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A small gene family in maize encodes a family of β-glucosidase aggregating factor (BGAF)-like proteins, and the product of the *bgaf2* gene also aggregates β-glucosidase

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In certain maize genotypes called "null", β-glucosidase, a major defense related enzyme, forms large insoluble aggregates when tissue integrity is compromised (Biochem. Genet. 28:31-36, 1990). We have shown that a protein called β-glucosidase aggregating factor (BGAF) is responsible for β-glucosidase aggregation and hence the β-glucosidase null-phenotype (Plant Physiol. 122:563-572, 2000). BGAF is a modular protein containing an N-terminal dirigent domain and a C-terminal jacalin-related lectin (JRL) domain (J. Biol. Chem. 282:7299-7311, 2007). BGAF is a lectin; it shows high preference for galactose but binds other carbohydrates as well. BGAF specifically interacts with maize β-glucosidases (isozymes Glu1 and Glu2), forming large insoluble complexes (J. Biol. Chem. 282:7299-7311, 2007). Aggregation of β-glucosidase by BGAF does not affect enzyme activity (unpublished results) nor does bound β-glucosidase interfere with the ability of BGAF to bind carbohydrates.

Proteins sharing sequence similarity and modular architecture with BGAF are also reported from wheat, rice, barley, sorghum and creeping bentgrass *Agrostis stolonifera.* In wheat, rice, and barley, a small family of genes encodes BGAF-like proteins, four genes each in wheat and rice, and three in barley. The products of these genes from wheat (Plant Physiol. Biochem. 43:185-192, 2005; Plant Physiol. 147:1412-1426, 2008) and one of the genes from rice (Toxicon. 47:133-139, 2006) are shown to be lectins with monosaccharide preference for mannose. No protein-aggregating activity was, however, reported for these proteins. Recently, we showed that an ortholog of BGAF from

sorghum is a GalNAc-specific lectin, but it lacks protein-aggregating activity (Glycobiol. 19:277-287, 2009). Although the above observations suggest that the β-glucosidase aggregating activity is unique to maize BGAF alone, the occurrence of other BGAF-like proteins in maize with protein aggregating activity cannot be ruled out. We predicted that multiple genes encoding BGAF-like proteins must be present in maize also, since its close relatives wheat, rice, and barley each have a small family of genes encoding BGAF-like proteins.

Searching the maize genome database (www.maizeGDB) using the maize BGAF cDNA sequence as query led to identification of at least six genes. The gene encoding BGAF, which we described earlier (J. Biol. Chem. 282:7299-7311, 2007) is located on chromosome 7 and was designated as *bgaf1*. The remaining genes were denoted as *bgaf2*, *bgaf3*, *bgaf4*, *bgaf5* and *bgaf6*, where the order reflects their divergence distance in sequence from *bgaf1*. One gene (*bgaf* 7) predicted from EST sequences was not found in the maize genome sequence. The remaining genes are located on chromosomes 2 (*bgaf2*), 6 (*bgaf3*, *bgaf4*, and *bgaf5*) and 8 (*bgaf6*), respectively.

The predicted protein products of genes *bgaf2*, *bgaf3*, *bgaf4*, *bgaf5* and *bgaf6* share 69%, 44%, 45%, 43%, and 41% sequence identity, respectively, with BGAF1, and vary in size from 32 to 35 kD. Maize EST database searches identified EST clones AY105022, AY104689, AY103569, BT016225 and BT042436 whose sequences matched with the sequences of *bagf2*, *bgaf3*, *bgaf4*, *bgaf5*, and *bgaf6* genes, respectively. Moreover, we were able to construct complete cDNA coding sequences corresponding to seven different *bgaf* genes including *bgaf7* using sequence overlaps among EST clones in the database. To investigate if the products of these genes (other than *bgaf1*) participate in protein-protein interactions, an EST clone (AY105022) corresponding to the *bgaf2* gene was selected. Complete sequencing of EST AY105022 indicated that it contained a 948 bp open-reading frame encoding a 315 amino acid long polypeptide, consisting of a predicted N-terminal dirigent domain and a C-terminal JRL domain. The predicted protein was identical to the product of *bgaf2* gene located on chromosome 2.

Both full-length BGAF2 protein and its JRL domain (produced in *E. coli*) showed binding to maize Glu1. In the pull-down assay, precipitable complexes of maize Glu1 were obtained with full-length protein, whereas no such complexes were observed with

its JRL domain. In the gel-shift assay, in the presence of full-length protein, the Glu1 activity zone showed smearing, extending from the sample well to the boundary between stacking and resolving gel (Fig. 1, lane 4), indicating formation of large aggregates. The JRL domain also showed a distinct activity band of mobility slower than Glu1, suggesting formation of a smaller, soluble JRL-Glu1 complex (Fig 1, lane 5). The above results clearly indicate that the product of the *bgaf2* gene is also a β-glucosidase aggregating factor, which we designated as BGAF2.

To investigate whether BGAF2 is expressed in maize, null-line H95 shoots were extracted with PBS (pH 7.4) and the BGAF2-β-glucosidase complexes were isolated after passing through a column of Nickel with immobilized rGlu1 on it, followed by affinity chromatography on lactosyl-agarose. SDS-PAGE of fractions obtained from the lactosyl-agarose column showed the presence of four bands of sizes 62, 60, 34 and 32 kD, respectively (Fig. 2, lane 4). The 60 and 62 kD bands are native and recombinant maize Glu1, respectively. The latter (rGlu1) is larger than native Glu1 because of the presence of a His-tag. The 32 kD band is native BGAF1. The ~34 kD protein band was found to be immuno-reactive with antisera raised against a BGAF2 specific peptide, suggesting that the 34 kD protein is in fact a BGAF-like protein, namely BGAF2. To establish the identity of the 34 kD protein band unequivocally, it was excised from the gel and subjected to LC-MS/MS analysis. Three peptides of sequence ANQAAILESK, FSGSTLEVR and VGPWGGSGGPMELTETETPMR were identified from LC-MS/MS analysis. When these were used as query to search the current (Nov 2008) release of the NR (NCBI) database, the product of the *bgaf2* gene received the highest hits, indicating that the 34 kD protein band is in fact BGAF2.

The β-glucosidase null phenotype in maize (H95 null-line) is not due to BGAF1 alone. BGAF2 is also responsible for β-glucosidase aggregation although its contribution is minor compared to BGAF1 because it is less abundant than BGAF1. There are at least eleven maize null-lines in which we have observed that BGAF1 is a major factor responsible for β-glucosidase aggregation (unpublished data). It is not known at this time whether *bgaf3, bgaf4, bgaf5* and *bagf6* genes are expressed in maize at the protein level, and if so, whether their products participate in protein-protein interaction. The fact there are cDNAs corresponding to each of the seven predicted *bgaf* genes in the maize EST

database indicates that these genes are transcribed and show both temporal and spatial expression patterns. For example, *bgaf1* and *bgaf2* are expressed in aerial organs, whereas *bgaf4*, bgaf5, and *bgaf6* are expressed in embryo and endosperm, respectively (http://www.ncbi.nlm.nih.gov/unigene).

The precise physiological role of β-glucosidase-BGAF interactions is not well understood at this time. We speculate that these interactions have a key function in plant defense responses. There is good reason to believe that these lectins deter the insect larvae from feeding onto plants by lodging the enzymes in the oral cavity or the midgut (by binding to glycoproteins) and causing a localized burst of toxic chemicals (aglycones and their break-down products) in these cavities. In fact, the protein product of the *Hfr-1* gene, a BGAF homolog from wheat, has been shown to deter Hessian fly larvae from feeding on resistant plants by binding to sensory receptors (Plant Physiol. 147:1412-1426, 2008). It is now becoming clear that there are at least two chimeric lectins in maize that specifically interacts with β-glucosidases. We postulate they help the plant to launch a powerful defense response to attack by pests.

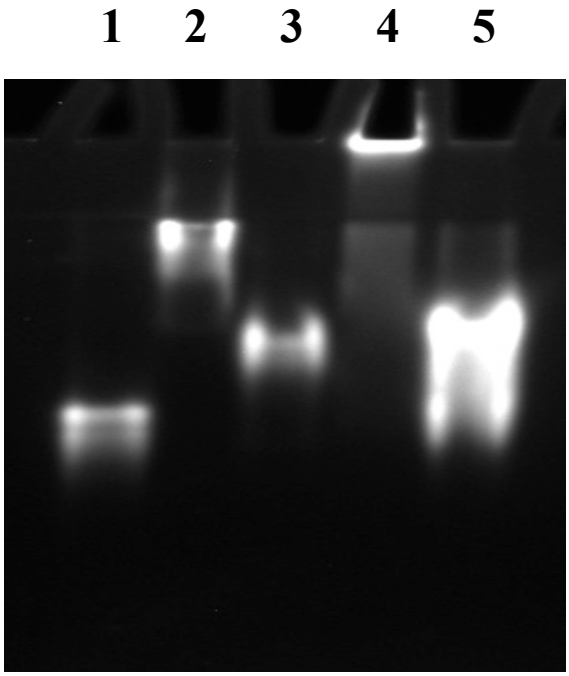

Fig. 1. Gel-shift assay to detect binding of recombinant BGAF2 and its JRL domain to maize Glu1. Maize Glu1 (125 nM) was mixed with BGAF1 (150 nM), its JRL domain (500 nM), BGAF2 (400 nM), and its JRL domain (10 μM), and incubated at room temperature for 30 min. Following incubation, 40 μl of reaction mixtures were mixed separately with 20 μl of sample buffer and electrophoresed on a 8% native gel. β-Glucosidase activity was detected by incubating gel with 4-methylumbelliferyl-β-D-glucopyranoside. *Lane 1*, Glu1; *lane 2*, Glu1+BGAF1; *lane 3*, Glu1+JRL domain of BGAF1; *lane 4*, Glu1+BGAF2; lane *5*, Glu1+the JRL domain of BGAF2.

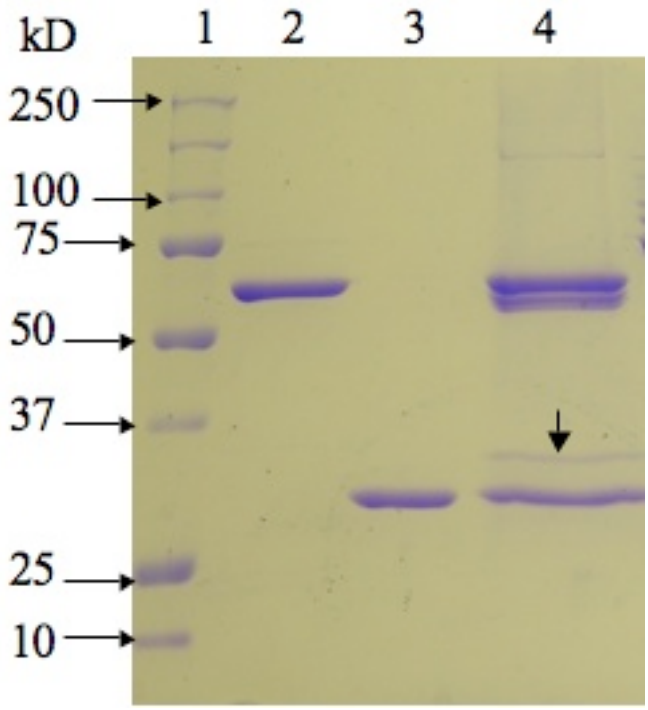

Fig. 2. SDS-PAGE profile of complexes of maize β-glucosidase with BGAF1 and BGAF-like protein isolated from maize null-line H95. *Lane 1*, Molecular weight markers; *lane 2*, recombinant maize Glu1 expressed with His-tag; *lane 3*, recombinant BGAF1; *lane 4*, β-glucosidase-BGAF complexes isolated from maize null-line H95. Note that there are four bands in lane 4, two of which are native (60 kD, lower band) and recombinant (62 kD, upper band) Glu1, respectively. The protein band of size similar to rBGAF1 is native BGAF1. The band (34 kD) immediately above native BGAF1, which was immuno-reactive with BGAF2 specific antibody, was excised and subjected to LC-MS/MS analysis to establish its identity.