag to the N-terminus of recombinant mutant β-glucosidases to speed up purification. All mutant β-glucosidases were expressed in high yield (1.0−5.0 mg of enzyme/1000 mL culture volume) in *E. coli* BL21 CodonPlus cells, and the level of purity achieved was 95% or better based on SDS−PAGE profiles (data not shown). The expression level and purity of BGAFs were comparable to those of recombinant mutant β-glucosidases.

Analysis of Glu1 Mutants by Gel-Shift Assay and Frontal Affinity Chromatography (FAC). The interaction between β-glucosidase and BGAF was detected and measured by the gel-shift assay and FAC. In the gel-shift assay, when wild-type or mutant β-glucosidases interact with BGAF, their electrophoretic mobility is reduced, producing either a smeared zone or discrete band of enzyme activity (Figure 3, lanes 2, 6, 8, 10, 16, and 20). In contrast, the electrophoretic mobility of wild-type Dhr2 or certain mutant maize β-glucosidases (i.e., Glu1 mutants), which do not bind to BGAF, remains unaltered (Figure 3, lanes 4, 12, 14, and 18) even in the presence of greater than 50-fold molar excess of BGAF. However, the gel-shift assay does not yield quantitative data to evaluate the contribution of individual amino acids within the predicted BGAF-binding site on Glu1. Therefore, we also used FAC to determine K_d values for interaction of mutant β-glucosidases with BGAF, and the data are summarized in Table 1.

Table 1: Frontal Affinity Chromatography (FAC) Used To Determine the Apparent Dissociation Constants ($K_{d(app)}$) of Wild-Type and Mutant β-Glucosidases[a]

β-glucosidase	BGAF concn (B_t) (nmol)	dissociation constant ($K_{d(app)}$) (nM)
Glu1	6.45	111 ± 11.3
Dhr2	6.45	NB[b]
I72V Glu1	2.08	277 ± 3.4
N75D Glu1	2.08	79 ± 0.7
K81A Glu1	2.08	231 ± 1.5
K81E Glu1	2.08	NB
T82E Glu1	4.87	317 ± 20.4
T82S Glu1	2.08	76 ± 1.4
T82Y Glu1	2.08	NB
N481E Glu1	4.87	232 ± 9.9
N483G Glu1	4.87	152 ± 1.8
T485E Glu1	4.87	177 ± 8.9
T485K Glu1	4.87	144 ± 1
Y487T Glu1	4.87	179 ± 1.1
E490R Glu1	4.87	145 ± 1
T500GA Glu1	4.87	4 ± 1.7
S16W Glu1	4.87	97 ± 5.1
S26P Glu1	4.87	54 ± 5.5
D27S Glu1	4.87	156 ± 7.7
Dhr2 mutant 1	4.87	NB
Dhr2 mutant 2	4.87	NB
Dhr2 mutant 3	4.87	NB
Dhr2 mutant 4	4.87	NB
Dhr2 mutant 5	4.87	NB
Dhr2 mutant 6	3.59	ND[c]

[a] The BGAF concentration on a lactosyl agarose column was determined from the elution profiles of Glu1. All of the values reported are an average of two measurements. $[A]_0$ = 250 nM. [b] NB: not binding. [c] ND: not determined.

The N-terminal peptide span I72−T82 of Glu1 is sufficient for BGAF binding. The sequence alignment indicates that

maize β-glucosidases differ from their dhurrinase homologues by four amino acid substitutions within this 11 amino acid long region (Figure 1). Therefore, we produced mutants of Glu1 in which these four amino acids are substituted with corresponding residues from the dhurrinase counterparts. The gel-shift assays showed that two of the single mutants (I72V and N75D) produced smeared zones of reduced mobility after incubation with BGAF (Figure 3, lanes 6, and 8, respectively), suggesting that neither I72 nor N75 is essential and sufficient for BGAF binding. However, the FAC data showed that the I72V had about 2.5-fold reduction in affinity (K_d = 277 nM) and N75D had slight increase in affinity (K_d = 79 nM) (Table 1), although these changes in affinity were not evident in the more qualitative gel-shift assays. These K_d value changes suggest that both I72 and N75 are involved in BGAF interaction but their relative contribution is not critical for BGAF binding.

The K81A single mutant also interacted with BGAF, yielding two discrete bands (Figure 3, lane 10). The top band is a K81A−BGAF complex, whereas the bottom band is free, unreacted K81A mutant (Figure 3, lane 10). The gel-shift data indicate that the K81A mutant interacts with BGAF, but its affinity for BGAF is lower than that of the wild-type Glu1. This observation is consistent with the FAC data in that the K_d for the K81A mutant (231 nM) is higher than that for wild-type Glu1 (Table 1). When we mutated K81 to E (as in Dhr2), the mutant showed no detectable interaction with BGAF in the gel-shift assay (Figure 3, lane 14) and no binding in FAC (Figure 4A, Table 1). In the K to E substitution, a longer, positively charged side chain is replaced with a smaller, negatively charged side chain, which could disrupt the Glu1−BGAF interaction. These results illustrate the critical importance of K81 to Glu1−BGAF binding. Substitution of T82 to the corresponding residue (E) in Dhr1 and Dhr2 abolished binding to BGAF even when the BGAF concentration was 52-fold higher than that of mutant (Figure 3, lane 12). However, the T82E mutant yielded a minor retarded band that was visible above the free, unreacted T82E band when the BGAF concentration was in about a 65-fold molar excess (Figure 3, lane 20, arrowhead), indicating that the T82E mutation drastically reduced the affinity of this mutant enzyme to BGAF when compared to wild-type Glu1. These data are consistent with the FAC data in that the T82E mutant had about a 3-fold lower affinity (K_d = 317 nM) than the wild-type Glu1 for BGAF. When examining other mutants at position 82, the T82S mutant bound to BGAF in the gel-shift assay, whereas the T82Y mutant did not bind at all (Figure 3, lanes 16 and 18, respectively), which is consistent with the FAC results in which the K_d of the T82S mutant was comparable to that of Glu1 (76 vs 111 nM) and the T82Y mutant showed no detectable binding (Figure 4A, Table 1). These results demonstrate the importance of the role of T82 in the binding of Glu1 to BGAF. Interestingly, the T82S mutant was as effective as wild-type Glu1 in binding to BGAF, suggesting the importance of a hydroxyl group in the interaction. However, T82Y did not bind to BGAF, probably due to the much bulkier side chain.

The extreme N- (S1−T29) and C-terminal (F466−A512) regions of Glu1 also play a role in BGAF interaction and formation of large, insoluble complexes. In the C-terminal region, the peptide span A501−A512 could not be resolved

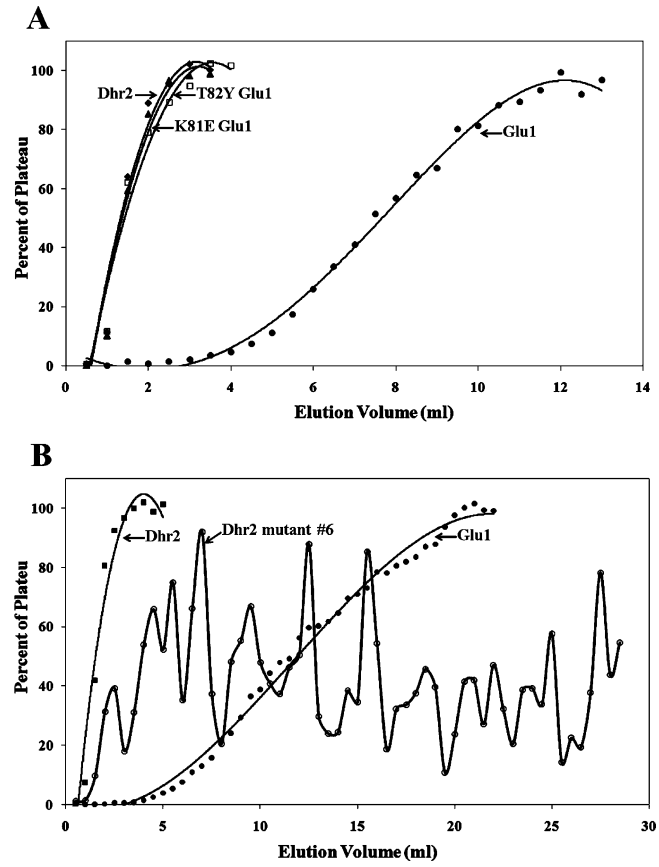

FIGURE 4: FAC profiles of wild-type and mutant β-glucosidases. (A) FAC profiles of Glu1 (●), Dhr2 (◆), K81E (▲), and T82Y (□). Note that the elution volume of K81E and T82Y is the same as that of Dhr2 (nonbinder). (B) FAC profiles of Glu1 (●), Dhr2 (■), and Dhr2 mutant 6 (○). Note that the shape of the FAC profile from the Dhr2 quadruple mutant is strikingly different from that of wild-type Glu1, and we are not able to calculate a K_d value for its interaction with BGAF.

in the 3D structure of Glu1 (Figure 5). Thus, the role of the region A501−A512 in binding of Glu1 to BGAF is not yet defined, but this region apparently does not mask the residues involved in the binding interaction (Figure 5). The peptide span F466−R480 in Glu1 is not likely involved in BGAF interaction and aggregation because it is in the aglycon binding site of the Glu1 active site (Figure 5) (*22*), which is away from the key BGAF-binding site (I72−T82) on Glu1. Thus we mutated the amino acids in the C-terminal peptide span N481−T500 of Glu1 that are different between Glu1 and Dhr1/Dhr2 and tested the mutants for BGAF binding by gel-shift assay. Each of these single Glu1 mutants (N481E, N483G, T485K, Y487T, E490R, and T500GA) except T485E appeared to have a high affinity for BGAF based on the gel-shift assay, producing patterns similar to that of wild-type Glu1 (Figure 6). In the case of T485E, a low concentration of BGAF (390 nM) was not sufficient for full interaction with T485E (Figure 6, lane 8). However, it produced a smeared gel-shift pattern similar to that of wild-type Glu1 when the BGAF concentration was raised to 7800 nM and above, which is at least a 52-fold molar excess (data not shown). Interestingly, most of these mutants differed in affinity by no more than a factor of about 2 based on K_d values derived from FAC, including the T485E mutant. We believe the discrepancies between the gel-shift and FAC data result from the qualitative nature of the gel-shift assays. Of

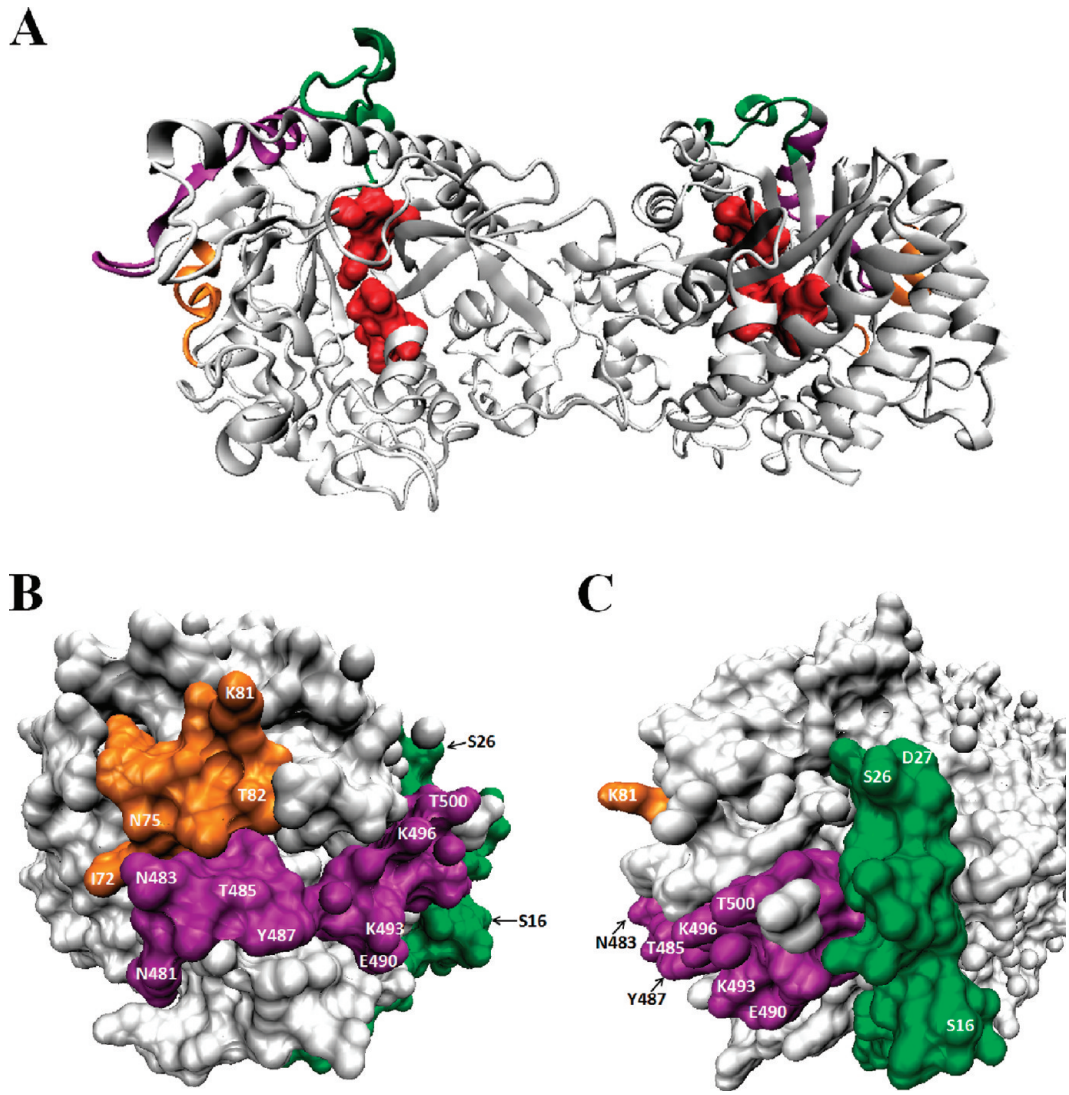

FIGURE 5: Three-dimensional structure of maize β-glucosidase isozyme Glu1 (PDB entry 1E1E). The three regions making up the BGAF-binding site on Glu1, extreme N-terminal ($Ser^{1}-Thr^{29}$), N-terminal ($Ile^{72}-Thr^{82}$), and C-terminal regions ($Asn^{481}-Thr^{500}$), are shown in green, orange, and purple, respectively. (A) Structure of Glu1 dimer showing the BGAF-binding site and the active site (shown in red; the two β-strands shown in red correspond to ^{188}TFNEP^{192} and ^{404}ITENG^{408} motifs, which form the active site in Glu1). (B, C) The amino acids that are postulated to be critical for BGAF binding are labeled. (B) Front view of Glu1. (C) Side view of Glu1. Visual Molecular Dynamics (VMD) was used to generate these images [newcartoon mode (A) and surface mode (B, C)].

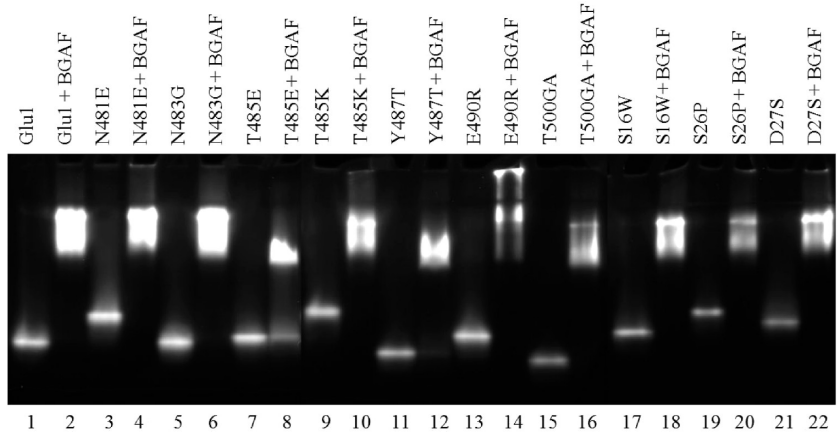

FIGURE 6: Gel-shift assay to detect the binding of wild-type and mutant Glu1 (mutated in the extreme N-terminal S1−T29 and C-terminal F466−A512 regions) to BGAF. The smeared or distinct zones of activity with reduced mobility in lanes containing enzyme + BGAF show the binding of enzymes to BGAF. Note that all single mutant β-glucosidases (refer to Figure 2) interact with BGAF.

particular note is the greatly enhanced affinity of the T500GA mutant (K_d = 4 nM) for BGAF. Based on the 3D structure of Glu1 (Figure 5B), T500 in Glu1 is located at a distance (16.2 Å) from the key contact residues (K81 and T82). The reason for the enhanced affinity is not obvious from the available data, but the results suggest that a hydrophobic

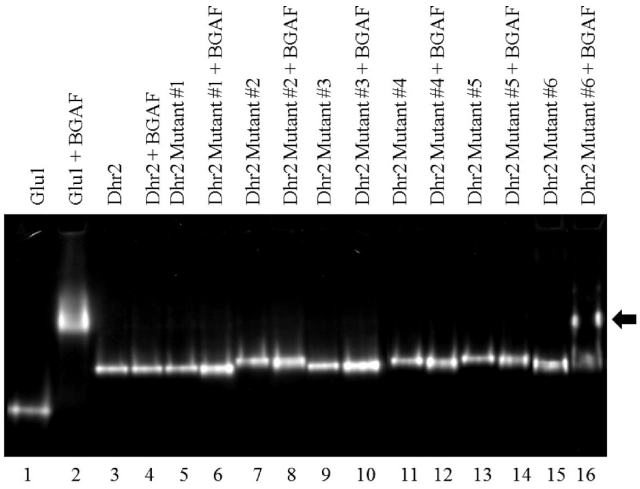

FIGURE 7: Gel-shift assay to detect the binding of wild-type and Dhr2 mutants to BGAF. Activity zones with reduced mobility in lanes (e.g., 2 and 16) containing enzyme + BGAF indicate binding to BGAF. Among six Dhr2 mutants (see Figure 2), a Dhr2 quadruple mutant (V72I/D75N/E81K/E82T) was the only one that bound to BGAF [lane 16, upper band (arrowhead)] albeit with low affinity as evident from the amount of unreacted (lane 16, lower band) mutant enzyme.

contact in the region of residue 500 in Glu1 may be important. In general, the results from the gel-shift assays and FAC experiments suggest that the residues in the peptide span N481−T500 of Glu1 contribute to, but are not essential for, binding to BGAF.

For the extreme N-terminal region S1−T29, we produced three single mutants of Glu1 (S16W, S26P, and D27S) by changing the amino acids at positions 16, 26, and 27 to their Dhr1/Dhr2 counterparts. Although these amino acids are not in the predicted BGAF-binding site, they are chosen because they represent nonequivalent and nonconservative substitutions between Glu1 and Dhr1/Dhr2. Gel-shift assay results indicated no discernible differences between the three single mutants (S16W, S26P, and D27S) and wild-type Glu1 in BGAF binding because they all produced a smeared enzyme activity as did wild-type Glu1 (Figure 6, lanes 18, 20, and 22), suggesting that these three sites individually do not play a role in the Glu1−BGAF interaction. In addition, the K_d values for the binding of these mutants to BGAF (Table 1) do not differ by more than about 2-fold from that for the wild-type Glu1, suggesting that these residues are not essential for the interaction.

Analysis of Dhr2 Mutants by Gel-Shift Assay and FAC. To confirm the importance of the two key amino acids (i.e., K81 and T82 of Glu1) in the peptide span I72−T82 in Glu1 for BGAF binding, we produced a number of Dhr2 mutants by SDM (Figure 2). Initially, we postulated that if K81 and T82 are essential for BGAF binding, mutating the corresponding amino acids of Dhr2 (i.e., E81 and E82) to K and T should confer BGAF-binding ability to Dhr2. The results showed that neither the single mutant E82T nor the double mutant (E81K/E82T) gained the ability to bind to BGAF even in the presence of an 80-fold molar excess of BGAF (39000 nM) (Figure 7, lanes, 6 and 8, respectively). Moreover, no evidence of binding was observed in the FAC assay. Another Dhr2 double mutant (V72I/D75N) involving two additional amino acids that differentiate Glu1 from Dhr2 in the peptide span I72−T82 also failed to bind to BGAF (Figure 7, lane 10), and binding was not observed in the FAC assay. This finding was not surprising because the V72I/D75N Dhr2 mutant lacks the two key amino acids (K81 and T82) of Glu1 for BGAF binding. On the basis of these results, we concluded that K81 and T82 are not sufficient to confer BGAF-binding ability to Dhr2 and thus additional amino acids (i.e., I72 and N75) from Glu1 are required. Consequently, we introduced additional amino acid substitutions into the E81K/E82T Dhr2 double mutant in order to confer to it BGAF-binding ability. Neither triple mutants V72I/E81K/E82T or D75N/E81K/E82T showed any BGAF-binding activity based on gel-shift assays (Figure 7, lanes 12 and 14) or FAC. Finally, we produced a quadruple mutant of Dhr2, V72I/D75N/E81K/E82T, and this mutant showed interaction with BGAF (Figure 7, lane 16). However, the quadruple mutant had lower affinity for BGAF than wild-type Glu1, as is evident from a large amount of the unreacted Dhr2 quadruple mutant enzyme with a 78-fold molar excess of BGAF (Figure 7, lane 16, arrowhead). This result suggested that although four amino acids (I72, N75, K81, and T82) on Glu1 are sufficient for BGAF binding, other regions [extreme N-terminal (S1−T29) and C-terminal (F466−A512) regions] on Glu1 are also important to enhance Glu1 affinity to BGAF. However, we could not obtain K_d data for the quadruple Dhr2 mutant [Dhr2 mutant 6 (V72I/D75N/E81K/E82T)] because of its irregular FAC elution pattern (Figure 4B). This elution pattern may be due to rapid binding and dissociation of the mutant enzyme from the column. Nevertheless, it binds to the column and elutes after Dhr2, indicating that four unique Glu1 amino acids (I72, N75, K81, and T82) are required to confer to Dhr2 BGAF-binding ability.

Superimposition of the Structure of Glu1 and Dhr1. Superimposing the structure of Glu1 and Dhr1 allows us to speculate as to the nature of the Glu1−BGAF interaction (Figure 8). Although we showed that BGAF is a chimeric lectin consisting of two domains (*18*), we do not know the location and nature of the β-glucosidase binding site on the JRL domain. Studies to map the site using SDM and domain swapping are currently in progress. Because our data show that the K81E mutant abolishes BGAF binding and the T82E, N481E, and T485E mutations reduce affinity (K_d = 317, 232, and 177 nM, respectively), it is likely that these mutations disrupt and modify ionic and hydrogen-bonding interactions between β-glucosidase and BGAF in their respective binding sites. We expect that the actual β-glucosidase binding site on BGAF has one or more acidic amino acids (D or E) and these acidic amino acids play a key role in the β-glucosidase−BGAF interaction.

Sequence Comparison of Plant β-Glucosidases. When we aligned and compared sequences of β-glucosidases from other plants with the maize β-glucosidase isozymes Glu1 and Glu2, we noted that only maize β-glucosidases have threonine at position 82 within the N-terminal peptide span I72−T82 (Figure 9). This position is occupied by a highly conserved glutamic acid in other plant β-glucosidases. Position 81, which is occupied by lysine in Glu1, is less conserved, in that other plant β-glucosidases have either lysine or glutamic acid at this position. As for I72 and N75, both of these amino acids are important but not to the extent that K81 and T82 are; they are essential for BGAF binding of the Dhr2 quadruple mutant (Figure 7, lane 16). Based on

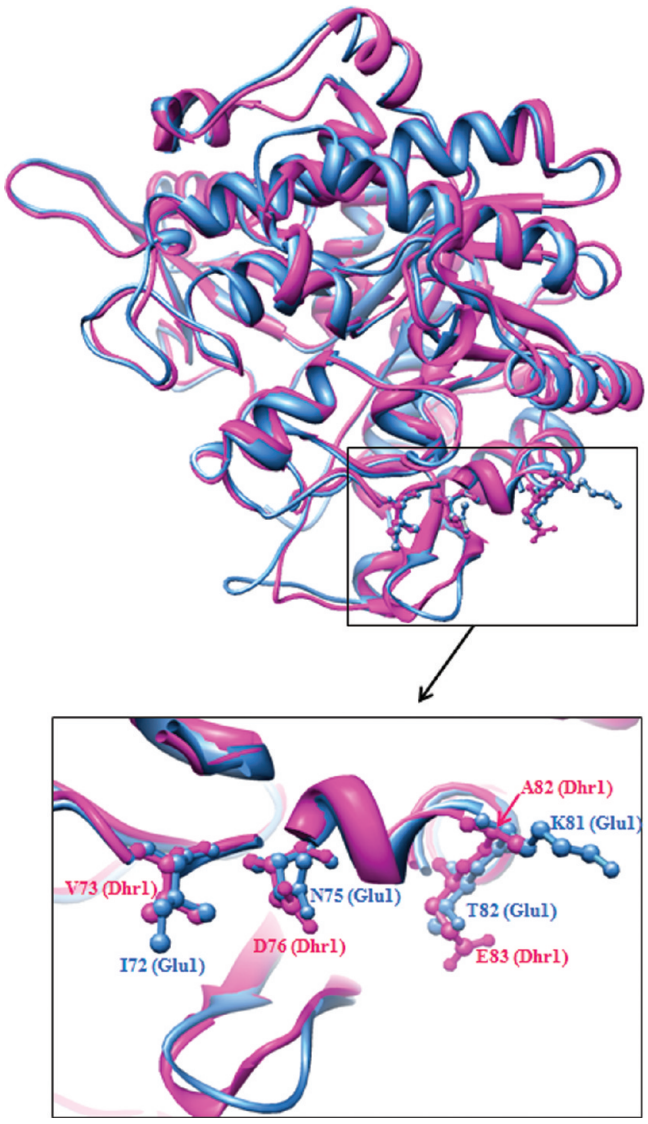

FIGURE 8: Superimposition of the structure of maize Glu1 (blue; PBD entry 1E1E) and sorghum Dhr1 (red; PDB entry 1V03). I72, N75, K81, and T82 of Glu1 are replaced by V73, D76, A82, and E83 in Dhr1, respectively, which account for the lack of BGAF binding to Dhr1. UCSFChimera was used to generate this image.

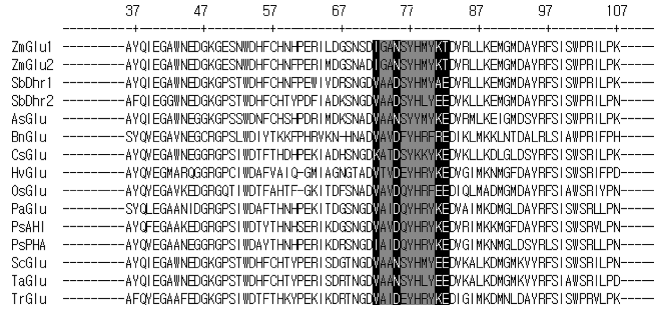

FIGURE 9: Sequence alignment of selected plant β-glucosidases. Residue numbering is based on the sequence of ZmGlu1. The BGAF-binding region I72−T82 is in gray background, and I72, N75, K81, and T82, the four amino acid residues that play a critical role in BGAF-binding, are shown in black background. Only maize β-glucosidases have threonine at position 82, and the other plant β-glucosidases have a highly conserved glutamic acid at the same position. Key: ZmGlu1, *Zea mays* β-glucosidase 1; ZmGlu2, *Z. mays* β-glucosidase 2; SbDhr1, *Sorghum bicolor* dhurrinase 1; SbDhr2, *S. bicolor* dhurrinase 2; AsGlu, *Avena sativa* β-glucosidase; BnGlu, *Brassica napus* β-glucosidase; HvGlu, *Hordeum vulgare* β-glucosidase; OsGlu, *Oryza sativa* β-glucosidase; PaGlu, *Prunus avium* β-glucosidase; PsAHI, *Prunus serotina* amygdalin hydrolase isoform AH I; PsPHA, *P. serotina* prunasin hydrolase isoform PH A; ScGlu, *Secale cereale* β-glucosidase; TaGlu, *Triticum aestivum* β-glucosidase; TrGlu, *Trifolium repens* β-glucosidase.

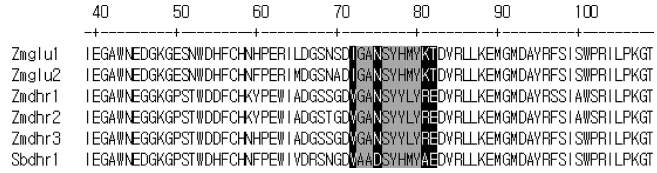

FIGURE 10: Sequence alignment of maize Glu1 and Glu2 with the predicted amino acid sequences of dhurrinase-like maize β-glucosidases and Dhr1. Residue numbering is based on the sequence of ZmGlu1. The BGAF-binding site on β-glucosidases is shown in gray background, and the key amino acids within this region are shown in black background.

the sequence alignment of plant β-glucosidases, there are no other plant β-glucosidases that have all four of the amino acids (I72, N75, K81, and T82) that are directly involved in BGAF binding. Thus, the sequence alignment results also support the hypothesis that the β-glucosidase−BGAF interaction is specific to the maize β-glucosidase isozymes, Glu1 and Glu2.

Recently, three more maize β-glucosidase genes, *Zmdhr1*, *Zmdhr2*, and *Zmdhr3*, whose predicted amino acid sequences are highly similar to dhurrinases, and are highly expressed in immature leaves, were reported by Coneva et al. (*26*). They aligned the predicted amino acid sequence of these gene products with Glu1, Glu2, and Dhr1 and found that the predicted proteins are more closely related by sequence to sorghum dhurrinase than maize β-glucosidase isozymes, Glu1 and Glu2 (*26*). The most interesting difference between these three *Zmdhr* gene products and Glu1 and Glu2 relates to the BGAF-binding site (I72−T82) on maize β-glucosidases. The three key amino acids (I72, K81, and T82) for BGAF binding in Glu1 and Glu2 are substituted by V, R, and E, respectively, in putative Zmdhr1, Zmdhr2, and Zmdhr3 proteins (Figure 10). Therefore, we predict that all three putative Zmdhr proteins will not interact with BGAF.

The Physiological Function of BGAF. Interaction of other BGAF-like proteins with β-glucosidases has been reported in the literature. The best examples of these proteins are myrosinase binding proteins (MBPs) (*27*) and PBP1 (PYK10-binding protein 1) (*28*). MBPs are found in *Brassica napus* and other members of the family Brassicaceae (*27*). They have one or more jacalin-related lectin (JRL) domains (*29*), and their binding to myrosinases does not seem to affect enzyme activity (*27, 30*). Myrosinase and MBPs are localized separately in developing seeds. Myrosinase is present in the myrosin cells, whereas MBP is localized in all cells in the mature embryo, except myrosin cells, epidermis, and provascular tissue, but colocalized in later growth stages (*27, 31*). PBP1 is a β-glucosidase binding protein in *Arabidopsis thaliana* and also has a jacalin-like lectin domain (*28*). PBP1 and PYK10 (a β-glucosidase) are localized in the cytosol and ER bodies, respectively, and the two proteins encounter each other and interact when tissue is damaged (*28*). PBP1 interacts with inactive PYK10 (soluble form) but not active PYK10 (insoluble form) and is believed to play a role in activation of PYK10 (*28*). The physiological functions of

these β-glucosidase binding proteins (BGAF, MBPs, and PBP1) are not well understood. It has been speculated that they function in plant defense systems because they and β-glucosidase are initially localized separately but encounter each other and interact upon tissue damage due to herbivory and pathogen infection (*32*−*34*). Recently, ground-breaking studies on the role of a BGAF-like protein from wheat (HFR1) in plant defense have been reported by Subramanyam et al. (*19*). Both BGAF and HFR1 have an N-terminal dirigent and a C-terminal JRL domain as well as lectin activity. A high level of HFR1 is accumulated at the site of larva feeding, and it deterred the Hessian fly larva from feeding on resistant plants (*19*). It is conceivable that the function of BGAF is similar to that of HFR1 in that it deters the European corn borer, a major pest of maize. Thus, one of the foci of future research on BGAF should be determination of its role in defense against the European corn borer and other maize pests.

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