CHAPTER 2

Cloning and Sequencing of a cDNA Coding for a β-Glucosidase Aggregating Factor (BGAF) from *Zea Mays* L (Accession No. AF232008) (PGR00-044).

David Blanchard and Asim Esen*

Department of Biology
Virginia Tech, Blacksburg, Virginia 24061-0406.

*Corresponding author; FAX: 540-231-9307;
E-mail: aevatan@vt.edu

A truncated version of this chapter is published as:
David Blanchard and Asim Esen*. (2000). Plant Physiology 122 (3); pp985-986.

*Corresponding author

In certain maize genotypes (nulls), β-glucosidase occurs as part of large, insoluble aggregates. The β-glucosidase zymograms of nulls are devoid of enzyme bands (Stuber et al., 1977). Recently, we clearly demonstrated that the null β-glucosidase phenotype is due to a β-glucosidase aggregating factor (BGAF), which specifically interacts with the enzyme forming high mol. wt heterocomplexes (Esen and Blanchard, 2000). Here we report the isolation of the cDNA encoding BGAF and identification of BGAF as a protein belonging to the small heat-shock protein family.

Heat-shock proteins (Hsp) are a diverse group of proteins that are produced in response to a variety of abiotic stresses. A number of these proteins are now known to function as molecular chaperones (Georgppoulos and Welch, 1993; Boston et al., 1996). During high-temperature stress, it is believed that molecular chaperones function to prevent irreversible protein denaturation that would otherwise be detrimental to the cell (Parsell and Lindquist, 1993). Another group of Hsps that are not well characterized are the small heat-shock proteins (sHsps), which have monomeric molecular masses ranging from 15 to 40 kD, but are known to form oligomeric complexes (Lee and Vierling, 2000). According to Waters et al. (1996) sHsps are the most diverse and abundant Hsps that are synthesized by plants. Vierling (1991) reported that higher plants have at least 20 sHsps and some plant species may have as many as 40 different sHsps. In contrast, most other organisms have one or only a few small heat-shock proteins. There are 4 major Hsps in *Drosophila*, 3 in mammals including αA- and αB-crystallin, 1 in yeast, and 1 in *Saccharomyces cerevisiae* (Arrigo and Landry 1994). The divergence of the plant sHsps occurred after the split of the plant and animal lineages and suggests that the tremendous diversity of sHsps in plants may reflect adaptations to stresses unique to plants.

The plant sHsps can be divided into at least five gene families based on sequence similarity and cellular localization (Vierling 1991). One family of proteins localizes to the chloroplast, one to the endoplasmic reticulum, and two, class I and II, to the cytosol. The fifth class localizes to the mitochondria (Lenne and Douce 1994). The diversification of cellular localization of sHsps is unique to plants and is suggestive of a multitude of functional roles of sHsps. In contrast, all of the nonplant sHsps localize to the cytosol (Arrigo and Landry 1994).

We now report the isolation and identification of a near full-length cDNA from maize shoots encoding a small heat-shock protein (also referred to as a β-glucosidase aggregating factor, BGAF) known to specifically interact with β-glucosidase (Esen and Cokmus, 1990; Esen and Blanchard, 2000). The near full-length sHSP (BGAF) cDNA is 1086-bp long and its 912-bp open reading frame codes for a 304 amino-acid-long mature protein which shows sequence identity (34%-98%; both 5' and 3' EST sequences) exclusively with partial maize heat-shock protein sequences in the maize EST database.

Table I: Characteristics of the maize small heat-shock protein or BGAF.

Organism:

Zea Mays L. (Inbred line H95)

Gene Product:

Small heat-shock protein (sHsp) or BGAF

Clone Type:

cDNA clone

Source:

N-terminal sequence (Commonwealth Biotechnologies, Richmond, VA) from the purified protein isolated from 3.5-d-old etiolated shoots followed by RT-PCR on mRNA isolated from 2-d-old etiolated shoots.

Sequencing Strategy:

Sequencing of both strands using an ABI 377 automated DNA sequencer (Virginia Tech DNA sequencing facility).

Features of the cDNA:

The 1086-bp long cDNA sequence includes a 912-bp coding sequence, and a 174-bp 3' untranslated region.

Features of the Deduced Protein:

The open reading frame (the 912-bp coding region) encodes a 304 amino acid-long mature protein with a calculated molecular mass of 31.7 kD, which is similar to BGAF isolated from maize. BGAF has a calculated isoelectric point of 6, which is consistent with the experimentally determined isoelectric point.

Techniques:

Three-day-old etiolated H95 shoots were extracted 4 times with 50 mM sodium acetate, pH 5 buffer containing 30% ammonium sulfate. The fourth pellet containing predominantly free BGAF was extracted with 50 mM sodium acetate buffer, pH 5. The extract was centrifuged at 20, 000g for 15 minutes. To concentrate BGAF, soluble proteins were precipitated with 40% ammonium sulfate and resuspended in a final volume of 2 mL sodium acetate buffer. The concentrate was applied to a Sephacryl 200-HR gel filtration column (90cm x 16mm). Fractions were screened for BGAF with anti-BGAF serum (produced in rabbits) by ELISA and the BGAF containing fractions were pooled. Ammonium sulfate was added to 0.8 M and applied to a ToyoPearl-butyl 650M hydrophobic interaction chromatography column equilibrated with 50 mM sodium actetate containing 0.8 M ammonium sulfate. After the development of the column with a manual step gradient using 0.1 M increments from 0.8 M to 0.1 M AS, BGAF containing fractions were determined by ELISA and pooled for concentration on a 10 K cut-off spin column (Gelman Sciences). The purity of BGAF was checked by SDS-PAGE and approximately 250 ρmoles of BGAF were submitted to Commonwealth Bioetechnology (Richmond, Virginia) for N-terminal sequencing. The N-terminal sequence was determined to be: [V, ?] [I, E] [G, P] [N, L] YAPIGIGATV. Therefore, the peptide APIGIGAT was used to design two degenerate primers (BGAF-6, CCNATHGGNATHGGNGCNAC; BGAF-7, GCNCCNATHGGNATHGGNGC).

Messenger RNA was purified from total RNA from etiolated 2-d-old H95 shoots using oligo-dT coated magnetic beads. An Oligo-dT-primer (RT-3) was used for first strand cDNA synthesis with AMV-reverse transcriptase (Promega). To amplify BGAF, the degenerate primers (BGAF-6, BGAF-7) were individually paired with RT-3 in separate PCR reactions. The 1 kb PCR product was reamplified with pfu Turbo DNA polymerase, gel purified, and blunt-end cloned into pBluescript® II SK (+/-) for sequencing in both directions.

Method of Identification:

A BLAST search was performed on the maize EST database. The BLAST search exclusively identified heat-shock protein EST's, that matched to putative BGAF with similarities

ranging from 56% - 97%. Although the in vivo function of BGAF remains to be elucidated, BGAF shares 56.2% amino acid identity with 32 kD jasmonate-induced proteins from barley (Accession nos. U43496 and U43497), which are implicated in systemic acquired resistance. Another protein (Accession no. U32427) isolated from wheat, which is also implicated in the systemic acquired resistance response shares 38% amino acid identity with BGAF. The 5'-end of the BGAF cDNA was obtained by overlapping the 5'-end of BGAF (327 bp)with the highest match (97%; Genbank accession no. T70648) in the EST database and was confirmed by reamplification of the H95 cDNA using a primer designed from the extreme 5'-end of the EST clone (Genbank accession no**.** T70648), along with a BGAF specific 3'-end primer. The resulting clone was sequenced and the deduced BGAF protein, now 38 amino acids longer at its N-terminus showed 96% (142/147) amino acid identity to that deduced from T70648 in the overlapped region. The few nucleotide mismatches that were found were attributed to sequencing errors from the EST clone since these clones are subjected to single pass sequencing, and thus, contain errrors. In fact, 6 of the 18 nucleotide mismatches between the 5' BGAF sequence and the EST sequence in the overlap region spanning 441 bp were assigned N (any nucleotide) in the EST sequence due to sequencing errors. The identity of the putative BGAF cDNA was shown following its expression in *E. coli.* Western blots of *E. coli* expression extracts probed with anti-BGAF serum showed an immunoreactive band whose electrophoretic mobility and molecular size were identical to BGAF isolated from maize. Additionally, functional assays clearly showed the presence of BGAF in *E. coli* expression extracts, which tested positive for β-glucosidase binding and aggregating activity in gel-shift assays.

Literature Cited

Arrigo AP, Landry J (1994) Expression and function of the low-moleular-weight heat-shock proteins. *In* R Morimoto, A Tissieres, C Georgopolous, eds, The Biology of Heat Shock Proteins and Molecular Chaperones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 335-373.

Boston RS, Viitanen PV, Vierling E (1996) Molecular chaperones and protein folding in plants Plant Mol Biol **32:** 191-222.

Esen A, Blanchard DJ (2000) A specific βglucosidase aggregating factor (BGAF) is Responsible for the β-glucosidase null phenotype in maize. Plant Pysiol **122**(2): 563-573.

Esen A, Cokmus C (1990) Maize genotypes classified as null at the glu locus have β-glucosidase activity and immunoreactive protein. Biochem. Genet. **28:** 319-336.

Georgopoulos C, Welch WJ (1993) Role of the major heat shock protein as molecular Chaperones. Annu Rev Cell Biol **9:** 601-634.

Lee GJ, Vierling E (2000) A small heat shock protein cooperates with heat shock protein 70 systems to reactivate a heat-denatured protein. Plant Physiology **122:** 189-197.

Lenne C, and Douce R (1994) A low molecular mass heat-shock protein is localized to higher plant mitochondria. Plant Physiol. **105:** 1255-1261.

Parsell DA, Lindquist S (1993) The function of heat-shock proteins in stress tolerance: degradation and reactivation of proteins. Annu Rev Genet **27:** 437-496.

Stuber CW, Goodman MM, Johnson FM (1977) Genetic control and racial variation of β-glucosidase isozymes in maize (Zea mays L.). Biochem Genet **15:** 3/4: 383-394.

Vierling E (1991) The heat shock response in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **42:** 579-620.

Waters ER, Lee GJ, Vierling E (1996) Evolution, Structure and function of the small heat-shock proteins in plants. J Exp Bot **47:** 325-338.