Maize β-Glucosidase-aggregating Factor Is a Polyspecific Jacalin-related Chimeric Lectin, and Its Lectin Domain Is Responsible for β-Glucosidase Aggregation*

Received for publication, August 4, 2006, and in revised form, January 6, 2007. Published, JBC Papers in Press, January 8, 2007, DOI 10.1074/jbc.M607417200

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In certain maize genotypes, called “null,” β-glucosidase does not enter gels and therefore cannot be detected on zymograms after electrophoresis. Such genotypes were originally thought to be homozygous for a null allele at the glu1 gene and thus devoid of enzyme. We have shown that a β-glucosidase-aggregating factor (BGAF) is responsible for the “null” phenotype. BGAF is a chimeric protein consisting of two distinct domains: the disease response or “dirigent” domain and the jacalin-related lectin (JRL) domain. First, it was not known whether the lectin domain in BGAF is functional. Second, it was not known which of the two BGAF domains is involved in β-glucosidase binding and aggregation. To this end, we purified BGAF to homogeneity from a maize null inbred line called H95. The purified protein gave a single band on SDS-PAGE, and the native protein was a homodimer of 32-kDa monomers. Native and recombinant BGAF (produced in Escherichia coli) agglutinated rabbit erythrocytes, and various carbohydrates and glycoproteins inhibited their hemagglutination activity. Sugars did not have any effect on the binding of BGAF to the β-glucosidase isozyme 1 (Glu1), and the BGAF-Glu1 complex could still bind lactosyl-agaroose, indicating that the sugar-binding site of BGAF is distinct from the β-glucosidase-binding site. Neither the dirigent nor the JRL domains alone (produced separately in E. coli) produced aggregates of Glu1 based on results from pull-down assays. However, gel shift and competitive binding assays indicated that the JRL domain binds β-glucosidase without causing it to aggregate. These results with those from deletion mutagenesis and replacement of the JRL domain of a BGAF homolog from sorghum, which does not bind Glu1, with that from maize allowed us to conclude that the JRL domain of BGAF is responsible for its lectin and β-glucosidase binding and aggregating activities.

β-Glucosidases (β-D-glucoside glucohydrolase; EC 3.2.1.21) hydrolyze β-glycosidic linkage(s) in alkyl and aryl β-D-glucosides, glycoproteins, and glycolipids and that between two glucose residues in β-linked oligosaccharides. They are found in all three (Archaea, Eubacteria, and Eukarya) domains of living organisms and play important roles in various biological processes, such as degradation of cellulosic biomass, hydrolysis of glycoconjugates, cyanogenesis, and modification of secondary metabolites (1). In plants, β-glucosidase activity is shown to be involved or implicated in several processes, such as defense against pathogens and herbivores (2–3), hydrolysis of phytohormones (4), floral development (5), lignification, and cell wall metabolism (6). In maize, two isozymes of β-glucosidase have been isolated and characterized with respect to structure, including three-dimensional structure, and function (7, 8). The major function of maize β-glucosidases is in the defense against pathogens and herbivores by releasing toxic aglycones, such as hydroxamic acids, from their glucosides. The predominant hydroxamic acid glucoside in maize is DIMBOAGlc, whose aglycone DIMBOA is the primary defense chemical against aphids and the European corn borer (Ostrinia nubilalis). A number of studies have shown a high degree of correlation between DIMBOAGlc content of maize and the level of resistance to insects and pathogens (9, 10).

In certain maize genotypes, β-glucosidase occurs as a part of large insoluble complexes (11). Zymograms of such genotypes are devoid of enzyme bands. Originally, these genotypes were thought to be homozygous for a “null” allele at the glu1 (maize β-glucosidase isozyme Glu1) gene. However, we have shown that the so-called “null” genotypes have β-glucosidase activity when assayed in solution. The enzyme is not detected on zymograms, because it occurs as large quaternary complexes whose native molecular masses may exceed 1.5 × 106 Da. Furthermore, we have shown that the null phenotype is due to a 32-kDa protein called β-glucosidase-aggregating factor (BGAF) (GenBankTM AA011261), which specifically binds and aggregates maize β-glucosidase, yielding high molecular weight complexes (12).

Recently, we cloned the cDNA encoding BGAF and identified it as a protein belonging to the jasmonic acid-induced protein family (13). BGAF is a modular protein consisting of an N-terminal disease response or dirigent domain and a C-termi-

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*This work was supported by National Science Foundation Grant MCB-0417143 (to A. E. and D. R. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2The abbreviations used are: DIMBOAGlc, 2-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one; PBS, phosphate-buffered saline; BGAF, β-glucosidase-aggregating factor; nBGAF, native BGAF; rBGAF, recombinant BGAF; JRL, Jacalin-related lectin; mJRL, mannospecific JRL; gJRL, galactose-specific JRL; HPLC, high performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; PSM, porcine stomach mucin; 4-MUGlc, 4-methylumbelliferyl-β-D-glucoside; MIC, minimum inhibitory concentration; MS, mass spectrometry; Neu5Ac, N-acetyl-neuraminic acid; Sb, Sorghum bicolor; Zm, Zea mays.
nal jacalin-related lectin (JRL) domain. Dirigent proteins exclusively occur in plants and have been implicated in defense and lignin biosynthesis (14, 15). There are 92 dirigent proteins in the pfam data base (available on the World Wide Web at www.sanger.ac.uk/Software/Pfam/). Of these, 83 are stand-alone, and nine are modular. Interestingly, it is only the JRL domain that occurs as a fusion partner in modular dirigent proteins, and they all come from the family Poaceae. Members of the JRL family are lectins and are thought to be involved in plant defense (16–18). Presently, there are 312 JRLs in the pfam data base. The majority of them come from plants, a few occur in animals, and one occurs in bacterium. Some members of the JRL family have modular structure comprising multiple copies of JRL domains in tandem (2–6 copies) or fused to other proteins, such as dirigent, Kelch (galactose oxidase), NB-ARC (nucleotide-binding proteins involved in ubiquitination), FBA (F-BOX), endonuclease, and a peptidase. Of these 312 JRLs, only a few have been characterized with respect to sugar specificity and structure, including three-dimensional structure. They include Jacalin (Artocarpus integrifolia), Artocarpin (A. integrifolia), Heltuba (Helianthus tuberosus), Calsepa (Calystegia sepium), Banlec (Musa paradisica), MPA (Maclura pomifera), and Parkia platysphera (19–25). Based on sugar specificity, JRLs are subdivided into two groups: mannose-specific (mJRLs) and galactose-specific (gJRLs) lectins (26). Another major difference between mJRLs and gJRLs concerns their biosynthesis and processing. For example, gJRLs undergo co-/post-translational modification of a carbohydrate and β-glucosidase-binding sites reside in the JRL domain, and these two sites do not overlap.

**EXPERIMENTAL PROCEDURES**

**Materials**—The maize null line H95 was grown in a growth chamber at temperatures of 30 °C (night) and 24 °C (day) in darkness. After 3–4 days, etiolated shoots were harvested by cutting at the mesocotyl region below the node and stored at −80 °C until use. All sugars and glycoproteins for affinity chromatography and hemagglutination inhibition tests were purchased from Sigma. Lactosyl-agaroase lectin affinity matrix was from EY Laboratories (San Mateo, CA). Sephacryl-HR 200 and nickel-Sepharose™ Fast Flow were purchased from GE Healthcare. The BioSep-SEC-S 2000 gel filtration column was purchased from Phenomenex (Torrance, CA). Pfu DNA polymerase and the plasmid vectors pBlueScript SK +, pET 21a, and pET 15b were from Stratagene (La Jolla, CA) and Novagen (Madison, WI), respectively.

**Purification of BGAF from Maize H95 Inbred Line**—After discovering that the JRL domain could compete with full-length BGAF for the binding site on maize Glu1, we used the recombinant JRL domain to remove Glu1 from the BGAF-Glu1 complex and to purify BGAF in free form. Briefly, 15 g of maize H95 shoots were homogenized with 45 ml of 10 mM phosphate-buffered saline (PBS), pH 7.4, and centrifuged. The supernatant was loaded onto a 0.5-ml lactosyl-agaroase column, which had been equilibrated with PBS. The unbound proteins were removed by washing, and then the matrix (lactosyl-agaroase) was transferred to a 2-ml microcentrifuge tube. To this, 1.0 ml of recombinant JRL protein (2 mg/ml) was added, and the suspension was incubated on a rocking shaker overnight at 4 °C. After incubation, the suspension was centrifuged at 3000 × g, the supernatant was removed, and the matrix was repacked into a column (0.5 × 5 cm). Unbound JRL and JRL-Glu1 complex were removed by washing with PBS. Bound BGAF (free) and a minor amount of BGAF-Glu1 complex were eluted with 100 mM lactose in PBS. Fractions containing free BGAF and complex were identified by β-glucosidase activity (both elute together at 100 mM lactose concentration). To separate free BGAF from the minor BGAF-Glu1 complex contaminant, the above fractions were pooled, dialyzed, and loaded onto a Sephacryl HR 200 gel filtration column (80 × 1.6 cm) that had been equilibrated with PBS. The column was developed at a flow rate of 0.5 ml/min. Fractions containing free BGAF were identified by enzyme-linked immunosorbent assay, pooled, and concentrated using a Vivaspin20 spin column (Vivascience, Hannover, Germany).

**Purification of β-Glucosidase-BGAF Complex**—We discovered that the insoluble portion of the BGAF-Glu1 complex could be solubilized when Gal or lactose is included in the extraction buffer. To isolate the complex, the maize H95 null line pellet left after BGAF extraction was suspended in PBS containing 25 mM lactose and incubated on ice for 1 h. The suspension was centrifuged at 12,000 × g for 30 min. The supernatant was decanted and dialyzed against PBS overnight to remove lactose. The dialysate was then loaded onto a lactosyl-agaroase column, which had been equilibrated with PBS. The column was washed with PBS, and the bound complex was...
eluted with 100 mM lactose. Fractions containing β-glucosidase activity were pooled, dialyzed against PBS, and stored at −20 °C until use.

**Hemagglutination and Inhibition Assay**—Fresh whole rabbit blood (5.0 ml) was washed three times and suspended in 15 ml of PBS, pH 7.4. Trypsin (50 μl, 10 mg/ml solution in PBS, pH 7.4) was added to the washed erythrocytes and incubated at 37 °C for 20 min. The trypsinized erythrocytes were washed three times with PBS and suspended in the same buffer to give a 2% suspension. Hemagglutination activity of native free BGAF, rBGAF, and BGAF-Glu1 complex was determined by a 2-fold dilution procedure using trypsin-treated rabbit erythrocytes. The hemagglutination titer was defined as the reciprocal of the highest dilution exhibiting hemagglutination. Inhibition of hemagglutination by saccharides and glycoproteins that visibly decreased agglutination was defined as the minimum inhibitory concentration (MIC).

**SDS-PAGE**—SDS-PAGE was performed according to the method of Laemmli (30), using minigels (0.8 mm thick, 1.5-cm stacking gel and 6.5-cm separating gel). Samples were heated in the presence and absence of 2-mercaptoethanol in a boiling water bath for 5 min, cooled, and loaded (4 μg of protein) onto a 12% SDS-polyacrylamide gel. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250.

**Mass Spectrometry of Native BGAF after Trypsin Digestion**—After SDS-PAGE and staining with Coomassie blue, the band corresponding to native BGAF was excised and digested with trypsin. The peptide fragments were analyzed by MALDI-TOF-MS spectrometry on a Micromass MALDI spectrometer (Waters Corp., Milford, MA) using a matrix of α-cyano-4-hydroxy cinnamic acid. Peptide masses obtained by MALDI-TOF-MS were compared with the peptide masses obtained by the theoretical trypsin digestion of BGAF using the ExPASy proteomics tool “PeptideMass.”

**Molecular Mass and Structure of Native BGAF**—The molecular mass and quaternary structure of BGAF were determined by SDS-PAGE and gel filtration chromatography, respectively. Gel filtration was carried out on a BioSep-SEC-S 2000 HPLC column (7.8 × 300 mm) equilibrated and developed with PBS at a flow rate of 0.5 ml/min operating at room temperature on a Waters Breeze HPLC system (Waters). The column was calibrated with the following molecular mass standards: thyroglobulin (669 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carboxic anhydrase (29 kDa), and myoglobin (17 kDa).

**Protein and Carbohydrate Estimations**—Estimates of protein concentration were made with Bradford reagent using crystalline bovine serum albumin as a standard (0–1.0 mg/ml concentration range). The microassay format in 96-well flat bottom plates was employed as described by the manufacturer (Pierce). After 10 min of incubation of the dye and protein mixture, the absorbance was read at 595 nm on a VersaMax plate reader (Molecular Devices, Sunnyvale, CA). Total neutral sugars were determined by the phenol/sulfuric acid method (31).

**Metal Ion Requirements for Lectin Activity**—To examine the cation requirement for lectin activity, native BGAF (0.1 mg/ml) was incubated with 10 mM EDTA for 1 h at room temperature and dialyzed against 1 liter of PBS overnight. To the demetalized samples, 1 mM (final concentration) CaCl₂, MgCl₂, MnCl₂, or ZnCl₂ were separately added and incubated at room temperature for 1 h. Then hemagglutination activity was measured as described above.

**Construction, Cloning, and Expression of Wild-type BGAF, Chimeric BGAF, Truncated BGFAs, and Dirigent and JRL Domains**—Full-length BGAF cDNA was recloned and expressed in *E. coli*, using the primers, template, and vectors described previously (13). DNA sequencing, however, revealed an unintended mutation in the stop codon UGA to UGG (Trp), which adds 3 amino acids and a hexahistidine tag to the C terminus. As a result, the expressed protein had a His tag at the C terminus. Chimeric BGAF was generated using overlap extension PCR (32). The chimera contained the dirigent domain of the sorghum (residues 1–153) BGAF homolog (GenBank™ DQ866804), which does not bind maize Glu1, and the JRL domain of the maize BGAF (residues 152–306). To generate N-terminally truncated BGFAs and dirigent and JRL domains, cDNA fragments encoding the desired amino acid sequences (see Table 1) were amplified using primers incorporating Ndel and BamHI sites to the 5’ and 3’ ends of PCR products, respectively. PCR was carried out in a PTC-100 thermal cycler (MJ Research Inc., Alameda, CA) using the high fidelity DNA polymerase *pfu*. All PCR products were gel-purified, separately cloned into pBluescript SK⁺ vector, and transformed into *E. coli* DH10B cells. Positive clones were identified by colony PCR, and the plasmids isolated from them were sequenced by the dideoxy method to confirm the accuracy of the expected insert DNA sequence. The above constructs were digested with Ndel and BamHI. The inserts were gel-purified, ligated to the expression vector pET 15b, which had been previously digested with the same set of enzymes, and transformed into *E. coli* BL-21 CodonPlus competent cells. Selection was carried out with ampicillin and chloramphenicol-containing LB plates. Truncated BGFAs, dirigent domain, JRL domain, and chimeric BGFAs were expressed with a His tag at the N-terminal end. For expression, bacterial colonies harboring cDNA encoding full-length BGAF and its truncated forms were grown in 2-liter LB medium supplemented with ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) under shaking (150 rpm) at 37 °C. In the exponential phase at *A*₆₀₀ = 0.6, expression was induced by adding isopropyl 1-thio-β-β-D-galactopyranoside to a final concentration of 0.6 mM, and incubation was continued overnight at 24 °C. Cells were harvested by centrifugation, washed with PBS, pH 7.4, resuspended in the same buffer containing DNase I (40 μg/ml), 2 mM MgCl₂, 2 mM MnCl₂, and 0.2 mM phenylmethylsulfonfluoride, and lysed using a French press.

**Purification of Full-length Recombinant BGAF (rBGAF), Truncated BGFAs, Sbdirigent-ZmJRL Chimera, Dirigent, and JRL**—Full-length rBGAF was purified by affinity chromatography on a lactosyl-agarose column. Bacterial cell lysate (25 ml) was applied to a lactosyl-agarose column (1 × 5 cm) previously
TABLE 1
Oligonucleotide primers used in PCR to prepare various BGAF cDNA constructs used in this study

| Construct | Identification number of PCR primers used for insert amplification and chimeric protein construction | Amplified BGAF cDNA region |
|-----------|--------------------------------------------------|-----------------------------|
| Sense*    | Antisense*                                       |                             |
| ZmBGAF Δ1–38b |                                       |                             |
| ZmBGAF Δ1–63  | 57  40                                        | 190–918                     |
| ZmBGAF Δ1–88  | 58  40                                        | 265–918                     |
| ZmBGAF Δ1–113 | 59  40                                        | 340–918                     |
| ZmBGAF Δ1–138 | 60  40                                        | 415–918                     |
| ZmDirigent 1–156 | 37  38                                        | 1–468                       |
| ZmJRL 153–306 | 39  40                                        | 457–918                     |
| SbDirigent (1–153) | 32, 64                                        | 1–459, 453–918              |
| ZmJRL (152–306) | 32, 64                                        | 40, 66, 1–459, 453–918      |

Primer sequence numbering system was 5′ to 3′. Underlined bases indicate where the restriction end site (NdeI and BamHI at 5′ and 3′ ends, respectively) was introduced, whereas bases in italic type (primers 64 and 65) show overlapping regions used for overlap extension PCR.

*Numbers in the sense and antisense columns represent primer numbers used in PCR.

bAmino acid numbers deleted from the N-terminal end of intact BGAF.

RESULTS

Purification of BGAF from Maize H95 Null Line—The purification method used was different from that described previously (13). Since BGAF bound to lactosyl-agarose lectin affinity column and the JRL domain competed with intact BGAF for binding sites on β-glucosidase, free BGAF and BGAF-Glu1 complex (PBS extract of H95 tissues contains a considerable amount of soluble BGAF-Glu1 complex) were adsorbed onto a
lactosyl-agarose affinity matrix. The recombinant JRL domain was used to remove Glu1 from the complex (Fig. 1), leaving behind free BGAF for elution. The purification procedure is summarized in Table 2. Purified BGAF gave a single band with an apparent mass of 29 kDa on SDS-PAGE (Fig. 2) with (lane 2) or without (lane 3) reduction with 2-mercaptoethanol. SDS-PAGE analysis of purified BGAF-Glu1 complex showed two bands with apparent molecular mass of 60 and 29 kDa, corresponding to β-glucosidase and BGAF, respectively (Fig. 2, lane 5).

The molecular mass of native BGAF obtained by SDS-PAGE was smaller than the one calculated from its amino acid sequence (31.7 kDa). To determine whether BGAF underwent proteolysis during purification, it was subjected to MALDI-TOF analysis after in-gel trypsin digestion. The MALDI-TOF mass spectrum of purified native BGAF (Fig. 3) gave a peptide mass fingerprint consistent with the predicted polypeptide of 306 amino acids. The MALDI-TOF-MS spectrum of native BGAF showed the presence of molecular ions at m/z 2018 and 1396, respectively (2022 and 1396.76, theoretical mass), which can only be obtained with intact N and C termini, suggesting that there was no proteolysis.

The molecular mass of BGAF was also estimated by gel filtration chromatography on a BioSep-SEC-S 2000 HPLC column, which gave a single symmetrical peak at an elution volume corresponding to an estimated molecular mass of 64 kDa (Fig. 4). Gel filtration on Bio-gel P100 (data not shown) confirmed the result from that on BioSep-SEC-S 2000. Together with SDS-PAGE analysis, these results indicate that the native BGAF molecule occurs as a homodimer of two 32-kDa polypeptides. Neutral sugar analysis by the phenol/sulfuric acid method did not detect any sugars in purified BGAF, and this result was confirmed by the MALDI-TOF mass spectral data that indicated no glycosylated peptides.

**Hemagglutinating Activity and Inhibition of Hemagglutination**—BGAF agglutinated sheep and rabbit erythrocytes. Table 3 shows the inhibition of hemagglutinating activity of BGAF by mono-, di-, and oligosaccharides and glycoproteins. Among the monosaccharides tested, the most potent inhibitors were Gal, followed by GalN, Man, GalNAc, and N-acetylneuraminic acid (Neu5Ac) (Table 3). Glc and Fru were also inhibitory but at higher (125 mM) concentration. In contrast, none of the aldopentoses tested showed inhibition. Inhibition assays revealed that BGAF had about 8-fold greater preference for the α-anomer of Gal (MIC = 3.9 mM) than the β-anomer (MIC = 31.2 mM). Among di- and oligosaccharides tested, N-acetyllactosamine, lactose, raffinose, and stachyose were all inhibitory at concentrations ranging from 1.9 to 7.8 mM. However, the most dramatic differences were observed when the glycoproteins asialofetuin, ovalbumin, and porcine stomach mucin (PSM) were compared with free Gal as inhibitors in agglutination assays. The comparison showed that the glycoproteins were 6000 times more inhibitory than free Gal. The data from experiments testing the effect of chelating agents (i.e. EDTA) on hemagglutination activity indicated no requirement for any cations for the lectin activity of BGAF. Furthermore, cations failed to enhance hemagglutination titer (data not shown).

**Purification and Characterization of Recombinant BGAF**—Following expression in E. coli, rBGAF was purified to homogeneity by lactosyl-agarose lectin affinity chromatography, yielding a single band on SDS-PAGE (Fig. 2, lane 4). The lectin properties of BGAF were investigated by determining its hemagglutinating activity and sugar-binding specificity. The minimum concentration of rBGAF required for hemagglutinating trypsin-treated rabbit erythrocytes was ~0.08 μg/ml, which was similar to that of native BGAF (0.1 μg/ml). Carbohydrate specificity of rBGAF was also similar to that of native BGAF (Table 3) in that lactose, raffinose, stachyose, Gal, and Man were the best inhibitors among the mono-, di-, and oligosaccharides tested (see above). Among the glycoproteins tested, asialofetuin, BSM, and ovalbumin gave MIC values similar to that of native BGAF. This, together with the results of hemagglutination inhibition assays, show that BGAF is a polyspecific chimeric lectin with preference for Gal and Gal-containing oligosaccharides.

**TABLE 2**

| Stage          | Volume | Total protein | Titer | Total activity | Specific activity | Purification | Yield |
|---------------|--------|---------------|-------|----------------|------------------|-------------|-------|
|               | ml     | mg            |       | Al units       | AU/mg            | x-fold %    | %     |
| Crude extract | 35     | 70            | 512   | 17920          | 256              | 1           | 100   |
| Affinity      | 3.4    | 0.15          | 256   | 870            | 5800             | 23          | 4.8   |
| SEC           | 9.6    | 0.03          | 32    | 307            | 10233            | 40          | 2.0   |

* Titer was defined as the reciprocal of end point dilution exhibiting visual hemagglutination.

**AU, agglutination units (volume × titer).**
Effect of Binding of Glu1 on Hemagglutination Activity of BGAF—To examine the effect of Glu1 binding on lectin activity of BGAF, the hemagglutination activity and sugar-binding specificity of the BGAF-Glu1 complex were evaluated. The BGAF-Glu1 complex agglutinated trypsin-treated rabbit erythrocytes, and the minimum concentration required was 0.125 μg/ml, a value similar to that of native and recombinant BGAF. In the hemagglutination inhibition assay, only Gal and its derivatives were inhibitory, and their MIC values were significantly elevated (relative to free BGAF) (Table 3). Man and Neu5Ac acid did not inhibit the hemagglutinating activity of the BGAF-Glu1 complex even at 125 mM concentration (Table 3). With glycoproteins, however, no significant differences in the MIC values were observed, indicating that binding of β-glucosidase to BGAF does not alter the affinity of BGAF for sugars that are on glycoproteins.

Effect of Sugars on β-Glucosidase Aggregating Activity of BGAF—To investigate whether the carbohydrate-binding site in BGAF overlaps with the Glu1 binding site, β-glucosidase aggregation was studied at increasing concentrations of Gal and lactose. Pull-down assays indicated a significant inhibition of precipitation in the presence of Gal and lactose (Fig. 5A). Both sugars showed a hyperbolic curve with half-maximum saturation values at ~2 mM concentration. Sucrose, which had no affinity for BGAF (see Table 3), showed no such effect (Fig. 5A), suggesting that only sugars that bind to BGAF can inhibit β-glucosidase precipitation. At first, these results suggested that the binding of Gal or lactose to BGAF inhibited its binding to Glu1. However, gel shift assays still showed higher molecular weight BGAF-Glu1 complexes even in the presence of saturating concentrations of lactose (Fig. 5B, lanes 3–9). The smeared pattern of the β-glucosidase activity zone in the presence of lactose (Fig. 5B, lanes 3–9) was indistinguishable from the sample containing no lactose.

Construction, Isolation, and Characterization of Dirigent and JRL Domains of BGAF—To address the question as to which of the two domains of BGAF is involved in β-glucosidase binding and aggregation, predicted cDNA sequences encoding the dirigent (residues 1–156) and JRL (residues 152–306) domains of the BGAF molecule were expressed separately in E. coli. Following purification (Fig. 2, lanes 6 and 7), the ability of these domains to bind and aggregate β-glucosidase was evaluated by pull-down and gel shift assays. In the concentration range 0–120 nM (Fig. 6A) and subsequently in the range 0–1000 nM (Fig. 6B), neither dirigent nor JRL was able to form aggregates with Glu1 that were large enough to precipitate by centrifugation in the pull-down assay. Note that intact BGAF at 14 nM (1:4 ratio) exhibited maximum pulldown (Fig. 6A). A mixture of dirigent and JRL domains failed to form precipitable complexes both in the lower and upper range of concentrations tested (Fig. 6, A and B), suggesting that intact BGAF is required for β-glucosidase aggregation.

In the gel shift assay, surprisingly, the JRL domain showed retardation of Glu1 mobility (Fig. 6C). Unlike full-length BGAF, the JRL domain gave a smaller, soluble complex with Glu1 that yielded a distinct band on native gels (Fig. 6C, lane 4). Since both dirigent and JRL were expressed with a His tag at the N terminus, to rule out a possible interference from His tag, dirigent and JRL were digested with thrombin, and their ability to bind Glu1 was evaluated. Consistent with previous results, only JRL was able to bind and reduce the mobility of Glu1 in the gel (results not shown), showing that the His tag did not affect the binding of the JRL domain to Glu1. The role of JRL in Glu1 binding was further confirmed by a competitive binding assay in which the JRL domain was used as a com-
petitive inhibitor. As seen in Fig. 7, at 60 nM concentration, the JRL domain was able to compete out 50% of the \( \beta \)-glucosidase aggregating activity of intact BGAF, whereas the dirigent domain showed a significantly lower inhibitory effect (12%) at the same concentration. These results together with the gel shift assay showed that the JRL domain of BGAF contains a binding site for Glu1.

**Effect of Truncation on \( \beta \)-Glucosidase Aggregating Activity of BGAF**—Our previous studies have suggested that the N-terminal region (residues 1–38) of BGAF is not required for \( \beta \)-glucosidase aggregation (13). In the present study, we generated four additional deletion mutants (Table 1) to further delineate the regions of BGAF required for \( \beta \)-glucosidase binding and aggregation. All of the four truncated BGAFs, upon incubation with Glu1 followed by centrifugation, led to loss of \( \beta \)-glucosidase activity from the supernatant, indicating the formation of large insoluble complexes (data not shown). These results were confirmed by gel shift assays where all of the four truncated mutants produced large complexes with Glu1 (Fig. 8). It is interesting to note that BGAF\( \Delta 1–138 \), which is only 14 amino acids longer than the predicted JRL polypeptide (see Table 1 for details), produced complexes with Glu1 that are large enough to precipitate by centrifugation. Similar to full-length BGAF, the \( \beta \)-glucosidase activity zone (band) shifted, and a smear extending from the top of the resolving gel to the sample well in the stacking gel was present (Fig. 8, lane 7). Unlike the JRL domain, BGAF\( \Delta 1–138 \) agglutinated trypsin-treated rabbit erythrocytes (data not shown).

To investigate what effect the additional 14 amino acids had on the structure of the JRL domain, the hydrodynamic behavior of JRL and BGAF\( \Delta 1–138 \) was examined by gel filtration chromatography. The elution profiles (Fig. 9) showed symmetrical peaks at elution volumes corresponding to the size of the proteins with molecular masses of 17 and 650 kDa, suggesting monomeric and oligomeric structures for JRL and BGAF\( \Delta 1–138 \), respectively.

**Construction of Chimeric BGAF and Characterization of Its \( \beta \)-Glucosidase Aggregating Activity**—We have cloned and expressed in *E. coli* a BGAF homolog from sorghum, which shares 67% sequence identity with maize BGAF but does not

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**TABLE 3**

Inhibition of hemagglutination activity of native BGAF, recombinant BGAF, and BGAF-Glu1 complex by saccharides and glycoproteins

Inhibition of hemagglutination was assayed by serially diluting saccharide and glycoprotein solutions in the microtiter wells, followed by the addition of 4 units of the lectin and then the addition of a 2% suspension of trypsinized rabbit erythrocytes after 30 min. The lowest concentration of saccharides or glycoproteins that visibly decreased the extent of agglutination was defined as the MIC. The MIC values were obtained from two independent measurements.

| Saccharides              | Native BGAF (\( \mu M \)) | rBGAF (\( \mu M \)) | BGAF-Glu1 complex (\( \mu M \)) |
|-------------------------|---------------------------|---------------------|----------------------------------|
| Galactose               | 7.8                       | 1                   | 0.50                             | 0.25                             |
| Methyl-a-D-galactopyranoside | 3.9                      | 2                   | 1                               | 1                               |
| Methyl-\( \beta \)-D-galactopyranoside | 31.2                  | 0.25                | 0.25                            | 0.12                            |
| Galactosamine           | 15.6                      | 0.50                | 0.50                            | 0.12                            |
| N-Acetyl-D-galactosamine | 31.2                     | 0.25                | 0.25                            | 0.62                            |
| N-Acetylneuraminic acid | 31.2                      | 0.25                | 0.25                            | NI                              |
| Fucose                  | NI                        | 0.50                | 0.50                            | NI                              |
| Mannose                | 15.6                      | 0.50                | 0.50                            | NI                              |
| Methyl-a-D-mannopyranoside | 7.8                      | 1                   | 1                               | NI                              |
| Rhamnose               | NI                        |                     | NI                              | NI                              |
| Glucose                | 62.5                      | 0.12                | 0.06                            | NI                              |
| Fructose               | 125                       | 0.06                | 0.06                            | NI                              |
| Arabinose              | NI                        |                     | NI                              | NI                              |
| Xylose                 | NI                        |                     | NI                              | NI                              |
| Maltose                | 62.5                      | 0.12                | 0.06                            | NI                              |
| Sucrose                | NI                        |                     | NI                              | NI                              |
| Lactose                | 1.9                       | 4.1                 | 4.1                             | 1                               |
| N-Acetyl lactosamine   | 3.9                       | 2                   | 2                               | 1                               |
| Raffinose              | 1.9                       | 4.1                 | 4.1                             | 2                               |
| Stachyose              | 7.8                       | 1                   | 1                               | ND                              |

| Glycoproteins           | Native BGAF (\( \mu M \)) | rBGAF (\( \mu M \)) | BGAF-Glu1 complex (\( \mu M \)) |
|------------------------|---------------------------|---------------------|----------------------------------|
| Ovalbumin              | 0.0013                    | 6000                | 6000                             | 6000                             |
| Horseradish peroxidase | NI                        |                     | NI                              | NI                              |
| Asialofetuin           | <0.001                    | >7800               | >7800                           | >7800                           |
| PSM                    | 0.001                     | 7800                | 7800                             | 7800                             |

* Relative potency, relative to D-Gal with native BGAF.
* Isolated from maize H95 “null” line.
* NI, no detectable inhibition.
* ND, not determined.
bind maize Glu1.3 If the JRL domain of maize BGAF is solely responsible for β-glucosidase aggregation, we hypothesized that replacing the JRL domain of BGAF homolog from sorghum with that of maize would confer β-glucosidase binding and aggregating activity to it. To this end, we constructed Sbd irrigent-ZmJRL chimera, expressed it in E. coli, and tested it for β-glucosidase binding and aggregating activity. Pull-down assays indicated that the chimera formed large complexes that precipitate by centrifugation. Consistent with the above result, the gel shift assay showed complexes even larger than those observed in the presence of saturating concentrations of Gal (Fig. 10, lane 2). Note that same pattern is observed even in the presence of saturating concentrations of Gal, thus confirming the identity of the complexes.

**DISCUSSION**

The null phenotype for β-glucosidase in maize is due to aggregation of the enzyme by BGAF, which specifically interacts with maize β-glucosidase isozymes Glu1 and Glu2 and forms insoluble complexes (12). BGAF is a modular protein with two distinct domains, an N-terminal dirigent domain and a C-terminal JRL domain, each about 150 amino acids long and each implicated in defense-related functions. Although the JRL domain occurs in multiple tandemly arranged copies in a number of polyproteins or in fusion with other protein domains, BGAF and its homologs are the only proteins in which the JRL domain occurs in fusion with the dirigent domain. Apparently, the original gene fusion that created the dirigent-JRL modular gene occurred in a grass ancestor from which came wheat, barley, bent grass, rice, sorghum, and maize, since there are no BGAF homologs in dicots and nongrass monocots. It is apparent that these chimeric proteins have evolved from fusion of an ancestral stand-alone JRL domain with a dirigent domain and were selected to perform specialized functions (e.g. defense) in monocots. Although a wealth of information is available on the structure and function of stand-alone JRLs (19–25), very little is known about the lectin activity and carbohydrate specificity of this class of chimeric JRLs.

Although the lower estimate of molecular weight of native BGAF by SDS-PAGE suggested proteolysis, MALDI-TOF-MS analysis ruled out such a possibility (Fig. 3). The low estimate of monomeric molecular weight of BGAF is probably due to its high content of hydrophobic amino acids. Hydrophobic proteins tend to bind SDS at a higher ratio compared with hydrophilic ones, thereby migrating slightly faster on SDS-PAGE (34). The larger size (see Fig. 2, lane 4) of recombinant BGAF compared with native BGAF was due to the presence of 3 additional amino acids and a His tag at the C terminus and 5 additional amino acids at the N terminus. The latter were introduced due to the use of an Nhel restriction site. BGAF is a dimer, as evident from its elution profile on a BioSep-SEC-S2000 gel filtration column (Fig. 4), wherein it eluted in an elution volume corresponding to a molecular mass of 64 kDa. Furthermore, the two monomers in dimeric BGAF are held together by noncovalent interactions, since its mobility on SDS-PAGE is unchanged after treatment with 2-mercaptoethanol (Fig. 2, lane 2). The stand-alone dirigent and JRLs are reported to be dimeric (35) and oligomeric (19–25), respectively, and the dimeric/oligomeric assemblies are stabilized by noncovalent interactions. In the case of BGAF, it is not known which one of the two domains is responsible for dimerization, although there are indications that the dirigent domain might be required for dimerization (see below).

BGAF is a lectin, and the following evidence supports this conclusion. 1) BGAF binds to the lactosyl-agarose lectin affinity column, the binding is inhibited by lactose and Gal, and the bound BGAF is eluted from the column with lactose or Gal. 2) BGAF has hemagglutinating activity, as has been shown by its

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ability to agglutinate trypsin-treated rabbit erythrocytes and inhibition of this activity competitively by sugars and glycoproteins (Table 3). Hemagglutinating activity of BGAF requires that BGAF be minimally a dimer, because the recombinant JRL domain we produced in E. coli is a monomeric protein, and it lacks hemagglutinating activity. Its lectin activity does not require metal ions, because EDTA had no effect on the hemagglutinating activity (data not shown), which is consistent with results from earlier studies with other JRLs (19–25). Moreover, BGAF is a polyspecific lectin but with a marked preference for Gal. Among the monosaccharides tested, Gal was the most

FIGURE 6. Aggregation of Glu1 by rBGAF, dirigent, and JRL domains. In the pull-down assay, Glu1 (58 nm) was incubated with increasing concentrations (A, 0–120 nm; B, 0–1000 nm) of rBGAF (○), dirigent domain (●), JRL domain (□), and dirigent plus JRL domains (▲) at room temperature in PBS, pH 7.4. Following incubation for 2 h, the remaining β-glucosidase activity in the supernatant was determined as described under “Experimental Procedures.” Note that only BGAF forms precipitable aggregates with Glu1 and maximally when 58 nM Glu1 is mixed with 14 nM BGAF. In contrast, dirigent and JRL domains do not form precipitable complexes even at 1000 nM concentration (B). C, gel shift assay. Glu1 (58 nm; lane 1) was incubated with rBGAF (14 nm; lane 2), dirigent (100 nm; lane 3), JRL (100 nm; lane 4), and dirigent plus JRL (100 nm each; lane 5) at room temperature for 2 h in PBS, pH 7.4. Following incubation, samples were mixed with dye and electrophoresed on an 8% native gel. β-Glucosidase activity was detected by staining with 4-MUGlc as described previously (13). Note that only the JRL domain retards the mobility of Glu1 (lanes 4 and 5).

FIGURE 7. Competitive effect of dirigent and JRL domains on β-glucosidase aggregating activity of BGAF. Glu1 and rBGAF in PBS (58 and 14 nm, respectively) were mixed together and allowed to stand at room temperature for 2 h. This was then added to microcentrifuge tubes containing increasing concentrations of dirigent (●) and JRL (○) domains (0–1000 nm) in PBS, pH 7.4. Following incubation at room temperature for 2 h, the remaining β-glucosidase activity in the supernatant was determined as described under “Experimental Procedures.” The BGAF activity competed out (percentage) was computed from the β-glucosidase activity remaining in the supernatant.

FIGURE 8. Gel shift assay to detect binding of truncated BGAF mutants to maize Glu1. Maize Glu1 (58 nm) was incubated with truncated BGAF mutants (400 nm) at room temperature for 2 h in PBS, pH 7.4. Following incubation, 25 μl of reaction mixture was mixed with 10 μl of sample buffer and electrophoresed on an 8% native gel. β-Glucosidase activity was detected by staining with 4-MUGlc as described previously (13). Lane 1, Glu1; lane 2, Glu1 plus full-length rBGAF; lane 3, Glu1 plus BGAFΔ1–38; lane 4, Glu1 plus BGAFΔ1–63; lane 5, Glu1 plus BGAFΔ1–88; lane 6, Glu1 plus BGAFΔ1–113; lane 7, Glu1 plus BGAFΔ1–138.
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FIGURE 9. Elution profile of JRL domain and truncated BGAF,1–138 on BioSep-SEC-S2000 gel filtration column. The JRL domain eluted at 17.1 min, whereas BGAF,1–138 eluted at 11.1 min. The arrows indicate positions at which calibration standards eluted (see Fig. 4 for details). AU, absorbance units.

potent inhibitor, followed by Man, Neu5Ac, and Glc (Table 3). Inhibition of hemagglutinating activity of BGAF by galactose derivatives, such as GalN and GalNAc, suggest that the carbohydrate-binding site in BGAF is large enough to accommodate sugars with bulkier side chains. The higher inhibitory effect of α- and β-methyl galactosides and mannosides as compared with Gal and Man indicate that methylated sugars are preferred ligands. The configuration at the anomeric carbon of Gal and Man appears to be important for binding, as demonstrated by the preference of α- over β-anomer. Furthermore, the -OH group at the C-6 position in Gal and Man appears to be critical for recognition and binding, since L-Fuc (6-deoxy-Gal) and L-rhamnose (6-deoxy-Man) were not inhibitory (Table 3). The higher inhibitory effect of lactose (β-D-Galp-(1→4)-α-D-Glc), raffinose (α-D-Galp-(1→6)-α-D-Glcp-(1→2)-β-D-Fru), and N-acetylactosamine (β-D-Galp-(1→4)-N-acetyl-D-Glcp) is probably due to the presence of subterminal Glc and Gal residues. Note that Glc is a poor inhibitor and that sucrose (β-D-Fru-(2→1)-α-D-Glcp) by itself does not inhibit hemagglutinating activity of BGAF (Table 3). It is, therefore, reasonable to assume that when Glc or Gal is present next to a terminal Gal residue, they enhance the inhibitory effect of saccharides.

The higher inhibitory potency (~6000 times relative to Gal) of glycoproteins (asialofetuin, PSM, and ovalbumin) (Table 3) is probably due to higher affinity of BGAF for certain types of oligosaccharide structures within the glycan chains. For example, asialofetuin contains 11 terminal N-acetyllactosamine residues (36), and it is possible that BGAF binds to these structures with much higher affinity than it does to Gal. Note that N-acetylactosamine is a more potent inhibitor of BGAF hemagglutinating activity than Gal (Table 3). An interesting observation is that peroxidase, a glycoprotein rich in Man-type glycan chains (37), had no effect on the hemagglutinating activity of BGAF, whereas ovalbumin, which also contains mannose-rich glycan chains (38), inhibited its lectin activity. Thus, it is possible that BGAF recognizes and binds with high affinity to mannose chains with certain types of linkages only. Lectins are known to exhibit such preferences for specific oligosaccharide structures within glycan chains (39). The results of hemagglutination inhibition tests with free sugars and glycoproteins clearly show that BGAF binds both Gal and Man. Unlike free BGAF, the carbohydrate binding specificity of the BGAF-Glu complex was distinctly different, as evident from lack of inhibition by methyl-α-D-Man, Man, and Neu5Ac (Table 3). The MIC values for Gal derivatives were also elevated, suggesting that binding of β-glucosidase to BGAF reduces the affinity for free sugars, possibly by causing a limited conformational change in the carbohydrate-binding site. Surprisingly, the affinity of the BGAF-Glu complex toward glycoproteins was unaffected (see Table 3) in that the complex behaves like free BGAF. At present, the reason for the reduced affinity of the BGAF-Glu complex for methyl-α-D-Man, Man, and Neu5Ac is not known.

The carbohydrate binding specificity of BGAF clearly resembles that of jacalin and MPA lectins. JRLs, based on carbohydrate-binding specificity, are subdivided into gJRLs and mJRLs (26). gJRLs (jacalin and MPA lectins) in addition to Gal bind GalNAc, Neu5Ac, and MurNAc (19, 20), whereas mJRLs display an exclusive specificity for Man (40–43). Gal binding and broader specificity of gJRLs is believed to be due to con-/post-translational processing of their precursor polypeptide chain, which generates a larger α-chain and a smaller β-chain (27). The amino group of Gly at the newly created N terminus (β-chain) has been shown to interact with O-4 of Gal and is believed to confer galactose-binding specificity to jacalin and MPA lectins (19, 24). Astoul et al. (44) proposed that the differ-
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ence in the carbohydrate-binding specificities of mJRLs and gJRLs is due to the differences in the size and shape of their carbohydrate-binding site. As mentioned above, BGAF is a polyspecific lectin with a marked preference for Gal (Table 3), but unlike gJRLs, it does not undergo proteolytic processing. How do we then explain the galactose-binding specificity of BGAF? One possibility is that fusion of a stand-alone JRL domain with the dirigent domain at the N terminus has created a carbohydrate-binding site whose shape and size are similar to that of jacalin or MPA lectins.

Our data unequivocally show that both the lectin and the β-glucosidase binding/aggregating activities of BGAF reside in its JRL domain, and the sites responsible for these two activities are distinct. The evidence for this conclusion is as follows. 1) Both the BGAF-Glu1 complex and the free BGAF bind to a lactosyl-agarose column and can be eluted with either lactose or Gal, and the presence of the BGAF-Glu1 complex in the eluate can be shown in gel shift assays. 2) Lactose or Gal, when included in extraction buffers, solubilizes the BGAF-Glu1 complex from the pellet fraction of tissue homogenates. The solubility of the complex is not due to disruption of the BGAF and Glu interaction but due to its solubility upon binding of lactose or Gal, because the complexes can still be detected by gel shift assays. Likewise, the BGAF-Glu1 complexes are still formed when BGAF and Glu1 are mixed and incubated in the presence of increasing concentrations of Gal, and gel shift assays show no differences between controls (no Gal added) and that to which the highest concentration (125 mM) of Gal is added (Fig. 5, lane 9). 3) The BGAF-Glu1 complex agglutinates trypsin-treated rabbit erythrocytes, and its hemagglutination activity is inhibited competitively by saccharides and glycoproteins (Table 3). 4) Free recombinant JRL domain binds β-glucosidase, as evident from the formation of a single band of enzyme activity with slower mobility in gel shift assay (Fig. 6C). 5) Free recombinant JRL domain disrupts the BGAF-Glu1 complex bound to a lactosyl-agarose column and elutes Glu1 from the column, leaving behind BGAF bound to the column. 6) The chimeric BGAF that has the dirigent domain of sorghum BGAF homolog fused with the JRL domain of maize BGAF binds β-glucosidase, whereas the one that has the dirigent domain of maize BGAF fused with the JRL domain of sorghum BGAF homolog does not bind β-glucosidase. Furthermore, our results suggest that the carbohydrate- and β-glucosidase-binding sites in BGAF are not allosterically linked, since the addition of sugar has no effect on BGAF-Glu1 interaction (Fig. 5B), although the pull-down assay (Fig. 5A) at first suggested overlapping of two sites. Certain lectins possess a second type of binding site through which they bind to noncarbohydrate ligands (45). Binding of sugars, however, dissociates the noncarbohydrate ligands from the lectin (46); this is not the case with the β-glucosidase-BGAF complex.

As can be seen from Fig. 6C, mixing and incubating the JRL domain with β-glucosidase resulted in a distinct band of β-glucosidase activity with reduced electrophoretic mobility instead of a smear. The distinct band is thought to correspond to the JRL-Glu1 complex comprising a Glu1 dimer and one or two JRL monomers. This is consistent with our gel filtration chromatography data (see Fig. 9, peak 2 eluting at a retention time of 17.1 min), which show that the recombinant JRL domain is monomeric. These data also suggest that the JRL domain of BGAF has only one β-glucosidase-binding site. We know from our previous studies that native and catalytically active β-glucosidase is a dimer, in which each monomer has one BGAF (or JRL) binding site. Thus, when JRL and Glu1 interact, the interaction is likely to be between a Glu1 dimer and two JRL monomers, producing a distinct band with a slower mobility than the Glu1 dimer alone on zymograms but faster than the BGAF-Glu1 complexes (Fig. 6C). In contrast, the dirigent domain, when mixed and incubated with β-glucosidase, did not alter the mobility of Glu1 in gel shift assays, but it did exhibit some inhibitory effect on aggregating activity of BGAF in solution assays (Fig. 7). Although the reason for this inhibitory effect is not clear at this time, it is possible that the dirigent domain is required to form large precipitable BGAF-Glu1 complexes (Fig. 6, A and B). The monomeric JRL domain failed to agglutinate trypsin-treated rabbit erythrocytes and did not bind to the lactosyl-agarose lectin affinity column (data not shown), indicating that it does not have a functional carbohydrate-binding site.

We know from our gel filtration experiments that JRL is monomeric (Fig. 9, peak 2 eluting at 17.1 min), and dimerization might be required for lectin activity. By definition, lectins are sugar-binding proteins of nonimmune origin that agglutinate cells and glycoconjugates, and to form detectable aggregates, both the lectin and the glycoconjugates must be multivalent (47).

Our N-terminal deletion mutagenesis and β-glucosidase binding data show that amino acids involved in β-glucosidase binding and aggregation are located in the region spanning residues 139–306 of BGAF, as evident from the ability of the Δ1–138 BGAF mutant to bind and form large insoluble complexes with β-glucosidase (Fig. 8, lane 7). Note that the deletion mutant Δ1–138 BGAF is only 14 amino acids longer than the predicted JRL domain (residues 152–306) used in the present study, which binds to β-glucosidase but does not aggregate it. Therefore, we postulate that the 14-amino acid region is required for the JRL domain to oligomerize. This is supported by gel filtration data (Fig. 9, peak eluting at a retention time of 11.1 min), which show that Δ1–138 BGAF is an oligomer. Unlike the JRL domain, Δ1–138 BGAF behaves like wild type BGAF, suggesting that the oligomerization of the JRL domain is required for both β-glucosidase aggregation and lectin activity. Moreover, the chimeric BGAF in which the JRL domain of a BGAF homolog from sorghum (nonbinder) was replaced with the JRL domain (residues 152–306) of maize BGAF (binder) not only binds to maize β-glucosidase but also forms aggregates even larger than the maize BGAF (Fig. 10, lane 4). This indicates that the amino acids critical for β-glucosidase binding are in region 152–306 of BGAF, whereas the additional 14 amino acids are required for the oligomerization of the JRL domain.

The facts that BGAF is a dimer and that there is one binding site for β-glucosidase on the JRL domain of each BGAF monomer suggest that BGAF is bivalent. If BGAF were monovalent, it could bind only one β-glucosidase dimer, resulting in quaternary association with a size of ~180 kDa. In our previous report (13), based on the presence of two octapeptide repeats in the primary structure of BGAF, we had proposed that BGAF was
monomeric and bivalent and each bivalent monomer bound to two β-glucosidase dimers, linking β-glucosidase dimers in a linear chain in which monomeric BGAF with two binding sites and dimeric β-glucosidase alternate. Our present studies on BGAF and the results from mapping of amino acid residues on maize Glu1 involved in BGAF binding by site-directed mutagenesis do not support the involvement of the two octapeptide repeats in BGAF in β-glucosidase binding. The present consensus is that native BGAF is a homodimer with one β-glucosidase binding site per monomer (in the JRL domain). This means that one BGAF dimer would bind two β-glucosidase dimers, connecting β-glucosidase dimers in a linear chain in which the BGAF dimer with two binding sites and β-glucosidase dimer alternate (Fig. 11).

Currently, the precise physiological role of the BGAF-Glu1 interaction in maize is not known. However, there are several intriguing possibilities. First, dirigent proteins are induced during insect and pathogen attack (14), and they catalyze the stereo- and regio-specific coupling of monolignol radicals, precursors for lignin synthesis (15). Lignans, their derivatives, and lignin are thought to play important roles in plant defense functions (48). Likewise, mJRLs and gJRLs have been implicated and shown to be involved in defense (16–18). For example, the product of the RTM1 gene in Arabidopsis thaliana is a JRL and has been shown to inhibit specifically long distance movement of tobacco etch virus in the phloem tissue of transgenic tobacco plants (16). The Hfr-1 (Hesian fly response gene-1) gene in wheat, whose product is a BGAF-like protein, has been shown to inhibit Hesian fly larval feeding and killing of larvae (17).

Similarly, benzo-(1,2,3)-thioleole-7-carbothioic acid S-methyl ester treatment induced systemic acquired resistance in wheat (18). Benzo-(1,2,3)-thioleole-7-carbothioic acid S-methyl ester protects wheat against powdery mildew infection by induction of several genes (chemically induced genes, WCI genes), which include a gene encoding a BGAF-like protein. In the case of BGAF and its homologues, the fusion of the dirigent domain with a lectin domain brings together two different defense proteins that have overlapping or complementary roles to mount a coordinated defense response. Thus, a fusion protein with dual functions will be more advantageous to the plant not only for coordinate regulation but also for possibly the stabilizing effect of two protein domains on each other against proteases of pathogens, insects, and herbivores. The facts that binding of BGAF to β-glucosidase does not affect the enzyme activity (13) and that the carbohydrate-binding site in the complex is free to bind sugars suggest a specific role for the BGAF-β-glucosidase interaction to protect the maize plant against pests, such as the European corn borer. During insect feeding, the BGAF-β-glucosidase complex formed as a result of tissue disruption would bind to glycoproteins in the oral cavity, and/or it may pass down to the peritrophic matrix (PM), where it would bind glycoproteins. In the former case, it would prevent further feeding by causing a local burst of DIMBOA from hydrolysis of DIMBOA-Glc in the oral cavity. In the latter case, however, the BGAF-β-glucosidase complex is likely to damage PM (by forming voids) by binding to proteinaceous matrix (glycoproteins). Feeding of wheat germ agglutinin to European corn borer larvae has been shown to damage PM (49). Additionally, BGAF might protect β-glucosidase from insect proteases.

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Covalently linked dirigent and JRL domains. The small orange circle on each JRL domain represents β-glucosidase binding. The small orange circles represent the JRL domain covalently linked to the dirigent domain (blue circles).

Figure 11. A model for β-glucosidase-BGAF interaction leading to β-glucosidase aggregation and precipitation. The model is based on studies showing the presence of one binding site per JRL monomer. Additionally, free BGAF exists as dimer (based on gel filtration data) and thus must be bivalent. The circles in blue represent the JRL domain covalently linked to the dirigent domain (yellow circles). The small orange circle on each JRL domain represents the β-glucosidase-binding site.

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