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Binding-protein expression is subject to temporal, developmental and stress-induced regulation in terminally differentiated soybean organs

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Abstract. Binding protein (BiP) is a widely distributed and highly conserved endoplasmic-reticulum luminal protein that has been implicated in cotranslational folding of nascent polypeptides, and in the recognition and disposal of misfolded polypeptides. Analysis of cDNA sequences and genomic blots indicates that soybeans (*Glycine max* L. Merr.) possess a small gene family encoding BiP. The deduced sequence of BiP is very similar to that of other plant BiPs. We have examined the expression of BiP in several different terminally differentiated soybean organs including leaves, pods and seed cotyledons. Expression of BiP mRNA increases during leaf expansion while levels of BiP protein decrease. Leaf BiP mRNA is subject to temporal control, exhibiting a large difference in expression in a few hours between dusk and night. The expression of BiP mRNA varies in direct correlation with accumulation of seed storage proteins. The hybridization suggests that maturing-seed BiP is likely to be a different isoform from vegetative BiPs. Levels of BiP protein in maturing seeds vary with BiP mRNA. High levels of BiP mRNA are detected after 3 d of seedling growth. Little change in either BiP mRNA or protein levels was detected in maturing soybean pods, although BiP-protein levels decrease in fully mature pods. Persistent wounding of leaves by whiteflies induces massive overexpression of BiP mRNA while only slightly increasing BiP-protein levels. In contrast single-event puncture wounding only slightly induces additional BiP expression above the temporal variations. These observations indicate that BiP is not constitutively expressed in terminally differentiated plant organs. Expression of BiP is highest during the developmental stages of leaves, pods and seeds when their constituent cells are producing seed or vegetative storage proteins, and appears to be subject to complex regulation, including developmental, temporal and wounding.

Key words: Binding protein – Development (leaf, pod, seed) – Endoplasmic reticulum – Gene expression – *Glycine* (binding protein)

Introduction

The endoplasmic reticulum (ER) of eukaryotes possesses a group of proteins termed the reticuloplasmins (Koch 1987) that function in the cotranslational processing of newly synthesized nascent proteins. Among these proteins are molecular chaperones, protein-disulfide isomerase and calcium-binding proteins (Pelham 1989 for reviews). Although unrelated to each other, the reticuloplasmins share a common carboxyterminal sequence motif KDEL or HDEL sequence (see Denecke et al. 1992 for plant-specific results). This sequence functions as a retrieval signal that is recognized by a *cis*-Golgi receptor that mediates the recycling of the reticuloplasmins from the bulk flow of proteins exiting the ER to return the reticuloplasmins to the ER lumen (Pelham 1988; Semenza et al. 1990; Lee et al. 1993). Binding protein (BiP) is a conserved molecular chaperone resident in the ER lumen. Binding proteins, cDNAs and genomic clones have been isolated and characterized from animal, yeast and plant cells (Haas and Meo 1988; Ting and Lee 1988; Wooden et al. 1988; Normington et al. 1989; Rose et al. 1989; Denecke et al. 1991; Fontes et al. 1991; Giorini and Galili 1991; Anderson et al. 1993), and the deduced sequences of BiP from all of these sources exhibit considerable sequence similarity.

Binding protein has been shown have two related functions. As a molecular chaperone, BiP facilitates the cotranslational transfer of nascent polypeptides into the

The sequences reported in this paper have been submitted to GenBank and are identified with the accession numbers BiP-A (U08384), BiP-B (U08383), BiP-C (U08382) and β-1,3 glucanase (U08405)

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Abbreviations: BiP = binding protein

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ER and presumably mediates folding into the correct conformation (Nguyen et al. 1991; Sanders et al. 1992; Nicchitta and Blobel 1993). This function is apparently essential to maintain protein synthesis and processing by the endomembrane system. Binding protein is essential to yeast cells, and the disruption of BiP gene expression has been shown to be lethal (Rose et al. 1989; Normington et al. 1989; Vogel et al. 1990). Binding protein also appears to have an essential role in the recognition of misfolded or malformed proteins that are posttranslationally unstable and degraded shortly after synthesis. Inhibition of cotranslational glycosylation by tunicamycin induces BiP gene expression (Lee 1987). Correct protein folding, which is dependent upon calcium availability within the ER lumen, is mediated by KDEL-tailed calcium-binding proteins. Disruption of calcium flow with ionophores induces misfolding of nascent polypeptides (Booth and Koch 1989). Calcium ionophores induce BiP gene expression (Lee 1987). Misfolded proteins are recognized by BiP, which tightly binds to malformed protein (Chang et al. 1987; Hurtley et al. 1989; Kassenbrock et al. 1988; Kozutsumi et al. 1988; Pelham 1989 for review) prior to its disposal within the pre-Golgi endomembrane system. In-vivo inhibition of cotranslational glycosylation of the bean storage protein phaseolin by tunicamycin results in an interaction between BiP and the misglycosylated nascent polypeptide (D'Amico et al. 1992). Binding protein also appears to interact with a mutant phaseolin which is unable to form correct trimeric oligomers because the carboxyterminal 59 amino acids has been deleted (Pedrazzini et al. 1994). The sequence of events that occurs after BiP binding to malformed or misglycosylated proteins remains to be elucidated.

Because of its universal requirement in protein synthesis by the ER, and as the result of other experimental observations, the expression of BiP is widely regarded as constitutive in animal and yeast cells (see Gething and Sambrook 1992 for review). Expression of BiP in plants exhibits tissue- and organ-specific variability. Denecke et al. (1991) examined BiP expression in a variety of different tobacco organs and found that not only is BiP expression enhanced in rapidly growing and differentiating organs but it is also correlated with the abundance of the ER. Gibberellic-acid induction of barley aleurones results in massive proliferation of the ER and concurrent accumulation of BiP protein (Jones and Bush 1991). Binding protein is overexpressed in the endosperm in the maize mutants termed *Fluory-2* (Boston et al. 1991; Fontes et al. 1991). These mutants exhibit reduced zein synthesis and aberrant protein morphology (Christainson et al. 1974; Jones 1978; Lending et al. 1989). Binding protein has been shown to facilitate the assembly of ER intraluminal prolamine protein bodies (Levanony et al. 1992; Li et al. 1993). The overexpression of BiP is proposed to be directly related to its role in mediating protein folding and assembly.

Plant tissues are particularly useful models for examining the regulation of putative constitutively expressed genes. Individual organs of higher plants constitute large populations of terminally differentiated cells that are subject to further developmental, temporal and/or environmental regulation. We have examined the expression of BiP in several different terminally differentiated soybean organs, including leaves, pods and cotyledons, and in leaves subject to wounding stress. We have correlated BiP gene expression with the steady-state levels of BiP protein and found that the two parameters appear to be independently regulated.

Materials and methods

Materials. Leaves, pods and developmentally staged seeds of greenhouse-grown soybean plants (*Glycine max* L. Merr, cv. Century) were used in all experiments. For whitefly experiments, the plants were placed in a small greenhouse containing a colony of several hundred whiteflies (*Trialeuroides vaporarorium* Westwood) at 27°C. Germinating seeds were grown by imbibing seeds on wet paper towels in the dark at room temperature. The rec A$^-$ *Escherichia coli* host strain XL1-Blue and R408 helper phage were from Stratagene Cloning Systems (Stratagene, La Jolla, Calif., USA). A tobacco BiP polyclonal antibody elicited against a fusion protein (Hofte and Chrispeels 1992) was a gift from Dr. M.J. Chrispeels, University of California at San Diego, USA. Anti-HDEL monoclonal antibody was the gift of H.R.B. Pelham, MRC Laboratory of Molecular Biology, Cambridge, UK. A random-primed DNA labeling kit was purchased from Boehringer Mannheim (Indianapolis, Ind., USA). A Sequenase Version 2.0 DNA Sequencing Kit was purchased from United States Biochemical Corporation. Radioisotopes were purchased from Amersham Corporation (Arlington Heights, Ill., USA) and NEN Research Products (Boston, Mass., USA). Restriction enzymes were purchased from Stratagene Cloning Systems and GIBCO BRL (Gaithersburg, Md., USA). The 0.24–9.5-kb and 0.16–1.77-kb RNA Ladders were purchased from GIBCO BRL. Lambda DNA-Hind III/ΦX-174 DNA-Hae III Digest was purchased from Pharmacia LKB Biotechnology (Piscataway, N.J., USA). Rainbow protein-molecular weight markers were purchased from Amersham Corporation. All chemicals unless otherwise noted were obtained from Sigma Chemical Company (St. Louis, Mass., USA).

Nucleic-acid isolation. Cellular DNA was isolated from leaves by phenol/chloroform extraction followed by purification on a CsCl gradient as described by Kalinski et al. (1986). Phagemid DNA was isolated using the alkaline/SDS method (Birnboim and Doly 1979), RNase digestion and then phenol/chloroform extraction.

Total RNA was isolated from the tissue ground in liquid nitrogen with a pestle and mortar. The powder was suspended in 100 mM Tris-HCl (pH 8.0), 200 mM aurintricarboxylic acid, 100 mM EDTA, 100 mM β-mercaptoethanol at a ratio of 500 mg of tissue to 1 ml buffer. Purification of RNA was essentially done as described by Wadsworth et al. (1988). Polyadenylated RNA was separated from total RNA on a Poly (A$^+$) Quik column (Stratagene Cloning Systems) according to a supplied protocol. The amount of nucleic acids was determined from spectrophotometric readings at wavelengths of 260 nm and 280 nm (Sambrook et al. 1989).

Microsome isolation. Three grams of mid-maturation soybean leaves was homogenized in 9 ml of 50 mM Tris-HCl, 10 mM KCl, 0.2 mM $MgCl_2$, 12% (w/v) sucrose on ice. The homogenate was filtered through two layers of cheesecloth and then centrifuged for 30 min at 12 000 rpm with an SS-34 rotor in a Sorvall (Norwalk, Conn., USA) RC-2b centrifuge. The supernatant was then centrifuged for 3 h at 38 000 rpm in a Beckman (Fullerton, Calif., USA) SW41 rotor and L2-70 M ultracentrifuge to pellet the microsome fraction. The microsomes were suspended in 250 µl of SDS sample buffer and heated to 90°C for 5 min prior to analysis by SDS/PAGE-immunoblot using anti-BiP or anti-HDEL antibodies.

Immunoblots. To determine profiles of BiP-protein expression the following samples from greenhouse-grown soybeans cv. Century

were obtained: leaves at various stages of development (1/8, 1/3, 1/2 and fully expanded); de-seeded pods from 35-mg, 85-mg, 155-mg, and 220-mg maturing seeds; fully expanded leaves collected at sunrise, midday, sunset, and at 1 h after sunset; cotyledons from 35-mg, 85-mg, 130-mg and 230-mg maturing seeds; 1-, 2-, 3-, 4-, 5-, 7-, and 12-d germinating seedlings. The samples were quickly frozen in liquid N_2, and stored at −80°C until processed. Frozen samples were ground with a pestle in a mortar at 4°C with sample buffer (50 mM Tris-HCl, pH 6.8; 2% w/v SDS; 10% v/v glycerol; 100 mM DTT). Sample buffer was added at a ratio of 1 ml per gram fresh weight. After grinding, samples were centrifuged for 5 min at 5000 rpm, at 4°C, in a Sorvall SS34 rotor. The supernatants were transferred to new tubes. Aliquots of the samples were fractionated by SDS/PAGE (12% resolving gel) (Laemmli 1970). Immunoblotting onto nitrocellulose was performed as previously described (Towbin et al. 1979; Herman 1987). Replicate lanes were stained with Amido Black (Bio-Rad Laboratories, Richmond, Calif., USA) to visualize total proteins, or processed for immunoblotting. The blots were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS; 50 mM Tris-HCl pH 7.4, 0.15 M NaCl). The blots were incubated for 16 h in 5% nonfat milk in TBS with a 1:2600 dilution of anti-tobacco-BiP antibody. Other replicate blots were probed with the culture superatent of the anti-HDEL monoclonal antibody 2E7 diluted 1:15 in 5% nonfat milk in TBS. The immunoreactive protein was visualized with goat anti-rabbit or anti-mouse immunoglobulin G (IgG) alkaline phosphatase when appropriate, as described previously (Kalinski et al. 1992).

Leaf λ ZAP II cDNA library and immunological isolation of BiP cDNAs. Total RNA (3.3 mg) was isolated from 14 g of green mature leaves. Approximately 30 µg of poly(A)$^+$RNA was used for the synthesis of cDNAs and cloning into the Eco RI site of the λ ZAP II DNA vector by Stratagene Cloning Systems (Short et al. 1988). Approximately 5×10^4 recombinants were immunologically screened for *iso*propyl-β-D-thiogalactopyranoside-induced expression of BiP fusion proteins using a tobacco BiP polyclonal antibody. Immunoscreening was done as previously described (Kalinski et al. 1990) except that the nitrocellulose filters were incubated overnight in 1:2600 dilution of a polyclonal antibody. After two rounds of rescreening of the library, thirteen positive recombinant λ plaques were isolated and were used for in-vivo excision of the pBluescript plasmid from the λ ZAP II vector (Short et al. 1988). Restriction maps of the isolated clones indicated that there were three distinct classes among the thirteen clones. The longest representive clone of each class was selected for further analysis.

Northern and Southern blots. The RNA (8–20 µg) for northern blots was denatured at 65°C for 10 min in 50% formamide, 2.15 M formaldehyde, 20 mM 3-(N-morpholino)ethanesulfonic acid (Mops), 5 mM sodium acetate, 0.5 mM EDTA (pH 7.0) containing ethidium bromide at concentration of 0.03 µg per 1 µg RNA in the sample (Gong 1992). Samples were cooled on ice prior to electrophoresis on a 1% agarose/formaldehyde gel. The ethidium-bromide-stained agarose gel was visualized under UV light for evaluation of equal RNA loading in each lane. Transfer of RNA to nitrocellulose filters, prehybridization and hybridization were performed as described in Kalinski et al. (1992). For homologous sequences the RNA blots were washed at high stringency for 30 min at 65°C in 0.1 × SSPE (1 × SSPE = 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 0.1% (w/v) SDS. A final post-hybridization wash was done for 30 min at 49–58°C for partially homologous sequences.

Southern blots of soybean cellular DNA from leaves were performed as described by Kalinski et al. (1990). The DNA blot was washed at 68°C for 30 min in 0.1 × SSPE, 0.1% SDS (w/v).

DNA sequencing. Sequencing of double-stranded DNA by the chain-termination method (Sanger et al. 1977) was done according to the protocol provided by the manufacturer of the kit.

Results

Specificity of anti-BiP antiserum. The anti-BiP antiserum used in our studies was elicited against a BiP fusion protein expressed in *E. coli* (Hofte and Chrispeels 1992). Other studies have shown that this antiserum appears to be specific for BiP in the ER fraction of oat root tips fractionated on continuous sucrose gradients (Herman et al. 1994) and by immunoprecipitation (Hofte and Chrispeels 1992; Pedrazzini et al. 1994). In order to further examine the specificity of the anti-BiP serum for BiP, SDS/PAGE-immunoblots of leaf microsomal proteins were prepared. Replicate lanes were stained for total protein or labeled with anti-BiP or anti-HDEL antibodies. The anti-HDEL monoclonal antibody labeled a polypeptide of identical relative molecular mass (M_r) to anti-BiP cross-reactive protein, as well as two other minor bands that are presumably other reticuloplasmins (data not shown). A split lane labeled by either anti-BiP or anti-HDEL antibodies labeled a single common band. Based on this assay and previously available data, we conclude that this particular antiserum preparation is directed at BiP epitopes, which is consistent with a protein associated with the microsomal fraction possessing the carboxyterminal HDEL retention sequence.

Cloning of BiP cDNAs. Recombinant phages containing BiP cDNAs were isolated by immunologically screening a lambda ZAP library contructed from fully expanded soybean leaf mRNAs. Highly immunoreactive plaques were isolated and the cDNA inserts subcloned. Three different restriction-map classes of BiP cDNAs were obtained and a member of each class was isolated and sequenced. The primary sequence of the longest cDNAs of 2.7 kb, termed BiP-A, encoded a single open reading frame of a protein of M_r 80 kDa (Fig. 1), and this clone was chosen as a probe for expression experiments described in subsequent sections of this paper. The other two leaf BiP isoforms identified as restriction-enzyme classes were represented by one clone encoding a complete open reading frame (BiP-B) and another containing a truncated open reading frame (BiP-C) beginning at amino acid No. 1 and extending through No. 456. Analysis of the nucleic-acid and deduced protein sequences with the sequences on deposit in GenBank indicated that the clones isolated were highly similar to tobacco (Denecke et al. 1991; GenBank No. JQ1360, JQ1361), tomato (GenBank No. LO8830), and maize (Fontes et al. 1991; GenBank No. JQ0966) BiPs. A gap alignment of the deduced protein sequence of the three soybean BiP isoforms and tobacco BiP (GenBank No. JQ1360) demonstrates the high similarity between these proteins (Fig. 2). Soybean BiPs are highly similar in most of the open reading frame. The BiP cDNAs characterized from tobacco (Denencke et al. 1991; GenBank Nos. JQ1360, JQ1361), tomato (GenBank No. LO8830), maize (Fontes et al. 1991; GenBank No. JQ0966) and soybean all possess deduced HDEL ER retention sequences (Pelham 1989, for review). The sequences immediately adjacent to the HDEL retention sequence exhibit variability between tobacco and soybean BiP-A and -B (Fig. 2), as well as the

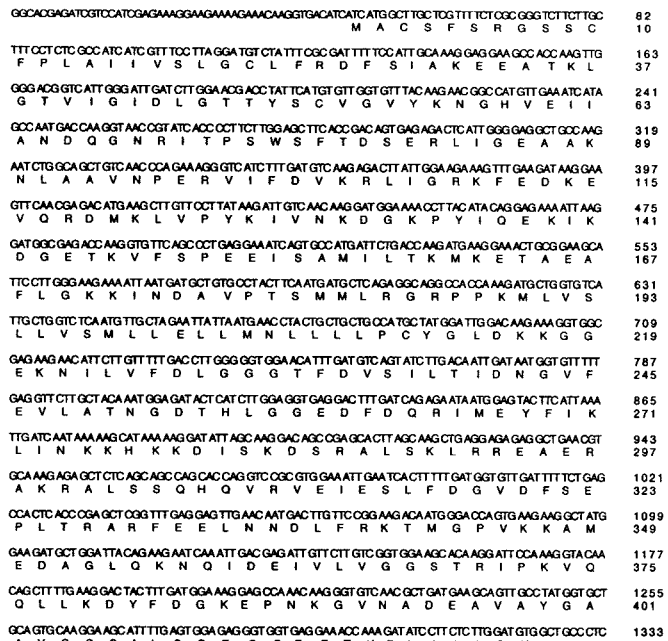

Fig. 1. Nucleotide and deduced amino-acid sequences of BiP-A cDNA. The nucleic-acid sequence and deduced protein sequence of soybean leaf BiP (clone A) is shown

HDEL-adjacent sequences of maize and tomato BiPs (not shown). The putative signal sequences of tobacco and soybean BiP exhibit little similarity (Fig. 2). There is a cotranslational glycosylation triplet at amino acid 615–617 of BiP-A that is conserved in an aligned location in BiP-B and tobacco BiP (Fig. 2). We have not yet determined if this glycosylation site is utilized.

The immunoselection of BiP cDNA clones did not result in the isolation of cDNA clones of other members of the heat-shock protein (HSP70) family of proteins. The isolation of BiP cDNAs and the exclusion of other

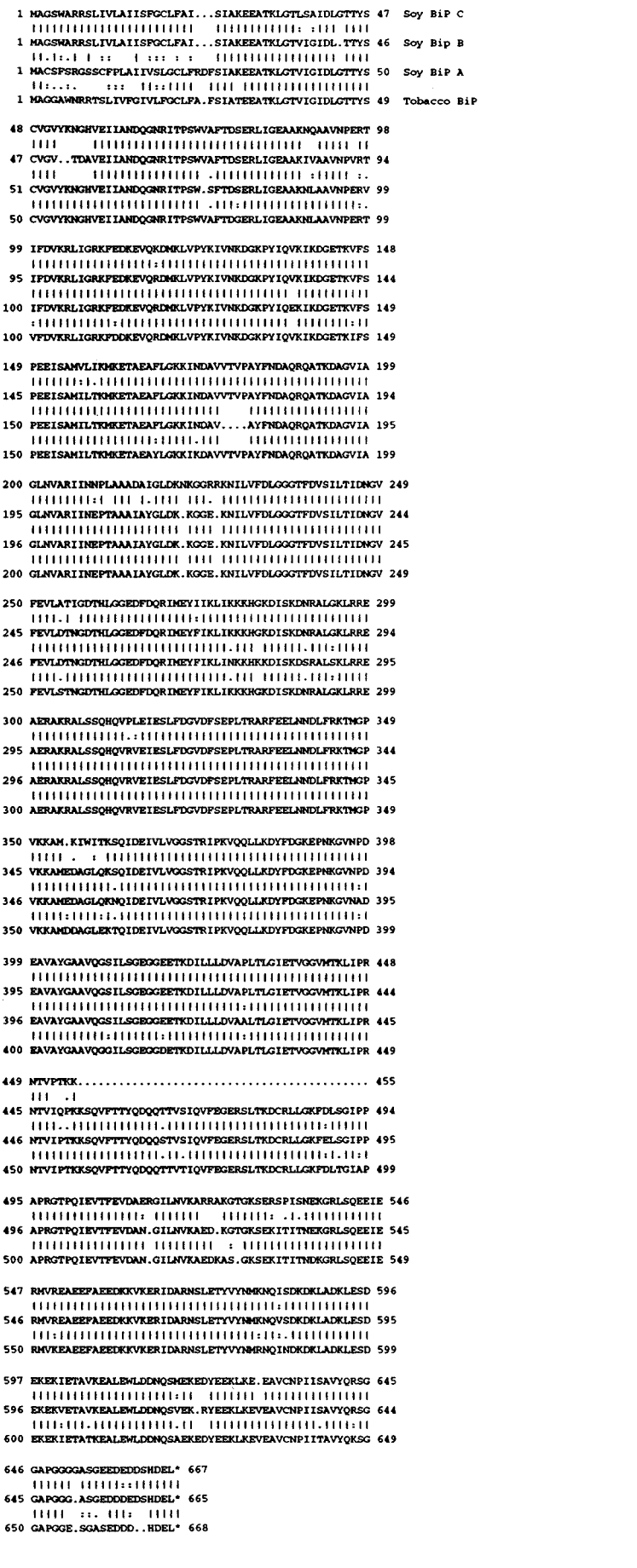

Fig. 2. Sequence alignment of BiP isoforms with tobacco BiP. A sequence alignment of the deduced protein sequences of soybean leaf BiP isoforms (A, B, C) and tobacco BiP (JQ1360) is shown. The BiP sequences of both of these plants are highly homologous

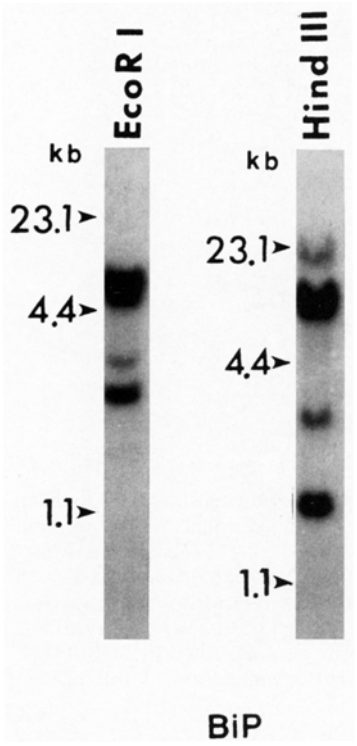

Fig. 3. Hybridization analysis of soybean genomic DNA. Total genomic DNA (10 μg/lane) was digested with either Eco RI or Hind III restriction enzymes, fractionated on a 0.8% agarose gel and transferred to a nitrocellulose membrane. The blot was hybridized with a random-primed labeled BiP cDNA and the final wash was at 68°C for 30 min in 0.1 × SSPE, 0.1% SDS. The hybridization pattern indicates that there are a few different BiP-related genes that constitute a small gene family

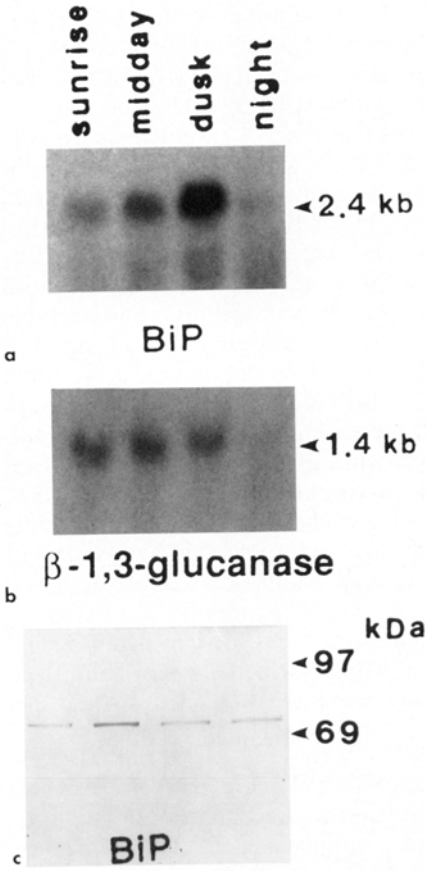

Fig. 4a–c. Diurnal regulation of BiP expression in leaves of soybean. The northern blots contain equal amounts of total RNA (20 μg/lane) isolated from mature and approximately the same size leaves that were taken from separate plants. The RNA blots were hybridized sequentially with BiP (Fig. 4a) and β–1,3-glucanase (Fig. 4b) cDNAs, and washed at high stringency (65°C/30 min). Protein extracts from temporally staged leaves were separated by SDS-PAGE and immunoblotted to visualize BiP (Fig. 4c). Expression of BiP is temporally regulated with peak expression at dusk. Accumulation of BiP protein does not follow the same temporal pattern

HSP70 family proteins is consistent with the apparent specificity of this antiserum.

During the isolation of leaf cDNA clones we inadvertently isolated a partial cDNA for an uncharacterized putative β–1,3 glucanase (sequence not shown). The β–1,3 glucanases are widely distributed plant cell proteins that includes both intravacuolar and secreted isoforms and can be induced by stress. We re-probed several of the Northern blots presented in this paper with the β–1,3 glucanase cDNA in order to have an additional assay of an mRNA of a soluble protein that is expressed in soybean leaves and encodes a protein synthesized by the ER.

Southern blot analysis of Eco RI and Hind III-digested genomic DNA hybridized with a BiP-A cDNA probe indicates that the BiP gene family is a small gene family (Fig. 3) in soybean. This interpretation is consistent with our isolation of three different leaf cDNAs.

Expression of BiP is temporally regulated. Leaves are subject to daily cycles of the environment, including the light-dark cycle. Plants have been shown to exhibit circadian and diurnal alterations in gene expression (Giuliano et al. 1988; Nagy et al. 1988; Taylor 1989; Linthorst et al. 1993) that could affect our analysis of developmentally and stress-regulated BiP gene expression. In order to test whether BiP gene expression may exhibit diurnal variations we harvested leaves at different times of day and used these to prepare RNA and protein samples. The RNA samples were normalized to the same physical mass of RNA. Potential loading errors were controlled by visualization of the fractionated RNA at the completion of the gel run. The gels were visualized again after completion of blotting to ensure complete capillary transfer of the fractionated RNA onto the nitocellulose membrane. Expression of the BiP gene exhibited considerable variation during the course of the day, with a peak in BiP-mRNA abundance occurring at dusk (Fig. 4a). The abundance of BiP mRNA declined to an almost undetectable level a few hours later, indicating that BiP expression is not constitutive in soybean leaves over short periods of time. It is important to note that the leaves assayed were fully mature, expanded leaves that were not exhibiting obvious growth or cell division. The variations in BiP expression were present over short time periods and independent of growth and development. We then examined expression of a putative β–1,3-glucanase on the same blot to provide a control of another soybean leaf

gene product. Although we did not experimentally determine whether this clone encodes a β–1,3 glucanase that is a secreted or vacuolar isoform, both isoforms are synthesized on the ER. This allowed us to assess the gene expression of protein that will initially be cotranslationally localized with BiP before its secretion. The β–1,3 glucanase mRNA exhibited a completely different pattern of gene expression from that of BiP, with almost identical levels of mRNA throughout the entire day (Fig. 4b). Like the expression of the BiP gene, the abundance of β–1,3 glucanase mRNA also declined to almost undetectable levels after night onset. The abundance of the BiP protein exhibited much smaller variations (Fig. 4c) than that demonstrated for BiP mRNA (Fig. 4a). There appeared to be a small increase in BiP abundance at midday. Levels of BiP protein between dusk and night appeared to be identical, in marked contrast to the extreme difference of BiP-mRNA levels at this time. These results clearly show that BiP gene expression is highly dependent upon temporal regulation and that the level of BiP protein in the leaf cells is independently regulated. In order to eliminate temporal variations in gene expression, all of the subsequently described developmental and insect wounding assays were accomplished on mid-day samples.

Developmental regulation of BiP expression. In order to test whether BiP expression and accumulation is constitutive in plant cells, we examined steady levels in soybean leaves, pods and seeds during development. Leaves, pods and seeds all share the common characteristic of termination of cell division early during formation followed by a period of expansion and maturation. This permited us to directly assess BiP expression and accumulation independently of cell division as a determining factor.

Expression of the BiP gene increases during leaf expansion. Immature leaves complete cell division early during formation and this is followed by a long period of expansion. During the early stages of expansion, leaves are net importers of carbon, switching to net export when the leaves are about half-expanded (see Dale 1988, Turgeon 1989 for reviews), the sink-source transition. Soybean leaves were staged based on the size of the leaf. Protein extracts and total RNA samples were prepared from 1/8-, 1/3-, 1/2- and fully expanded leaves. Northern blots of total leaf RNA hybridized by the BiP cDNA at high stringency indicated that the apparent gene expression of BiP increased during the course of leaf expansion, with fully mature leaves exhibiting higher levels of BiP mRNAs than immature leaves (Fig. 5a). It has been previously speculated that immature plant cells may express higher levels of BiP (Denecke et al. 1991) as a consequence of its role in assisting in protein synthesis essential to differentiation. Surprisingly, BiP expression in soybean leaves appears to be the opposite of this prediction with mature leaves expressing substantially higher levels of BiP than more-immature leaves. We then analyzed the level of BiP protein during leaf maturation to provide a comparison with the mRNA expression. The SDS-PAGE immunoblots of the leaf extracts demonstrated

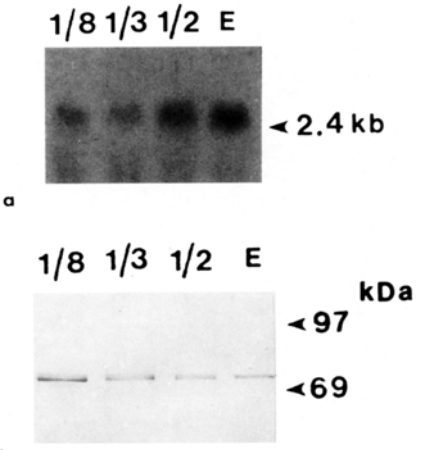

Fig. 5a, b. Expression of the BiP gene during maturation of soybean leaves. **a** The northern blot shown contains equal amounts of total RNA (20 μg/lane) isolated from 1/8-, 1/3-, 1/2-expanded and mature, fully expanded (*E*) leaves. The RNA blot was washed at high stringency (65°C/30 min). Expression of BiP mRNA increases during the course of leaf expansion and maturation. **b** Protein extracts from developmentally staged leaves were separated by SDS-PAGE and immunoblotted. The apparent accumulation of BiP protein declines during leaf maturation

that the relative abundance of BiP normalized to the fresh weight of the source material declined during the course of leaf expansion (Fig. 5b). However, some caution is warranted with regard to the interpretation that BiP-protein levels are the opposite of BiP-mRNA levels. The apparent decrease in BiP protein may result from the accumulation of proteins, cell walls and other constituents during the course of leaf expansion that dilutes the apparent concentration of BiP protein.

Soybean leaves accumulate a vacuolar glycoprotein, vegetative storage protein (VSP) in leaf cells (see Staswick 1994 for review). The VSP is apparently mobilized after the onset of reproductive growth and provides a nutritional source for the maturing seeds. The expression of VSP mRNA is greatly enhanced by depodding soybean plants, which removes the sink. The overexpression of VSP mRNA results in the accumulation of VSP protein. We tested whether the induction of VSP also induces the overexpression of BiP, which would provide positive evidence in favor of a requirement for BiP in vacuolar storage-protein accumulation. We compared the levels of BiP expression in the leaves of paired plants that were allowed to set seeds normally, or were depodded every other day. Paired samples of total RNA were prepared from leaves after three, four and five weeks of continuous depodding and from controls. This period of depodding has been previously shown to induce VSP overexpression, a finding that was verified for this set of leaf samples with SDS/PAGE-immunoblots using anti-VSP antisera (gift of Dr. Paul Staswick, University of Nebraska, USA) as a probe. Northern blots of the paired podded and depodded leaves exhibited little difference, indicating that the induction of the overaccumulation of VSP does not induce the simultaneous induction of BiP (data not shown).

Expression of BiP in maturing pods is not developmentally regulated. Soybean pods encasing the maturing seeds undergo considerable enlargement during seed fill. The pods have a critical role in conducting metabolites derived from the canopy to the seeds. In soybeans, transient storage of vegetative storage proteins temporally sequesters carbon and nitrogen resources directed to the seeds. Expression of the BiP gene does not appear to vary during the course of seed maturation (Fig. 6a). The relative levels of BiP protein declined during the course of seed maturation (Fig. 6b). In fully mature pods the level of BiP protein was much reduced, even though the level of gene expression was essentially unchanged from that of the more immature stages.

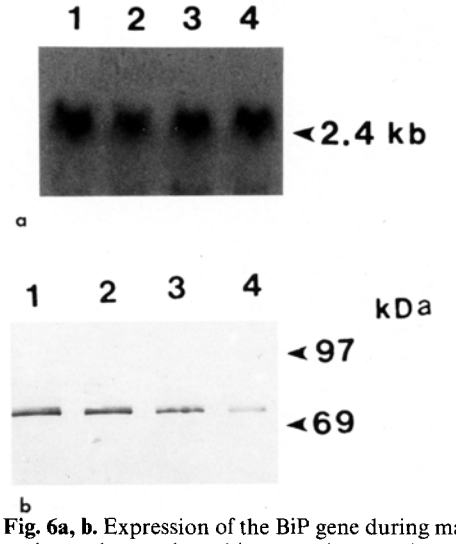

Fig. 6a, b. Expression of the BiP gene during maturation of soybean pods. **a** The northern blot contains equal amounts of total RNA (20 µg/lane) isolated from de-seeded pods that contained 40–50 mg (*lane 1*), 80–100 mg (*lane 2*), 150–180 mg (*lane 3*) and 250–300 mg (*lane 4*) of developing seeds. The RNA blot was washed at high stringency (65°C/30 min) and demonstrates that there is very little change in BiP expression during the course of pod maturation. **b** Protein extracts from pods at the same stages as used in **a** were fractionated by SDS-PAGE, blotted, and immunoreactive BiP polypeptides visualized. Levels of BiP protein declined at the later stages of pod maturation, in contrast to the continuing high level of BiP gene expression

Expression of BiP in maturing seeds is developmentally regulated. The ER is extensively elaborated (Harris 1979) during the course of seed maturation, consistent with its role in the synthesis and accumulation of storage proteins and oil. We tested whether BiP expression is correlated with the changes in the ER. Northern blots of maturing-seed mRNAs at two developmental stages corresponding to an age just prior to (15 mg fresh weight seeds) and the midpoint (150 mg fresh weight seeds) of storage-protein accumulation were probed with the BiP-A cDNA (Fig. 7a). Surprisingly little BiP mRNA was detected in northern blots washed at 65°C/30 min in total RNA samples of maturing seeds (Fig. 7a) even though electron microscopy indicates that the ER is extensively proliferated in soybean seed cells for storage-protein synthesis (Herman 1987 for example). In contrast, high levels of BiP expression were detected in northern blots washed at 65°C of total RNA from cotyledons of germinated seeds. A northern blot of mRNA prepared from four different stages of soybean seed development was then probed with the BiP cDNA and washed at 58°C/30 min (Fig. 7b). The abundance of BiP mRNA increased with the onset of storage-protein accumulation in seeds of fresh weight from 30 to 80 mg. The BiP mRNAs re-

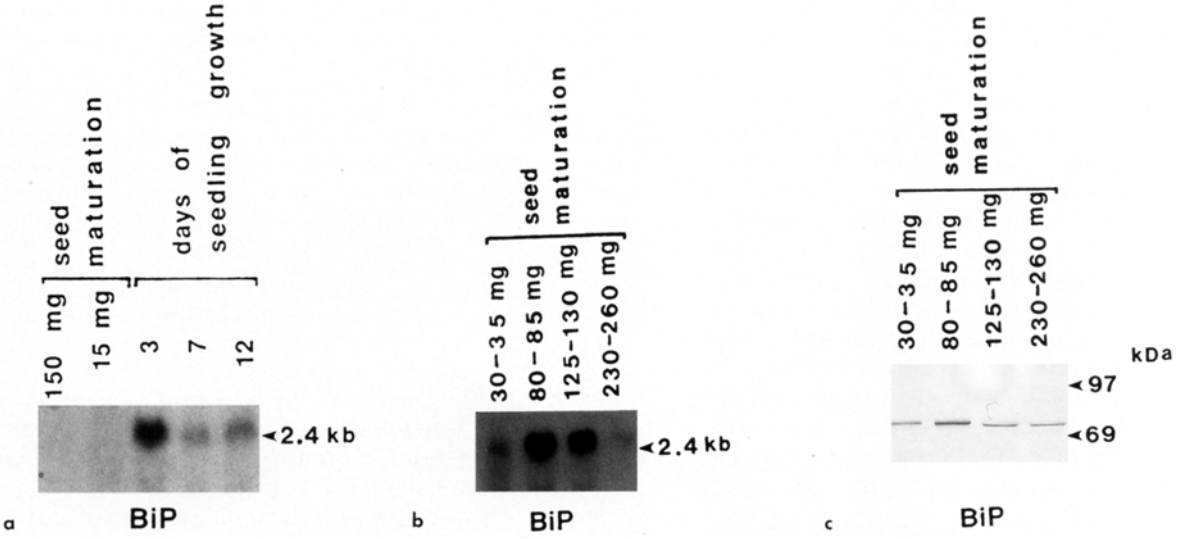

Fig. 7a–c. Expression of the BiP gene and accumulation of BiP protein during maturation and germination of soybean seeds. **a** The northern blot contains equal amounts of total RNA (15 µg/lane) isolated from maturing (15 and 150 mg fresh weight) and germinating (3-, 7-, 12-d-old seedlings) seeds. In blots washed at high stringency (65°C/30 min) no BiP mRNA was detected in maturing seeds while high levels of BiP mRNA were detected in 3-d-old seedlings. **b** A Northern blot containing 20 µg/lane of total RNA isolated from maturing seeds (30, 80, 125 and 230 mg fresh weight) was washed at moderate stringency (58°C/30 min). The BiP mRNAs detected exhibited a developmental variation with high levels of BiP detected at mid-maturation when storage protein and oil is accumulated. **c** Protein extracts from seeds at the same developmental stages as used in **b** were separated by SDS-PAGE, blotted and polypeptides immunoreactive with BiP antiserum visualized. Accumulation of BiP protein followed a similar pattern to that of BiP gene expression assayed at moderate stringency

mained abundant during active storage-protein accumulation (150 mg) but subsided to nearly undetectable levels after storage-protein accumulation was complete (225 mg). The detection of developmental variations in BiP abundance during seed maturation in a blot probed at moderate stringency is interpeted to indicate that the BiP gene(s) expressed are different members of the gene family. Further, these results suggest, but do not yet prove, that different BiP isoforms are probably expressed during seed maturation and germination. The germination BiP mRNAs are either very similar or identical to the leaf BiP mRNAs. An identical developmental series of seed protein extracts was fractionated by SDS/PAGE and blotted onto nitrocellulose membranes in order to test for the presence of BiP polypeptides. The BiP polypeptides were detected as a strongly immunopositive band at all developmental stages with a peak of immunoreactivity occurring in seeds of 85 mg fresh weight (Fig. 7c), which is the developmental stage of active storage-protein accumulation and subdivision of the central vacuole into protein-storage vacuoles. The BiP protein continued to be abundant throughout seed maturation to maturity. Although little BiP mRNA was detected in green mature soybean seeds even with a 58°C/30 min wash (Fig. 7b), BiP protein was still present at levels similar to those of more-immature seeds (Fig. 7c).

Adaption to persistent wounding by insects induces BiP overexpression. Inhibitors that induce synthesis of misfolded proteins, e.g. tunicamycin which inhibits cotranslational glycosylation, induce BiP overexpression. We have confirmed that tunicamycin induces massive overexpression of BiP in soybean leaves (data not shown). Temperature stress induces BiP in yeast cells (Normington et al. 1989; Rose et al. 1989) but not in tobacco plants (Denecke et al. 1991). Wound induction results in the synthesis of new proteins that function to mitigate the wound stress or function in defense roles. We studied whether wounding might induce BiP expression by investigating two different types of wounding of soybean leaves, persistent wounding resulting from insect feeding and single-event puncture wounding.

After an overnight exposure of the soybean plants to whitefly attack (see Byrne 1991 for whitefly review) there was an increase in the mid-day level of BiP expression (Fig. 8a). However, persistent long-term wounding induced by two weeks in the whitefly-infested greenhouse resulted in the overexpression of the mid-day BiP-mRNA level. The same blot was then reprobed with the β–1,3 glucanse cDNA. The β–1,3 glucanases are among the pathogen-related proteins that are often shown to be highly expressed in plants under fungal or bacterial attack. The β–1,3 glucanase mRNA also increased after 24 h of whitefly attack but, unlike BiP, there was little change in the mRNA level after persistent attack (Fig. 8b). The level of BiP protein examined at zero time, as well as after one and two weeks of persistent whitefly attack, only increased slightly (Fig. 8c). This appears to indicate that while BiP mRNA can be induced to be highly overexpressed by persistent wounding this does not result in a parallel large increase in BiP-protein levels.

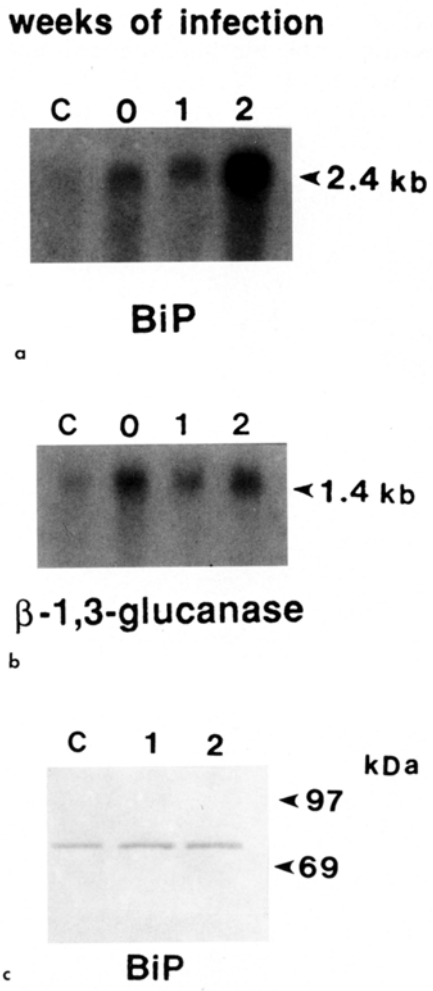

Fig. 8a–c. Persistent wounding of soybean leaves by whitefly induces BiP gene expression. Total RNA (8 μg/lane) from leaves whitefly-infested overnight (*O*), for one week (*1*) and two weeks (*2*) as well as control (*C*) noninfested (zero time) leaves were fractionated and blotted. **a** Hybridization of the BiP probe after a high-stringency wash (65°C/30 min). Note that after an overnight infestation there is a slight rise in BiP levels and after two weeks of persistent infestation BiP is overexpressed. **b** Reprobing with a β–1,3-glucanase probe indicates that there is a small increase in β–1,3-glucanase levels after overnight infestation that is maintained after 1 and 2 weeks of infestation. **c** Protein extracts from the infested and control leaves were separated by SDS-PAGE, blotted and the BiP immunocross-reactive polypeptides visualized. Persistent two-week whitefly infestation induces a small increase in BiP protein levels, but overexpression of BiP protein accumulation is not observed

Insect wounding and its induction of BiP gene overexpression occurs after long-term continuous wounding. We tested whether single-event wounding may produce similar enhancement of BiP expression. We punctured one leaflet of a trifoliate with the multiple-pin apparatus shown in Fig. 9a to simulate single-event insect wounding in several different plants, choosing leaves of identical age. No plant was used for more than one point time in order eliminate possible systemic signal-transduction events. The companion trifoliate leaflet was used as a parallel control. Wounding occurred at dawn and data for both the wounded and unwounded leaves were collected at various time points throughout the day and

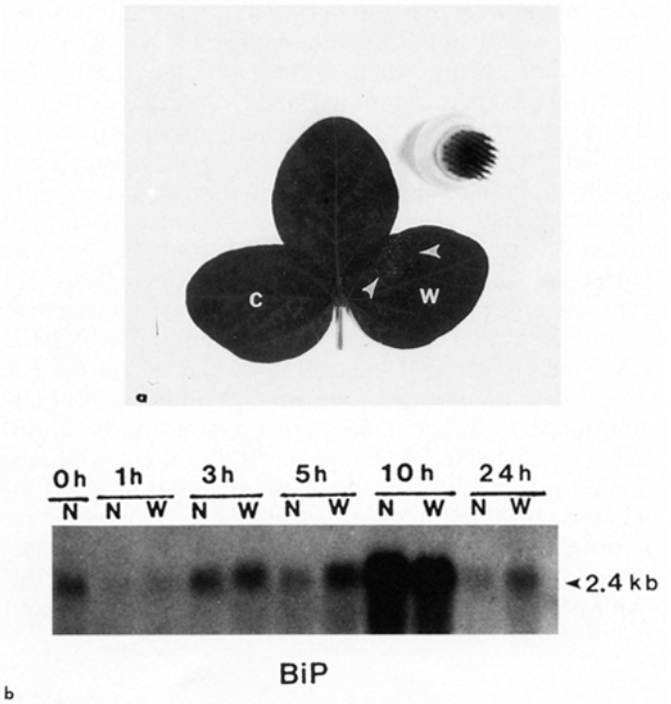

Fig. 9a, b. Puncture wounding of soybean leaves slightly enhances BiP gene expression. Leaves of identical age were punctured with a multipin device on one leaflet (*W*) of a trifoliate (**a**; *arrowheads*) at dawn (0 h) on plants maintained under the ambient day/night cycle. The companion leaflet (*C*) was used as an internal control for each time point. The northern blot (**b**) shows total RNA from the non-wounded (*N*) and wounded (*W*) leaves isolated at zero time and at 1 h, 3 h, 5 h, 10 h and 24 h. The RNA (20 μg/lane) was electrophoretically separated, blotted and probed with BiP cDNA, and the nitrocellulose blot was washed at high stringency (65°C/30 min). Single-event wounding slightly induces excess BiP expression 3 h after wounding. This excess expression is maintained 5 h after wounding and is still apparent after 24 h. However, the primary regulation of BiP expression is the temporal regulation, with very high levels of BiP mRNA present at dusk, 10 h after wounding. There is little or no difference in the BiP expression of wounded and nonwouned leaves at dusk

after 24 h. Levels of total RNA from both the wounded and control leaves were analyzed by northern blots (Fig. 9b). Both the wounded (Fig. 9b, labeled W) and control (Fig. 9b, labeled N) leaves exhibited a temporal change in BiP-mRNA expression, with the peak of expression occurring at dusk as previously shown in Fig. 4a. The wounded leaves exhibited a slight enhancement in BiP-mRNA levels beginning 3 h after wounding. However, the contribution of the wound-induced increase in BiP expression was dwarfed by the temporally regulated expression peaking at dusk.

Discussion

Binding protein is present as a highly conserved, small gene family. As visualized in Southern blots, soybean BiP exists in a small gene family with several different members expressed in the same tissues at the same developmental stage. Tobacco plants have also been shown to have multigene family encoding BiP isoforms (Denecke et al. 1991) while, in contrast, spinach plants appear to possess only a single BiP gene (Anderson et al. 1993). The sequence homology between tobacco (Denecke et al. 1991), maize (Fontes et al. 1991), tomato (GenBank No. LO8830) and soybean BiP indicates that this protein is highly conserved among plants. The primary differences between the tobacco and soybean sequences are in the region of the cotranslationally cleaved signal sequence and in the region immediately adjacent to the HDEL retention sequence. The variability of sequences adjacent to the HDEL is interesting because the lack of conservation at this site appears to indicate that these amino acids are not essential to display the HDEL for recognition by the retention receptor (Lee et al. 1993).

Although we have not yet cloned the BiP(s) expressed in maturing soybean seeds, the lack of hybridization after a high-stringency wash (65°C/30 min), while hybridizing with the BiP-A probe after a moderate-stringency wash (58°C/30 min), appears to indicate that the BiP(s) expressed in maturing seeds is different from those expressed in vegetative cells such as leaves and pods. We had considered the possibility that this hybridization is the consequence of HSP70 mRNAs rather than BiP mRNAs. However, by using an antiserum that appears to be specific, we have shown that BiP protein is abundant in maturing seed cotyledons.

The Southern blots shown in this paper were washed at high stringency and therefore may not show bands corresponding to the maturing-seed BiP genes. The immunological cross-reactivity indicates that maturing-seed BiP is identical in M_r to vegetative-cell BiP, and has epitopes in common. Whether the maturing-seed BiP(s) will prove to be an embryo-specific isoform of BiP is speculative for the present.

Expression of BiP is developmentally and temporally regulated in soybean plants. In this paper we have shown that BiP gene expression is variable and is dependent on development, diurnal time and wounding stress. To what extent various isoforms of BiP are expressed as the consequence of these regulating factors is for the present unknown and the subject of continuing research by this laboratory.

The pattern of BiP expression in seeds correlates very well with previous studies (Denecke et al. 1991) and qualitative observations of the proliferation of the ER during seed protein accumulation (Harris 1979). In soybeans the active phase of storage-protein accumulation correlates very with BiP expression. A functional role for BiP in mediating cotranslational folding of the soluble storage proteins synthesized by soybean seeds has not yet been established. Binding protein is not bound to normal phaseolin (Pedrazzini et al. 1994), although a transient association with BiP is possible, but BiP does appear to have a direct role in the aggregation of hydrophobic prolamine proteins (Levanony et al. 1992; Li et al. 1993) that are not translocated through the endomembrane system. However, BiP has been demonstrated to interact with underglycosylated phaseolin synthesized in the presence of tunicamycin (D'Amico et al. 1992) and with a deletion

mutant of phaseolin that is unable to form oligomers (Pedrazzini et al. 1994). This indicates that the function of BiP in plant cells includes a role in the recognition and disposal of malformed proteins.

We expected that BiP expression levels might be higher in immature leaves in the active stages of expansion and maturation, but the pattern of BiP expression clearly shows an increase in BiP expression as leaves mature. One potential function of BiP during the later stages of leaf development could be in the accumulation of those leaf storage proteins termed vegetative storage proteins (see Staswick 1991 for review). When we attempted to test this hypothesis by depodding soybean plants, a treatment that induces expression of vegetative storage-protein genes, we were unable to demonstrate any clear correlation with BiP expression. The temporal changes of BiP expression in leaves demonstrate that regulation of gene expression occurs over short time scales unrelated to growth and development.

Levels of BiP mRNA and protein appear to be independently regulated. There appears to be a lack of correlation between high levels of BiP expression and the accumulation of BiP protein. How can the differences between BiP-RNA expression and BiP-protein accumulation be reconciled? We have identified temporal and developmental stages where BiP mRNA exhibits wide variations in expression without parallel changes in the pattern of BiP-protein accumulation. Nothing is presently known about the turnover of BiP or potential translational regulation of BiP in plants. If BiP-protein levels are held relatively constant as it appears, then this implies that either the translational efficiency of BiP mRNA is regulated or that there is regulation of the half-life of BiP protein. Tobacco BiP is apparently unstable if expressed in yeast cells (Denecke et al. 1991), implying that there may be specific mechanisms for turnover of reticuloplasmins. Further, BiP may be posttranslationally regulated, resulting in patterns of BiP-protein accumulation that differ significantly from the patterns of BiP gene expression. The present data suggest many different lines of inquiry regarding the half-life and turnover of BiP, and its relationship to the function of the endomembrane system of plant cells. We are continuing our investigations with this general objective.

Expression of the BiP gene and accumulation of BiP protein are regulated by complex factors. The results we have presented in this paper demonstrate that soybean BiP is regulated in a complex pattern. There are two distinct interpretations that can be made with regard to the physiological consequences of the alteration of BiP-mRNA abundance. The changes in BiP abundance may reflect relative changes in the quantity of ER during the course of development, diurnal-time course or stress response. The increase of BiP expression in response to whitefly attack may result from increases in protein synthesis and/or turnover as the plants compensate for injury. Just such an interpretation was made of increases in BiP expression resulting from long-term sensitization training in the mollusk *Aplysia* (Kuhl et al. 1992). The changes in BiP expression during seed maturation correlate very well with this hypothesis. The ER has been documented as being extensively elaborated during the course of seed maturation to provide the capacity to synthesize seed storage proteins and oils, and BiP expression parallels this pattern. If this hypothesis is valid, then it would imply that ER synthesis and assembly is not only developmentally regulated but is also subject to diurnal and stress-regulation. This hypothesis could be tested by correlated studies involving morphometric analysis and expression of other intrinsic ER proteins. An alternative hypothesis is that the specific concentration of BiP in the ER lumen is variable, depending on the role of the ER during a particular developmental or physiological circumstance. In such a model, higher concentrations of BiP might be correlated with high levels of protein synthesis. Such a model could be tested by quantitative electron-microscopic immunocytochemistry. Much research remains to be accomplished to elucidate not only the regulation of BiP expression but also how the control of BiP expression is correlated with proliferation of the ER and its functional role in synthesizing proteins.

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