Identification of β-Glucosidase Aggregating Factor (BGAF) and Mapping of BGAF Binding Regions on Maize β-Glucosidase*

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In certain maize genotypes (nulls), β-glucosidase does not enter the gel and therefore cannot be detected on zymograms. Such genotypes were initially thought to be homozygous for a null allele at the glu1 gene. We have shown that a β-glucosidase aggregating factor (BGAF) is responsible for the null phenotype, and it specifically interacts with maize β-glucosidases and forms large insoluble aggregates. To understand the mechanism of the β-glucosidase-BGAF interaction, we constructed chimeric enzymes by domain swapping between the maize β-glucosidase isoforms Glu1 and Gu2, to which BGAF binds, and the sorghum β-glucosidase (dhurrinase) isoyme Dhr1, to which BGAF does not bind. The results of binding assays with 12 different chimeric enzymes showed that an N-terminal region (Glu1-Val145) and an extreme C-terminal region (Phe466-Ala515) together form the BGAF binding site on the enzyme surface. In addition, we purified BGAF, determined its N-terminal sequence, amplified the BGAF cDNA by reverse transcriptase-polymerase chain reaction, expressed it in Escherichia coli, and showed that it encodes a protein whose binding and immunological properties are identical to the native BGAF isolated from maize tissues. A data base search revealed that BGAF is a member of the jasmonate-induced protein family. Interestingly, the deduced BGAF sequence contained an octapeptide sequence (G(P/R)WGGSGG) repeated twice. Each of these repeat units is postulated to be involved in forming a site for binding to maize β-glucosidases and thus provides a plausible explanation for the divergent function of BGAF predicted from binding assays.

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) occurs ubiquitously in all three (archaea, eubacteria, and eu- karya) domains of living organisms. The enzyme catalyzes the hydrolysis of aryl and alkyl β-D-glucosides as well as glucosides with a carbohydrate moiety such as β-linked oligosaccharides (1). The occurrence and activity of β-glucosidase in maize is correlated with growth and certain desirable traits (2). The major function of maize β-glucosidase, however, may be in the defense of young plant parts against pathogens and herbivores by releasing toxic aglycones (e.g. hydroxamic acids) from their glucosides. Hydroxamic acids, derivatives of 1,4-benzoaxazin-3-one, are believed to be the major defense compounds in maize, wheat, rye, and wild barley (3). The predominant hydroxamic acid glucoside in maize is 2-glucopyranosyl 4-hydroxy-7-methoxy-1,4-benzoaxazin-3-one (DIMBOAGlc), whose aglycone DIMBOA is the primary defense chemical against aphids and the European corn borer (Ostrinia nubilalis). Several studies have shown a high correlation between DIMBOA content of maize genotypes and the level of resistance to or inhibitory affect on insects and pathogens (4–8).

In certain maize genotypes (nulls), β-glucosidase occurs as part of large insoluble quaternary aggregates (9). The β-glucosidase zymograms of such genotypes are devoid of enzyme bands (10). These genotypes were initially thought to be homozygous for a null allele at the glu1 gene. However, biochemical and immunological data from our laboratory established that the so-called null genotypes have β-glucosidase activity when assayed in solution, and have a 60-kDa polypeptide reacting specifically with anti-β-glucosidase sera on immunoblots (9). The enzyme is not detected on zymograms, because it occurs as large quaternary structures (>1.5 × 10^6 Da), which fail to enter the gel. After dissociation of these structures by SDS, the enzyme can be detected on gels (11). We have recently shown that the null phenotype is due to a β-glucosidase aggregating factor (BGAF), which specifically interacts with the enzyme, forming high molecular weight heterocomplexes (11).

We have identified BGAF as a protein belonging to the jasmonic acid-induced protein (JAp) family, and BGAF is solely responsible for β-glucosidase aggregation and insolubility, and thus, the apparent null phenotype. Jasmonic acid and salicylic acid are plant-signaling molecules that play an important role in induced disease resistance pathways. JAsps are believed to function in some step of these pathways. Blocking the response to either of these signals can render plants more susceptible to pathogens (12–17) and insects (18). Recently, it was shown that the jasmonic acid-dependent induced systemic resistance pathway and the salicylic acid-dependent systemic acquired resistance pathway are fully compatible, and they result in an additive effect on the level of induced protection (19).

In maize, cDNAs corresponding to the β-glucosidase genes (glu1 and glu2) have been cloned and sequenced (Refs. 20, 21; A. Esen and M. Shahid, direct submission GenBank® accession number U25157). The putative protein products of these cDNAs, Glu1 and Glu2, show 90% sequence identity with each other. Additionally, a β-glucosidase cDNA (dhr1) from sorghum has been sequenced, which shares 70% sequence identity with the cDNA of Dhr1 from sorghum.

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1 The abbreviations used are: BGAF, β-glucosidase aggregating factor; JAp, jasmonic acid-induced protein; PCR, polymerase chain reaction; AS, ammonium sulfate; PAGE, polyacrylamide gel electrophoresis; MUAGlc, 4-methylumbelliferyl-β-D-glucoside; EST, expressed sequence tag; DIMBOAGlc, 2-glucopyranosyl 4-hydroxy-7-methoxy-1,4-benzoaxazin-3-one; DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoaxazin-3-one.
Glu1 and Glu2 (22). Despite their high sequence identity, the maize and sorghum enzymes are functionally different with respect to BGAF binding. BGAF binds to both maize isoymes (Glu1 and Glu2) with high specificity but does not bind to their sorghum homolog Dhr1. Therefore, they provide an excellent system to study functional differences at nonconserved residues and elucidate the mechanism of BGAF-mediated enzyme aggregation and insolubility. The objective of the present study is to elucidate the mechanism of the β-glucosidase-BGAF interaction. To this end, we have generated a series of chimeras among Glu1, Glu2, and Dhr1 by domain swapping to identify the sites involved in BGAF binding. The binding properties of these chimeras enabled us to identify two separate and distinct polypeptide segments that together form a BGAF binding site on the surface of maize β-glucosidases. Finally, we have cloned and sequenced the BGAF cDNA and confirmed its identification by expressing it in E. coli and demonstrating the activity of its recombinant protein product unequivocally in binding assays.

**Experimental Procedures**

**Cloning and Expression of Parental and Chimeric β-glucosidase cDNAs**—The cloning and expression of cDNAs encoding Glu1, Glu2, and Dhr1 in E. coli were as described by Cieck and Eson (23). The cDNA encoding the mature sorghum β-glucosidase (dhurrinase-1) protein was amplified by polymerase chain reaction (PCR) using the same procedure and primers (sense, 5′-GCCAGCGTCAAGCGATAAGCTGGAG-3′ and antisense, 5′-AGCCAGCTGGTTAAGATGCTACTTCGCGTG-3′) containing Nhel (5′-end) and XhoI (3′-end) restriction enzyme sites (underlined), respectively. The construction and the expression of chimeric cDNAs were done using the wild-type parental plasmids as templates (i.e. glu1, glu2, and dhrl above). The peptide sequences from which the oligonucleotides were derived for chimeric cDNA construction by PCR are underlined in Fig. 1A (see below). Chimeric cDNAs (proteins shown in Fig. 1B) were constructed by the PCR-based technique of overlap extension (24). The internal oligonucleotides used to assemble chimeric β-glucosidases were: C-2, (sense) 5′-GGCTACTTCGCGTG-3′ and (antisense) 5′-AGAGACCAGCGGAAGTAGCC-3′; C-4, sense same as C-2 sense, and C-4 antisense same as C-2 antisense; C-15, (sense) 5′-GCCAGGTTGGTAGAATTATCC-3′ and (antisense) 5′-TTGAACCTCCTGCAACCTTGG-3′; C-16, (sense) 5′-GCCAGTGGTAAAGATGCTACTTCGCGTG-3′ and (antisense) 5′-TTGAACCTCCTGCAACCTTGG-3′; C-22, (sense) 5′-GGCTACTTCGCGTG-3′ and (antisense) 5′-AGAGACCAGCGGAAGTAGCC-3′; C-26, (sense) same as C-5 sense, and antisense, 5′-GTAAACTCTTTCAAC-3′.

**Identification and Mapping of BGAF**

![Image](308x331 to 554x730)

**FIG. 1.** A, sequence alignment showing the N- (1-214) and C-terminal (453-512) regions of maize (Glu1 and Glu2) and sorghum (Dhr1) β-glucosidases. The underlined peptides were used to design oligonucleotides to create the chimeric enzymes shown in B. The two regions (Glu1: Asn152 and Phe466-Ala512) that contain the residues forming a BGAF binding site are shown in green background. The residues that form the binding site are shown in red (invariant) and purple (variant), and those that are variant but equivalent or located outside the postulated boundary of the site are blue. See Fig. 4 for details. B, schematic representation of the 12 chimeric β-glucosidases constructs used to identify BGAF binding domains in maize (Glu1 and Glu2) and sorghum (Dhr1) β-glucosidases. The lengths of the domains in Glu1 or Glu2 replaced with their Dhr1 homolog by domain swapping are at the right of each chimera.

**Nonsorbert assay and pooled.** Ammonium sulfate was added to the pooled fractions to a final concentration of 0.8 M and applied to a Toyopearl-butyl 650 M hydrophobic interaction chromatography column equilibrated with 50 mM NaAc (pH 5.0) containing 0.8 M As. The column was developed with a manual step gradient using 0.1 M increments from 0.8 to 0.1 M As. β-glucosidase-containing fractions were determined by enzyme-linked immunosorbert assay and pooled, and concentrated on a 10,000 cut-off spin column (Gelman Sciences). The purity of BGAF was checked by SDS-PAGE, and 250 pmol of BGAF were subjected to N-terminal sequencing.

**β-Glucosidase—BGAF Binding Assays—**The interaction between BGAF and β-glucosidase was measured in a binding assay by mixing purified BGAF at 10-fold molar excess with purified β-glucosidases or their chimeras. In the case of chimeras C-5, C-6, C-18, C-19, and C-26, crude expression extracts rather than purified enzymes were used as β-glucosidase source in binding assays. The BGAF-β-glucosidase interaction is very specific and reminiscent of antigen-antibody interactions, therefore, crude expression extracts rather than purified enzyme could...
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RESULTS

BGAF Binding Assays—To examine the molecular basis of the BGAF-β-glucosidase interaction and define the location of the putative binding site(s), chimeric β-glucosidases were constructed (Fig. 1B). The interaction between BGAF and β-glucosidase is very specific and reminiscent of an antigen-antibody interaction, therefore, binding assays could be performed with both purified and unpurified ligands with no effect on assay sensitivity and specificity (11). Consequently, 7 (Dhr1, C-3, C-5, C-6, C-18, C-19, and C-26) out of 16 β-glucosidases were used as crude bacterial cell lysates in binding assays.

When we tested intact Glu1 and Glu2 and a Glu2/Glu1 chimera (C-3) for BGAF binding, the gel-shift assay yielded positive binding results as evident from the formation of BGAF-β-glucosidase complexes with reduced electrophoretic mobility on native PAGE gels. Thus, β-glucosidase activity zones (bands and smearing) were detected with MUGlc, purified Glu1. Additionally, BGAF expression extracts were electrophoresed on 12% (w/v) SDS gels (26) and electrolotted onto a polyvinylidene difluoride membrane for immunostaining. The membranes were incubated overnight with rabbit anti-BGAF serum. The rabbit anti-BGAF IgG (1st antibody) binding was detected with peroxidase-conjugated goat-anti-rabbit IgG (2nd antibody) using the peroxidase substrate 4-chloro-1-naphthol.

be used as ligand source. The enzyme-BGAF mixtures were incubated on ice for 1–2 h with occasional mixing. The reaction mixtures were then electrophoresed on 8% native gels, and the gels were equilibrated with two changes of 50 mM citrate/100 mM phosphate buffer (pH 5.8) for 5 min each after electrophoresis. β-Glucosidase activity was detected by incubating the equilibrated gels in a 1:1 solution of the fluorogenic substrate 4-methylumbelliferyl-β-D-glucoside (MUGlc) for 5–10 min. β-Glucosidase activity zones (bands) were visualized under UV light and documented using an AlphaImager 2000 documentation and analysis system (Alpha Innotech Corp., San Leandro, CA) (Fig. 2). The BGAF-Dhr1 binding assay was essentially the same as described above, except that mobility shifts of BGAF were analyzed by immunoblotting using anti-BGAF serum as a probe instead of enzymatic activity, because Dhr1 does not hydrolyze MUGlc. Initially, purified BGAF was incubated in 2-fold incremental concentrations ranging from 20 to 2.5 μg/ml with a fixed volume of a Dhr1 expression extract. Mixtures were electrophoresed on 8% native gels, blotted, and probed with anti-BGAF sera (Fig. 3). BGAF by itself and BGAF incubated with Glu1 served as negative and positive controls, respectively, in BGAF-Dhr1 binding assays.

Cloning and Sequencing of the BGAF cDNA—The N-terminal sequence of BGAF purified as described above was: (V/L)YAPIGIGATV. The peptide APIGIGAT was used to design two degenerative primers (BGAF-6, CCNATHGGNATHGGNGCN; BGAF-7, GNNCCNATHGGNATHGGNGCN). Messenger RNA was isolated from etiolated 2-day-old H95 shoots using oligo-dT-coated magnetic beads according to the vendor’s protocol (Dynal). An oligo-dT primer was used for first-strand cDNA synthesis with avian myeloblastosis virus-reverse transcriptase (Promega). To amplify the BGAF cDNA, the primers BGAF-6 and BGAF-7 were individually paired with the oligo-dT primer in separate PCR reactions. A PCR product of 1 kb was obtained, gel purified, and cloned into the Smal site of pBluescript II SK (+) for sequencing. A BLAST search of the maize EST data base “hit” three ESTs whose 3' ends overlapped with the 5'-end of the BGAF cDNA. Of these, the longest one (459 bp, GenBank™ T70648) had the highest match (97%) and a 327-bp overlap with the 5'-end of the BGAF cDNA. A primer (BGAF-16, 5'-CAGCTCTCATCTACAGTGTG-3') was derived from the extreme 5'-end of the EST T70648 and used with an extreme 3'-end primer (BGAF-12, 5'-CATTACAGTGCACCATCTGTG-3') to amplify, clone, and sequence the longest possible cDNA from H95 by RT-PCR.

Expression of BGAF cDNA in E. coli—The 1118-bp H95 BGAF cDNA sequence was utilized to design oligonucleotides to synthesize cDNA encoding the mature (BGAF-13, sense, 5'-CATATGGCTAGCCTATCAGATGGCAGAACAAAGGCGCGG-3'; BGAF-12, antisense, 5'-CTATGCTAGCCTATCAGATGGCAGAACAAAGGCGCGG-3') and the precursor (BGAF-15, sense, 5'-CATATGGCTAGCCTATCAGATGGCAGAACAAAGGCGCGG-3'; BGAF-14, antisense, 5'-CTATGCTAGCCTATCAGATGGCAGAACAAAGGCGCGG-3') BGAF polypeptides for expression in E. coli. The cDNAs were cloned into the expression vector pET21a. The E. coli host strain pLys S was transformed with the recombinant plasmid construct and used in expression studies. Expression of BGAF from the putative pBGAF cDNA was corroborated by functional binding assays as described previously except crude BGAF expression extracts were used to incubate with purified Glu1. Additionally, BGAF expression extracts were electrophoresed on 12% (w/v) SDS gels (26) and electrolotted onto a polyvinylidene difluoride membrane for immunostaining. The membranes were incubated overnight with rabbit anti-BGAF serum. The rabbit anti-BGAF IgG (1st antibody) binding was detected with peroxidase-conjugated goat-anti-rabbit IgG (2nd antibody) using the peroxidase substrate 4-chloro-1-naphthol.
yielded C-16, BGAF binding activity is mostly restored (Fig. 2, lane 12). Not surprisingly, replacing the extreme C-terminal 17 amino acids of Glu1 by the 23 amino acids of Dhr1 (C-15) showed BGAF binding activity (Fig. 2, lane 14). The binding data from chimeras C-21 and C-22 show that BGAF binding becomes tighter when the disruptive region (from Dhr1) spanning amino acids Ser466-Leu485 of C-16 is bisected, yielding C-21 and C-22 (Fig. 2, lanes 16 and 18). In the case of Dhr1/Glu1 or Dhr1/Glu2 chimeras, in which the N-terminal Glu1 or Glu2 regions were replaced with the N-terminal Dhr1 regions varying from 127 (C-26) and 205 (C-19) to 461 amino acids long (C-18, data not shown), no BGAF binding to any of these three chimeras was observed (Fig. 2, lanes 26 and 28).

In contrast, a Dhr1/Glu1 chimera (C-5) and a Dhr1/Glu2 chimera (C-6) in which the extreme N-terminal 29-amino acid-long segments of Glu1 and Glu2 were replaced with their Dhr1 homologue bind BGAF, indicating that this segment is not involved in BGAF binding (Fig. 2, lanes 22 and 24). However, C-5, which had the highest electrophoretic mobility (Fig. 2, lane 21), produced predominantly two distinct bands after interaction with BGAF (Fig. 2, lane 22). The region spanning amino acids Glu50-Phε205 was bisected through the construction of chimera C-26 in which the N-terminal Ser1-Asn127 region of Glu1 was replaced with its Dhr1 homolog. Interestingly, C-26 did not have any BGAF binding activity (Fig. 2, lane 28) similar to C-19 (Fig. 2, lane 26), which establishes that the polypeptide segment spanning amino acids Glu50-Asn127 must contain the other region(s) of Glu1 or Glu2 that is involved in forming the BGAF binding site. Binding assays show that BGAF does not bind to Dhr1 (Fig. 3, right panel) where the results were similar to those obtained with negative control BGAF by itself and BGAF plus E. coli lysate (data not shown). In contrast, the amount of BGAF detectable by immunoblotting decreased as the amount of BGAF reacting with the positive tester β-glucosidase (Glu1) increased, because the resulting complex was unable to enter the gel (Fig. 3, left panel). Furthermore, Dhr1 did not bind BGAF in coprecipitation assays (data not shown) performed as described previously (11).

Mapping BGAF Binding Regions—Our structural analysis relative to mapping was on the three-dimensional structure of Glu1 resolved by x-ray crystallography in collaboration with Bernard Henrisat’s crystallography group in Marseilles, France (27). Our initial finding that two regions (28 and 17 amino acids long) within the C-terminal 47 amino acids (based on data with C-15 and C-16) were each essential, but not sufficient, for BGAF binding led to the analysis of these regions on the surface of the Glu1 three-dimensional structure. It appears that the extreme 17-amino acid-long C-terminal region alone makes a greater contribution to BGAF recognition and binding than the 30-amino acid-long C-terminal region preceding it (cf. Fig. 2, lanes 12 and 14). To identify other polypeptide regions that are involved in BGAF binding, we scanned structural elements and amino acids located on the surface in the direct vicinity of the C-terminal 17 amino acids. Analysis of the three-dimensional structure of Glu1 indicates that the N-terminal region maps proximally to the C-terminal region (Fig. 4). On this basis, chimeras C-5, C-6, C-19, and C-26 were tested for BGAF binding activity. Chimeras C-5 and C-6 had binding activity, whereas C-19 and C-26 did not. Collectively, these binding data indicate that the 77-amino acid-long region comprising amino acids Glu50-Asn127 in the N-terminal half contain the other determinant(s) that is (are) involved in BGAF binding, and they map to the surface proximal to certain residues from the C-terminal 47-amino acid-long region. Both the binding data and the structural data corroborate the postulate that binding requires the formation of a site through folding by two distant regions of the primary structure.

Isolation and Identification of the BGAF cDNA—The BGAF cDNA was cloned and sequenced as described above and reported (GenBank accession number AF232008). The longest BGAF cDNA isolated from H95 is 1118 bp long and includes a 918-bp coding sequence and a 43-bp 5′- and a 157-bp 3′-untranslated region. Fig. 5 shows the deduced primary structure of the 306-amino acid-long putative BGAF precursor, which contains two octapeptide (GI/P/R/WGSGGG) repeats that are separated by 40 amino acids. These two repeats are postulated to play an essential role in forming the sites involved in binding to β-glucosidase (see below). The experimentally determined N-terminal sequence VISNKAPIGI of the mature protein starts 38 amino acids after the first methionine in the precursor. Thus, the BGAF precursor has a 38-amino acid-long presequence (i.e. signal peptide), which leaves a mature protein that is 268 amino acids long after the cleavage of the signal peptide.
Fig. 5. Primary structure of the putative BGAF precursor protein. The two octapeptide repeat regions postulated to be involved in binding to maize β-glucosidases are shown in underlined and italicized. The hydrophilicity plot (not shown) predicts that these regions reside on the surface. The N-terminal sequence (in boldface) determined by sequence analysis of a recombinant BGAF expressed in E. coli (no BGAF). Note that only the lanes containing recombinant E. coli lysate from the “null” maize inbred H95 following an 18-amino-acid-long signal peptide (underlined). The peptide sequence AFIGIGAT used for designing oligonucleotide sequences to amplify the original BGAF cdNA is underlined.

Fig. 6. Expression of the BGAF cdNA in E. coli. A, SDS-PAGE gel (12%, anode at bottom) blot of E. coli lysates probed with anti-BGAF serum. Lanes 1 and 2, total and soluble protein, respectively, from nonrecombinant pET21a containing E. coli cells (negative control); 3 and 4, total and soluble protein, respectively, from cells containing recombinant pET21a with the mature protein coding BGAF cdNA; 5 and 6, total and soluble protein, respectively, from cells containing recombinant pET21a with the precursor protein coding BGAF cdNA; 7, shoot extract from maize inbred H95 serving as a positive control. Note that the mature BGAF protein (lanes 3 and 4) produced in E. coli is smaller in size (nonglycosylated) than that produced in the maize plant (lane 7). B, BGAF binding to β-glucosidase detected on a native-PAGE gel (8%, anode at bottom) after incubation with MUGlc. Lane 1, lysate of E. coli cells containing nonrecombinant pET21a (negative control); 2, Glu1 (no BGAF); 3, expression extract from the mature protein coding BGAF clone (no β-glucosidase); 4, Glu1 + lysate from the mature protein coding BGAF clone; 5, lysate from the putative precursor protein encoding BGAF clone; 6, same as lane 5, but mixed and incubated with Glu1; 7, Glu1 + lysate from cells containing recombinant pET21a (no BGAF). Note that only the lanes containing recombinant mature (lane 4) and putative precursor (lane 6) show retarded β-glucosidase activity zones indicating the presence of a functional BGAF.

Discussion

The β-glucosidase null phenotype previously reported in maize is due to a specific interaction between the enzyme and an aggregating factor or BGAF (11). The specificity of the interaction is proven by the fact that BGAF does not bind β-glucosidases from fungi (Trichoderma and Aspergillus) and other plant sources (e.g., almond, black cherry, sorghum, rice, and oats). We have cloned and sequenced the cdNA encoding BGAF (GenBank accession number AF232008) and identified BGAF as a member of the Jap family (16, 17). It is possible that BGAF is also related functionally to small heat shock proteins, which (28–30) bind nonnative proteins, preventing their aggregation and maintaining them in a state competent for ATP-dependent refolding by other chaperones.

The BGAF-β-glucosidase system represents a highly specific protein-protein interaction, providing insights into the molecular mechanism of the interaction based on binding data from Glu1/Dhr1 and Glu2/Dhr1 chimeric enzymes. The BGAF binding assays with chimeras clearly show that replacement of the C-terminal 47 amino acids of Glu1 with its Dhr1 homolog abolishes binding (Fig. 2, lane 8), indicating that this region is essential for BGAF recognition and binding. The results of binding assays with chimeras C-2, C-4, C-16, C-15, C-21, and C-22 (Fig. 2, lanes 8, 10, 12, 14, 16, and 18) suggest that both the C-terminal 28-amino-acid-long (Phe466-Lys493) in C-16 and the extreme 17-amino-acid-long (Lys493-Ala512) in C-15 regions of Glu1 are individually capable of restoring BGAF binding, albeit not to the same extent. Thus, the Glu1 region Phe466-Lys493 is compatible with the Dhr1 region Glu492Asn514 as is the Dhr1 region Ser462Arg469 with the Glu1 region Lys496Ala512. This result is not surprising, because sites for most protein-protein interaction interfaces are confined to a surface patch that is usually composed of more than one stretch of the same polypeptide chain, which incrementally and combinatorially contribute to the interaction with the ligand. Within the region Phe466-Lys493 Glu1 and Glu2 differ from Dhr1 by eight amino acid substitutions where a given site in Dhr1 is occupied by a different and nonequivalent (except K493R) residue from that in Glu1 and Glu2 (Fig. 1A). Of these, P466S, Y467S, and K493R can be ruled out for involvement in BGAF binding, because the first three are buried in the active site cavity of the enzyme and the K493R substitution is likely to be equivalent. This narrows down the candidate sites for contribution to BGAF recognition and binding to N483G, T485E, Y487T, and E490R. These four amino acid substitutions separating Glu1 and Glu2 from Dhr1 are likely to have significant effects on BGAF recognition and binding, because they change the bulkiness, hydrophilicity, or charge of the side chain. Similarly, within the region Lys496Ala512, Glu1 and Glu2 differ from Dhr1 by six amino acid substitutions (K496Q, T500G, K502A, P504K, S505N, and K506N), an internal dipeptide (VE) addition and a terminal tetrapeptide addition (GQNL), each of which alone or in combination with others may affect BGAF recognition and binding (Fig. 1A). In short, both C-terminal regions (Phe466-Lys493 and Lys496-Ala512) are individually capable of complementing an N-terminal region (Glu50Val145, see below) to form a functional BGAF binding site. The finding that the BGAF binding site on Glu1 and Glu2 is made up of more than one stretch of polypeptide is also supported by the finding that chimeras C-18 (data not shown) and C-19 do not bind BGAF (Fig. 2, lane 26). Although the data indicate that the BGAF binding site is a surface patch that includes certain amino acids from the C-terminal (e.g., C-2, C-4) region, they alone are not sufficient to evoke BGAF binding. Moreover, BGAF showed no binding activity toward inactive, denatured Glu1 extracted from inclusion bodies (data not shown), indicating that the tertiary structure of the correctly folded enzyme is essential to form a functional binding site. The finding that chimera C-19 did not bind BGAF suggested that the N-terminal 205 amino acids contain the other region(s) that is (are) involved in BGAF binding. Analysis of the three-
dimensional structure of Glu1 (27) indicated that several segments from the N-terminal 205-amino acid-long region are located on the surface proximal to the C terminus (Fig. 4). The BGAF binding data from C-5, C-6, C-19, and C-26 together bracketed the region spanning Glu50-Asn127 as the other region contributing to the structure of the BGAF binding site. Again, within this region, Glu1 and Glu2 differ from Dhr1 by 13 amino acid substitutions. Of these, four sites (I72V, N75D, K81A, and T82A; highlighted in purple in Figs. 1A and 4) are likely to make a major contribution to the formation of the BGAF binding site, because they all cluster within a surface patch (Fig. 4). The patch includes four amino acids (N483G, T485E, Y487T, and E490R; purple in Figs. 1A and 4) from region Phe466-Lys493 and 2 (K496Q and T500G; purple in Figs. 1A and 4) of six from region Lys496-Ala512 from the extreme 47-amino acid-long C-terminal region. Four sites (K502A, P504K, S505N, and K506N) at which Glu1 and Glu2 differ from Dhr1 are not shown in Fig. 4, because they are within the last 11-amino acid-long free coil region at the extreme C terminus that could not be resolved in the crystal structure. Thus, the binding assays have identified two discontinuous segments (Glu50, Asn127 near the N terminus and Phe466-Ala512 at the C terminus) that are brought together on the surface of the β-glucosidase tertiary structure to form a functional BGAF binding site. This provides a plausible explanation for the lack of BGAF binding to Dhr1 and certain Glu1/Dhr1 or Glu2/Dhr1 chimeras (C-2, C-4, C-18, C-19, and C-26) in which local structural changes due to amino acid substitutions at one or more of the 11 plus postulated sites disrupt binding.

The N-terminal sequence data from a purified BGAF preparation and the three maize ESTs whose 3’-end overlapped with the 5’-end of the cloned BGAF cDNA were key to the isolation of a cDNA (1118 bp) with a full-length coding sequence from the maize inbred H95. The fact that the experimentally determined N-terminal sequence (VISNKPPIGATT) starts 38 amino acids after the first methionine in the deduced primary structure of the putative BGAF precursor suggests that the cDNA contains a full-length (918 bp) coding sequence (Fig. 5). Thus, the BGAF precursor is 306 amino acids long and has a 38-amino acid-long signal peptide. Thus, our experimentally determined N-terminal sequence belongs to a 268-amino acid-long mature BGAF protein whose calculated molecular mass and that of the one expressed in E. coli are similar (27.5 kDa) but is smaller than expected (~35 kDa), suggesting that the native BGAF isolated from maize is post-translationally modified (e.g. glycosylated). Interestingly, BLAST searches indicated that BGAF shared significant amino acid identity (58 to 61%) with three sylated). Interestingly, BLAST searches indicated that BGAF formed. There are other examples of β-glucosidase binding proteins in plants. β-Glucosidases from flax and oat occur in high molecular mass forms ranging from 245 to 1200 kDa (32–34). Additionally, Falk and Rask (35) reported two myrosinase (β-thioglucosidase)-binding proteins (50 and 52 kDa) from rapeseed.

It appears that binding of BGAF to β-glucosidase does not affect enzyme activity and kinetic parameters, suggesting that BGAF binding neither sterically blocks the active site nor changes the conformation to alter enzyme activity. This suggestion is corroborated by the finding that the postulated BGAF binding site (formed by residues in domains Glu50-Asn127 and Phe466-Ala512) on β-glucosidase is away from the active site (Fig. 4). One plausible function of BGAF-β-glucosidase interaction may be that BGAF plays a protective role for β-glucosidase, shielding the enzyme from endogenous proteases or proteases in the secretions of invading pests. Additionally, the BGAF-β-glucosidase interaction would keep active β-glucosidase at the wound site, preventing the enzyme from diffusing to other parts of the plant where it has been shown to elicit deleterious effects (36).
In conclusion, we have shown that maize BGAF is a member of the AP family that specifically interacts with β-glucosidase. Based on corroboratory binding and structural data we have identified two different regions in the primary structure of β-glucosidase, which form a BGAF binding site on the surface of the enzyme. We have also isolated the BGAF cDNA, deduced the sequence of its protein product, identified two octapeptide repeats in the sequence, and postulated that they form two binding sites each of which binds a monomeric unit of the β-glucosidase homodimer. We have confirmed the identity of the BGAF cDNA further by expressing it in E. coli and demonstrating that its recombinant protein products (mature and precursor BGAF) are functionally and immunologically identical to the native BGAF isolated from maize. The finding that BGAF shares significant amino acid identity (58–61%) with barley involved in systemic acquired resistance (GenBank® AF021258, U43496, and U43497) from barley involved in systemic acquired resistance and the fact that β-glucosidase also plays a role in defense suggest that the specific interaction between BGAF and β-glucosidase has physiological relevance. Future studies will focus on the precise identification of specific amino acids within the binding sites of BGAF and β-glucosidase and defining their roles using site-directed mutagenesis and x-ray crystallography, as well as understanding the physiological function of the BGAF-β-glucosidase interaction.

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