WATER-STRESS REGULATION AND MOLECULAR ANALYSIS OF THE SOYBEAN BIP GENE FAMILY[1]

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ABSTRACT- In order to gain more insight into the complexity of the BiP gene family in soybean, we have cloned a novel BiP cDNA and performed a molecular analysis of the soybean BiP gene family. Despite the high conservation of the soybean BiP cDNA's, they may be divided in three groups according to the level of nucleotide sequence homology among their 5' and 3' untranslated regions. As an endoplasmic reticulum molecular chaperone, the soybean BiP genes are regulated through the unfolded protein response pathway. However, unlike other plant BiP's, the soybean BiP genes are also induced by water-stress and heat shock. The difference in BiP gene copy number in plants may account, in part, for the differential regulation of their genes in response to stress conditions.

Additional index terms: molecular chaperone, soybean, stress induction.

REGULAÇÃO POR ESTRESSE HÍDRICO E ANÁLISE DA FAMÍLIA GÊNICA DE BIP DA SOJA

RESUMO- Com a finalidade de elucidar a complexidade da família multigênica de BiP da soja, um novo membro da família de genes BiP da soja foi clonado e a análise molecular dessa família multigênica foi conduzida. Apesar do elevado nível de conservação de estrutura primária entre os genes BiP da soja, esses podem ser divididos em três grupos de acordo com a homologia de seqüência de nucleotídeos das extremidades não traduzidas 5' e 3'. Como chaperones moleculares residentes no retículo endoplasmático, os genes BiP da soja são regulados por meio da via de resposta a proteínas mal dobradas. No entanto, diferentemente de outros BiP's de plantas, BiP da soja é induzido por estresse hídrico e choque térmico. Provavelmente, a diferença no número de cópias dos genes BiP em plantas contribui, parcialmente, para a regulação diferenciada dos seus genes em resposta a condições de estresse.

Termos adicionais para indexação: chaperone molecular, indução por estresse.

INTRODUCTION

The binding protein (BiP) is a member of the ubiquitous stress-induced family of heat shock proteins that transiently interacts with a wide variety of cellular proteins (Ellis & van der Vies, 1991). As a molecular chaperone, BiP has been described as a key mediator of translocation, folding, assembly and transport of newly synthesized secretory proteins within the endoplasmic reticulum (Denecke, 1996). BiP binds to nascent polypeptides as they enter into the endoplasmic reticulum lumen, forming intermediate complexes with the target proteins which disfavor misfolding and misaggregation (Gething & Sambrook, 1992; Vitale *et al.*, 1993; Fontes *et al.*, 1996; Pedrazzini & Vitale, 1996). This is achieved by reversibly binding to exposed hydrophobic stretches in the protein backbone of nascent polypeptides (Blond-Elguindi *et al.*, 1993). Because the intracellular transport depends upon correct tertiary and quaternary structures, BiP has been implicated in sorting and control of protein exit from the endoplasmic reticulum (Hurtley *et al.*, 1989; Knittler &

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Haas, 1992; Gomord & Faye, 1996; Halban & Irminger, 1996).

BiP is constitutively synthesized at low levels but can be induced upon accumulation of unfolded proteins in the endoplasmatic reticulum, as a result of the unfolded protein response (UPR) pathway (Mori *et al.*, 1992). Several components of the unfolded protein response signaling pathway have been described in *Saccharomyces cerevisiae*. The most upstream component characterized is an endoplasmatic reticulum transmembrane kinase protein, Ire1p, which is thought to be responsible for sensing and transmitting the unfolded protein signal to the appropriate downstream component (Mori *et al.*, 1993). The unfolded protein response element (UPRE) is a 22 pb upstream activating sequence that is necessary and sufficient to activate transcription of a linked promoter in response to accumulation of unfolded proteins within the endoplasmatic reticulum (Mori et al., 1992). Transcriptional activation is mediated by a basic-leucine zipper transcription factor, Hac1p, whose activity is controlled by regulated splicing of its mRNA through a novel splicing pathway, involving tRNA ligase (Cox & Walter, 1996; Sidrauski *et al.*, 1996).

As an endoplasmatic reticulum resident protein, BiP is synthesized as precursor protein containing a peptide signal that targets the protein to the secretory pathway and the carboxy-terminal tetrapeptide HDEL or KDEL, which constitutes an endoplasmatic reticulum retention signal (von Heijne, 1986; Pelham,1988). The retrieval of BiP to the endoplasmatic reticulum is mediated by interaction of a receptor protein localized in the cis-Golgi which recognizes the endoplasmatic retention signal, recycling BiP back to the endoplasmic reticulum (Lee *et al.*, 1993; Elmendorf & Haldar, 1993; Tang *et al.*, 1993).

The chaperone function of BiP has been examined in plants primarily by the detection of its association with storage proteins from seeds. BiP has been demonstrated to associate with underglycosylated phaseolins upon treatment of bean cotyledons with tunicamycin (D'Amico et al., 1992). Similarly, stable association of BiP was detected in protoplasts expressing an assembly-defective phaseolin mutant (Pedrazzini et al., 1994). Recently, BiP has also been shown to associate detectably with normal assembly competent subunits of soybean seed storage proteins (Fontes et al., 1996). Co-immunoprecipitation assays were used to detect association of BiP with the storage protein β-conglycinin from soybean. Either a maize BiP antibody or a β-conglycinin antibody co-immunoprecipitated the reciprocal protein in an ATP-depleted reaction. The role of BiP during the folding and assembly of seed storage proteins has been examined by pulse-labeling experiments followed by co-immunoprecipitation assays in cotyledons of common beans (Vitale *et al.*, 1993). Association of BiP with monomeric phaseolins precedes the oligomerization of the protein, as BiP was found associated with the monomeric polypeptides but not with fully assembled trimers. BiP has also been shown to associate in vitro with newly synthesized prolamins from rice (Li *et al.*, 1993).

In yeast and mammals, BiP is encoded by a single, essential gene (Normington et al., 1989; Ting & Lee, 1988). In contrast, tobacco BiP and soybean BiP are encoded by a multigene family (Denecke *et al.*, 1991; Kalinski *et al.*, 1994). In maize, two BiP isoforms can be separated by 2-D gel electrophoresis, suggesting the presence of a BiP gene family in this plant species (Fontes *et al.*, 1991). In fact, several copies of BiP genes have been identified in the maize genome (R. S. Boston, personal communication*). However, the existence of multiple closely related BiP-like genes in maize, tobacco and soybean is not a property of the plant kingdom, because spinach appears to have a single BiP gene (Anderson *et al.*, 1994). The biological significance of the difference in gene copy number in higher plants remains to be elucidated.

In soybean three distinct BiP cDNA's have been isolated from a leaf cDNA library (Kalinski *et al.*, 1994). Whether the BiP genes are functionally redundant in the soybean genome or exhibit distinct functions is unknown at present. A complete analysis of gene-specific expression profiles can only be performed when all the members of the soybean BiP family are isolated and gene-specific probes are available. In this paper, we report the molecular isolation of a new member of the soybean BiP family from a seed cDNA library and we perform a molecular analysis of the soybean BiP gene family. In addition, we show that, as an endoplasmic reticulum molecular chaperone, the soybean BiP is regulated by the unfolded protein response pathway, but, unlike other plant BiP's, it is induced by water-stress and heat shock.

MATERIAL AND METHODS

Isolation and sequencing of BiP cDNA clone

A maize BiP antibody was used to screen a λgt11 cDNA library prepared from RNA isolated of 55 days after flowering seeds from the Roonoke variety. Expression of the cloned sequences was induced after plaque formation by overlaying nitrocellulose filters treated with isopropyl-thiogalactoside. Duplicate

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nitrocellulose lifts of the lysed plaques were blocked with 1% serum albumin bovine (BSA), washed and incubated with a maize anti-BiP serum (Fontes *el al.*, 1991). The secondary antibody was goat anti-rabbit IgG conjugated to alkaline phosphatase. Screening of 5 10^6 plaques resulted in 4 positive clones, which were purified to homogenous populations. Positive clones were chosen for further analysis on basis of the insert size. The clone cDNAUFV1 was the only one harboring an insert whose size was large enough to encode a 70 kDa protein. The insert from cDNAUFV1 was subcloned into pUC119 and pUC118 for subsequent analysis by restriction enzyme digests, hybridization and sequencing.

The nucleotide sequence of the soybean BiP cDNA was obtained by sequencing multiple restriction enzyme fragments subcloned into pUC119 and pUC118. Double-stranded DNA sequencing was performed by the chain-termination method (Sanger *et al.*, 1977) with AutoCycle Sequencing Kit (Pharmacia) according to the protocol provided by the manufacturer. Reactions were performed with a Perkin-Elmer Cetus Thermo Cycler model 9600 programmed for the following setup: hot start at 94°C for 120 s, followed by 36 cycles of 94°C for 15 s; 55°C (M13 -40 primer) or 65°C (reverse primer) for 15 s and 72°C for 40 s. The programs were ended with 72°C for 10 min, followed by a soak file that held the temperature at 4°C. DNA sequencing was accomplished on an automated sequencer (A.L.F.™ DNA Sequencer).

DNA isolation and DNA gel blot analysis

Genomic DNA was extracted from young leaves essentially as previously described (Doyle & Doyle, 1990), except that polyvinilpirrolidone was omitted from the extraction buffer. Leaf genomic DNA from the soybean varieties Doko and IAC100 was digested overnight with several restriction enzymes, precipitated with ethanol 70% and separated on a TBE-0.8% agarose gel. The gel was washed with 250 mM HCl followed by alkaline denaturation (Sambrook *et al.*, 1989). After neutralization, the DNA was transferred to nylon membranes (Duralon-UV™ membranes, Stratagene) and UV fixed for gel blot analysis. Different regions of the cDNA BiP clone were labeled using the Illuminator™ Nonradioactive Detection System (Stratagene), according to the manufacturer's instructions, and used as probes. Membranes were prehybridized and hybridized at 65°C with QuickHyb hybridization solution (Stratagene) and washed two times for 15 min at 65°C with a high-stringency solution (0.1X SSC; 0.1% SDS). Block reaction was performed for 30 min at 60°C with gentle rocking. Antifluorescein-antibody-AP conjugate was incubated for 1 h at room temperature. The activity of the alkaline phosphatase was assayed with CSPD (Stratagene) during 5 min before exposure to X-ray film (Kodak X-OMAT - AR).

Production of anti-(BiP carboxy)-serum

All DNA manipulations were performed according to standard procedures (Sambrook *et al.*, 1989). The BiP coding region was amplified from cDNAUFV1 to create a *Bam*HI site just after the stop codon and cloned into the SmaI site of pUC118 to generate pUFV41. The nucleotide sequence delimited by positions +1048 to +2038, which encodes the last 329 amino acids of soybean BiP, was liberated from pUFV41 by digestion with *Nco*I and *Bam*HI and cloned into the pET16b (Novagene) expression vector, previously digested with *Nco*I and *Bam*HI. The BiP carboxy-terminus was synthesized under the control of the T_7 RNA polimerase promoter in *E. coli* strain BLE 21D which carries a T_7 RNA polymerase gene regulated by the lac promoter. The synthesis of the recombinant protein was induced by isopropylthio-β-D-galactoside (IPTG). The induced protein was resolved by electrophoresis on a 10% preparative SDS-PAGE gel (Laemmli, 1970), excised from the gel, vacuum dried, ground in liquid nitrogen and finally resuspended in TBS buffer [10 mM Tris-HCL, pH 7.6, 0.9% (m/v) NaCl]. Two rabbits were immunized through subcutaneous injections during 2-week intervals. Serum was recovered by bleeding 6 days after the third injection and stored at -20°C.

Cell culture and induction of BiP

Cotyledon cells from the variety IAC-12 were cultured as previously described (Finer & Nagasawa, 1988). Tunicamycin was added to cultures at 5 days after passage by dilution of a 5 g/l stock in DMSO to 10 mg/l in normal growth medium and incubated for 24 h. For the water stress, the normal growth medium was supplemented with 5% and 10% (m/v) polyethyleneglycol, which correspond to a water potential of -1.0 MPa and -1.4 MPa, respectively, and the cultured cells were incubated for 24 h. To mimic the acquired resistance response, the cultured soybean cells were treated with 10 μM, 50 μM, 250 μM or 500 μM salicylic acid for 24 h. For the heat shock treatment, the cells were incubated at 37°C for 1 h.

Immunoblot analysis

Cells or leaves were ground in liquid nitrogen, homogenized with extraction buffer [50 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl pH 7.5, 1% (v/v) Triton x-100, 1 mM phenylmethysulfonyl fluoride] and clarified by centrifugation at 16,000 g for 15 min. Equal amount of protein extract was loaded onto 10% SDS-PAGE polyacrylamide gel, electrophoresed and the proteins were blotted using a blot apparatus (Biorad), according to the manufacturer's instructions. Immunoblot analysis, in which anti-carboxy serum

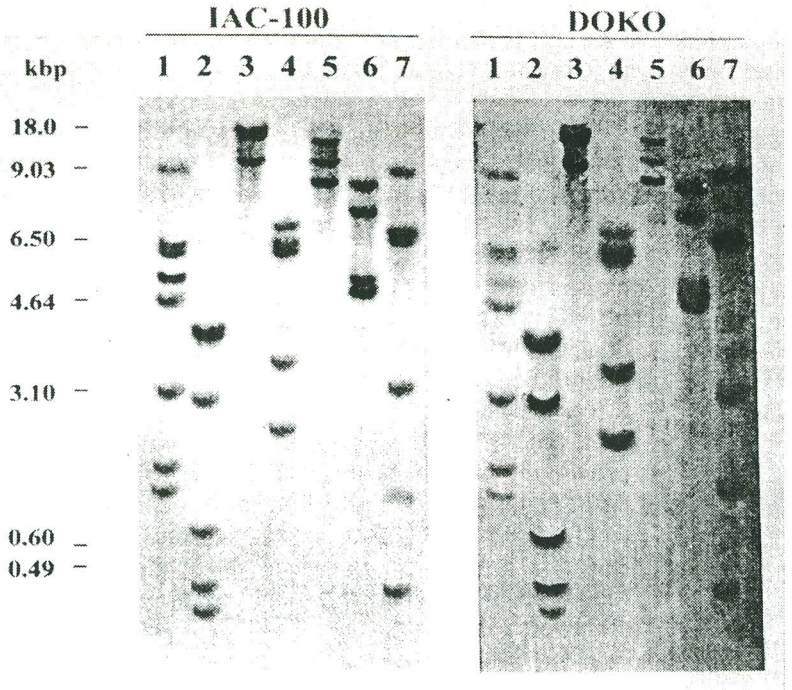

FIGURE 1- Southern blot of soybean genomic DNA. Leaf DNA from IAC100 and Doko varieties was restriction digested with *Eco*RI/*Bam*HI (lanes 1), *Eco*RI/*Bgl*II (lanes 2), SmaI (lanes 3), *Hind*III (lanes 4), XhoI (lanes 5), *Bgl*II (lanes 6) and *Eco*RI (lanes 7). Nucleotide sequence encoding the carboxy-terminus of BiP from soybean was fluorescein labeled and used as a probe.

was used at a 1:1000 dilution, was performed using a goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) at a 1:5000 dilution. The activity of the alkaline phosphatase was assayed using NBT (nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt) substrates.

RESULTS AND DISCUSSION

The BiP gene family is represented by 5-6 copies in the soybean genome

Although it has been reported that soybean BiP is encoded by a multigene family, the number of copies has not been estimated precisely because of the high conservation of nucleotide sequences among the members of the HSP70 family. Thus, the number of bands that hybridize on a genomic gel blot may overestimate the size of the BiP gene family. To overcome this problem, we conducted a search for regions of high conservation among BiP genes and least conserved with other members of the HSP70 gene family. In general, probes that cross-hybridize with BiP genes also cover conserved regions to the HSP70 superfamily. The most striking differences among HSP's and BiP are the 5' sequences encoding the signal peptide and the 3' sequences encoding the tetrapeptide HDEL which are unique to BiP within the members of the HSP70 superfamily. In fact, the probe covering the C-terminal encoding sequence, named 3' probe, was more effective to eliminate hybridizing bands that may correspond to other members of the HSP70 family. The 5' probe underestimated the size of the BiP gene family because the sequences encoding the N-terminal signal peptide are the least conserved between sBiPB and the other isolated BiP cDNA's, sBiPD, sBiPC, sBiPA (Figure 2). Thus, using the 3' probe, we performed an extensive DNA gel blot analysis of the soybean varieties Doko and IAC 100 (Figure 1). DNA digested with endonucleases that recognize one site in the cDNA, such as *Eco*RI, *Bgl*II and *Hind*III, resulted in similar banding patterns between the two varieties, in which more than two bands were detected. The endonuclease EcoRI yielded one major band and four bands of lower intensity (lanes 7); *Bgl*II digested resulted in four bands of high intensity (lanes 6); while *Hind*III (lanes 4) yielded three to four bands of high intensity and several bands of lower intensity. Even using the restriction endonucleases *Sma*I (lanes 3) and *Xho*I (lanes 5) which do not cut within the cDNA, we detected several higher molecular bands, poorly resolved on an 1% agarose gel. The number of BiP genes was further estimated to be 5-6 copies by reconstruction genomic DNA blots (data not shown), in which the copy number is estimated by comparison with the c DNA standard.

Isolation of a BiP cDNA clone and analyses of the soybean BiP gene family

A BiP cDNA was isolated from a seed expression library using a maize BiP antibody as a probe. The BiP cDNA, named sBiPD, encodes a polypeptide of 668 amino acid residues and Mr 85.729 kDa (Figure 2). The polypeptide displays several features shared by HSP70 proteins and specifically by BiP proteins. These features include an ATP binding site comprising the sequence LGIETVGGV (position 432-440) (Hendershot *et al.*, 1988), and the conserved peptide TVIGIDLGTTYSC (residues 36-48) found in members

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sBiPD  MAGSWARRSLIV-LAIISF-GCLFAI-SIAKEEATKLGTVIGIDLGTTYSCVGVYKNGHVEIIANNQGNRI
sBiPA  ...........-......-......-..................-....--TDA.........D.....
sBiPB  ..C.FS.G.SCFP....VSL....RDF.....................................D.....
sBiPC  ...........-......-......-...........LSA.....................D.....

sBiPD  TPSWVAFTDSERLIGEAAKNLAAVNPERTIFDVKRLIGRKFEDKEVQRDMKLVPYKIVNKDGKPYIQVKIK
sBiPA  ...................IV.....V.....................................................
sBiPB  ....-S.................ERV.....................................E...
sBiPC  .....................Q.........................K.....................

sBiPD  DGETKVFSPEEISAMILTKMKETAEAFLGKKINDAVVTVPAYFNDAQRQATKDAGVIAGLNVARIINEPTA
sBiPA  ..........................................K.....................
sBiPB  ..........................................----.....K.....................
sBiPC  ..............V.I.............................K...................N.L.

sBiPD  AAIAYGLDK-KGGE-KNILVFDLGGGTFDVSILTIDNGVFEVLATNGDTHLGGEDFGQRIMEYFIKLIKKK
sBiPA  .........-....-.............................D...........DK.............
sBiPB  .........-....-.............................D...........DK.........N..
sBiPC  ..D.I....N...RR.............................I...........DK.....I.......

sBiPD  HGKDISKDNRALGKLRREAERAKRALSSQHQVRVEIESLFDGVDFSEPLTRARFEELNNDLFRKTMGPVKK
sBiPA  ..........................................................................
sBiPB  .K......S...S.....................................................
sBiPC  ............................K.PL.....................................

sBiPD  AMEDAGLQKSQIDEIVLVGGSTRIPKVQQLLKDYFDGKEPNKGVNPDEAVAYGAAVQGSILSGEGGEETKD
sBiPA  ..........K.....................................................
sBiPB  .........N.....................................A.....................
sBiPC  ..-KIWIT..K.....................................................

sBiPD  ILLLDVAPLTLGIETVGGVMTKLIPRNTVIPTKKSQVFTTYQDQQTTVSIQVFEGERSLTKDCRLLGKFDL
sBiPA  ...............................QP.....................................
sBiPB  .......A.....................QP.............S.....................E.
sBiPC  ............................-....

sBiPD  SGIPPAPRGTAQIEVTFEVDAN-GILNVKAED-KGTGKSEKITITNEKGRLSQEEIERMVREEKDFAEEEK
sBiPA  ..........P..........ER.......RRA.......RSP.S.................AEE....D.
sBiPB  ..........P..........-.........-.............................AEE....D.

sBiPD  KVKERIDARNSLETYVYNMKNQVSDKDKLADKLESDEKEKIETAVKEALEWLDDNQSMEKEDYEEKLKEVE
sBiPA  ......................I.....................................................-.
sBiPB  ..........................................................V..-R.......-.

sBiPD  AVCNPIISAVYQRSGGAPGGGGASGEEDEDDSHDEL
sBiPA  ................................
sBiPB  ......................-....D.DE......
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FIGURE 2- Sequence comparison analysis of soybean BiP cDNA's. The first line represents the amino acid sequence deduced from our seed cDNA. The three lines below represent the deduced amino acid sequence of three-leaf cDNA's. Only the amino acid residues that differ from the first sequence are indicated. Dots in the first sequence correspond to gaps that were introduced to permit the best alignment of the sequences. The sequences in bold are consensus sequences shared by BiP genes or HSPgenes.

of the HSP family which are stress induced (Chapell *et al.*, 1986; Chang *et al.*, 1987; Munro & Pelham, 1986). Although this consensus sequence could be considered a structural characteristic of molecular chaperones, its functional significance remains to be determined. In addition, soybean BiP has the amino acid sequence NRALGKLRRECERAKRALSSQ corresponding to the putative calmodulin-binding domain (Steveson & Calderwood, 1990). Finally, the deduced soybean BiP contains hydrophobic N-terminal signal peptides as predicted by the method of von Heijne and ends with the C-terminal HDEL endoplasmic reticulum retention sequence (von Heijne, 1986; Pelham, 1990).

Amino acid sequence comparison with other previously sequenced soybean BiP cDNA's (genebank accessions n0 U08382; U08383; U08384) showed about 86%-96% identity among them (Figure 2). The high identity of sequences among the predicted polypeptides encoded by the soybean BiP cDNA's was not surprising considering the striking conservation within HSP70 gene class. In fact, two HSP70 clones in Drosophila, which were mapped to two different loci, showed 97% identity (Craig, 1989). At the nucleotide level, the high conservation of the soybean BiP genes drops within the 5' and 3' untranslated regions. While the 5' and 3' untranslated regions of sBiPA, sBiPB, and sBiPC clones are quite dissimilar, these regions are more conserved between sBiPB and sBiPD clones. Based on the level of conservation among these regions, the four isolated genes may be classified in three groups. The BiP gene family in tobacco was also divided in different groups according to the same parameter (Denecke *et al.*, 1991). The low conservation of the 5' and 3' untranslated regions among these soybean clones further supports the argument that the sBiPD clone is a different BiP gene of soybean.

Water-stress regulation of the soybean BiP genes

Several studies have evaluated the regulation of plant BiP gene expression in response to

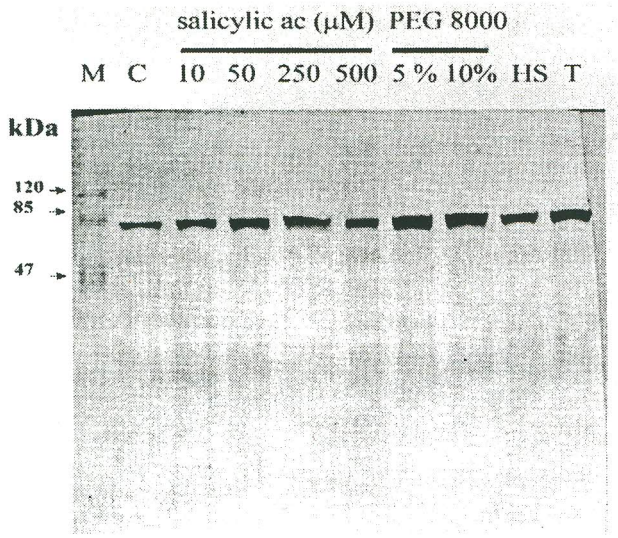

FIGURE 3- Induction of BiP synthesis by stress conditions in cultured cotyledon cells. Twenty-milliliter cultures of cotyledon cells were incubated in the presence of 10, 50, 250 or 500 μM salicylic acid, 5 or 10% (m/v) PEG and 10 mg/l tunicamycin (lane T) for 24 hr. For the heat shock treatment (lane HS), cultured cells were incubated at 37°C for 1 hr. Crude protein extracts were fractionated by SDS-PAGE and immunoblotted with an anti-(BiP-carboxy) serum. Lane C is the result of control cells. The sizes and positions of protein molecular mass markers are shown on the left in kDa.

environmental stimuli and to the accumulation of misfolded proteins in the endoplasmatic reticulum (Vitale *et al.*, 1993 and Anderson *et al.*, 1994). In order to gain insight into differences and similarities between the regulation of the soybean BiP multigene family and other plant BiP's, we investigated the soybean BiP gene expression under several stress conditions. For the analyses of BiP induction, we prepared antibodies against the BiP carboxy-terminus which covers a region of the protein that is the least conserved between BiP and the other members of the HSP70 family of stress-related proteins. While the antibody cross-reacted with a protein induced by tunicamycin (Figure 3, lane T) and salicylic acid (lanes 10, 50, 250 and 500) in soybean cultured cells, heat shock did not promote induction of the cross-reacting polypeptide to the same extent as expected for the cytosolic heat-shock induced HSP70 protein (lane HS). These results suggest that the anti-carboxy sera discriminate between BiP and HSP70. In addition, they demonstrated that, in contrast to the other plant BiP's so far examined, the accumulation of soybean BiP is 3-4 fold higher in heat shock treated cells than it is in unstressed cells (compare lanes C and HS).

Treatment of cultured soybean cells with PEG (lanes 5% and 10%), which mimics the water stress

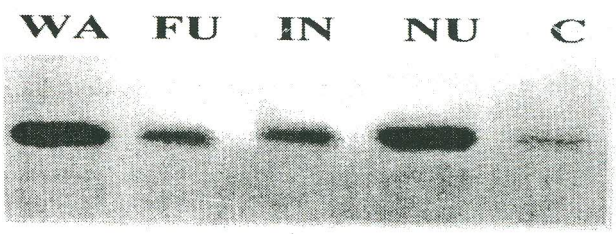

FIGURE 4- Water stress induction of BiP synthesis in soybean leaves. Total protein extracts of leaves from water-stressed (lane WA), fungus-infected (lane FU), insect-infested (lane IN), nutritional-depleted (lane NU) and control (lane C) soybean plants were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with an anti (BiP-carboxy) serum. The BiP immunocross-reactive polypeptides were visualized using a goat anti-rabbit IgG alkaline phosphatase conjugate and the substrates NBT and BCIP.

condition, induced BiP synthesis to the same extent as tunicamycin treatment (lane T). However, the synthesis of spinach BiP, which is a product of a single gene, has been shown to be unaffected by water stress and heat shock (Anderson *et al.*, 1994). In fact, in spinach, both treatments result in disappearance of the BiP mRNA, although the level of protein remains unaltered. Because the water stress was analyzed in spinach plant, to strengthen our results the water stress induction of soybean BiP was further analyzed in soybean plants. As shown in Figure 4, the response of BiP to stress conditions follows the same pattern of induction as in cultured cotyledon cells. In water-stressed leaves, BiP synthesis is up regulated to the same extent as in nutritional-stressed leaves (compare lanes WA and NU). Likewise, the level of BiP induction in response to pathogens (lanes FU and IN) is similar to the response of BiP to salicylic acid in cultured soybean cells.

Our results demonstrated that the pattern of BiP accumulation in response to stress conditions is different in soybean and spinach. Because the ratio of free BiP and BiP associated with polypeptides is thought to control BiP synthesis, one may argue that the failure of spinach BiP to respond to water stress is due to the high concentration of basal BiP under the conditions of the experiment. Tissues with high basal mRNA levels do not show detectable stress induction of plant BiP's (Denecke *et al.*, 1991). However, evidence argues against this hypothesis. First, spinach BiP was induced by tunicamycin (Anderson *et al.*, 1994). The tunicamycin-mediated induction of BiP has been proposed to be achieved by a feedback mechanism through the unfolded protein response pathway (Mori et al., 1992). Second, water stress caused a down regulation of spinach BiP mRNA

accumulation, showing that the conditions of stress were somehow effective. Finally, in soybean, we found that water stress was as effective as tunicamycin treatment for BiP induction (Figure 3). An alternative explanation for these results is that a signal transduction pathway, in addition to the unfolded protein response pathway, may exist to induce the synthesis of different soybean BiP's under specific stress conditions. The genomic cloning of the members of the BiP gene family will provide the necessary target promoters to study the differential regulation of the BiP gene family.

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