The Phosphorylation State and Expression of Soybean BiP Isoforms Are Differentially Regulated following Abiotic Stresses*

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The mammalian BiP is regulated by phosphorylation, and it is generally accepted that its unmodified form constitutes the biologically active species. In fact, the glycosylation inhibitor tunicamycin induces dephosphorylation of mammalian BiP. The stress-induced phosphorylation state of plant BiP has not been examined. Here, we demonstrated that soybean BiP exists in interconvertible phosphorylated and nonphosphorylated forms, and the equilibrium can be shift to either direction in response to different stimuli. In contrast to tunicamycin treatment, water stress condition stimulated phosphorylated state of BiP species in soybean cultured cells and stressed leaves. Despite their phosphorylation, we demonstrated that BiP isoforms from water-stressed leaves exhibit protein binding activity, suggesting that plant BiP functional regulation may differ from other eukaryotic BiPs. We also compared the induction of the soybean BiP gene family, which consists of at least four members designated soyBiPA, soyBiPB, soyBiPC, and soyBiPD, by tunicamycin and osmotic stress. Although all soybean BiP genes were induced by tunicamycin, just the soyBiPA RNA was up-regulated by osmotic stress. In addition, these stresses promoted BiP induction with different kinetics and acted synergistically to increase BiP accumulation. These results suggest that the soybean BiP gene family is differentially regulated by abiotic stresses through distinct signaling pathways.

The endoplasmic reticulum (ER) provides the folding environment that facilitates the acquisition of proper folding and assembly of secretory proteins, a prerequisite for them to move further through the secretory apparatus (for reviews, see Refs. 1–4). A set of ER-resident proteins, including molecular chaperones and folding enzymes, associates with newly synthesized polypeptides to assist proper folding and assembly of oligomeric secretory proteins (reviewed in Refs. 1, 3, and 5). The binding protein (BiP) represents one of the best-characterized molecular chaperones from the ER.

The mechanism of BiP binding to and release from nascent polypeptide is believed to be analogous to that described for the cytosolic HSP70 protein (6, 7). HSP70-related proteins exist in equilibrium between self-assembled forms and monomers in which the binding site is either free or associated with other proteins (8). This equilibrium is regulated by cycles of ADP/ATP binding and hydrolysis as well as cycles of protein substrate binding and release (9). BiP also exists in interconvertible oligomeric and monomeric forms and is post-translationally modified and regulated by ADP-ribosylation and phosphorylation (10, 11). These modifications occur upon the ATP-dependent release of BiP from associated proteins and can be reversed under stress conditions that increase the level of unfolded polypeptides in the ER. Because phosphorylation and ADP-ribosylation appear to be restricted to oligomeric forms of mammalian BiP that are not bound to nascent polypeptides, the monomeric, unmodified form of BiP is thought to be the biologically active species (12). In spinach, three forms of BiP, the 85-kDa monomer, a 280-kDa multimeric form, and a 650-kDa oligomeric form, have been described (13). However, only the oligomeric form of BiP is phosphorylated in vitro, suggesting that, like mammals, the level of functional plant BiP is regulated by post-translational modification.

The ER molecular chaperone proteins are expressed constitutively at low levels in all cells but are induced upon accumulation of unfolded protein in the lumen of the ER (5, 7). The expression of folding-defective mutant secretory proteins or treatment of cells with agents that impair protein folding activates a signaling cascade to allow communication between the ER and nucleus (14). This signal transduction pathway, designated the unfolded protein response (UPR) pathway, is characterized by the coordinated transcriptional up-regulation of BiP and other ER proteins that are involved in folding and assembly of nascent proteins. The inter-organelle signaling cascade, which has been elucidated in yeast, involves an ER transmembrane kinase and a basic leucine zipper transcription factor, Hac1p, whose level is modulated by a regulated spliceosome-independent mRNA splicing event (reviewed in Ref. 15).

In plants, like mammals and yeast, the expression of BiP is regulated according to cellular requirements for chaperone activity. Thus, both the increase of secretory activity and the accumulation of unfolded proteins within the ER result in the induction of BiP synthesis in plants (reviewed in Refs. 1 and 16). In the floury-2 mutant of maize, the synthesis of a zein-like storage protein variant, which contains an uncleavable signal sequence, is associated with increased accumulation of BiP (17–20). Expression of an assembly-defective mutant of the

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†† The abbreviations used are: ER, endoplasmic reticulum; UPR, unfolded protein response; RT-PCR, reverse transcriptase-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; PMSF, phenylmethylsulfonyl fluoride; bp, base pair(s).
bead storage protein phaseolin also induces BiP synthesis in tobacco leaf protoplasts (21). Furthermore, tunicamycin, a potent activator of the UPR pathway, efficiently induces BiP expression at both mRNA and protein level in several plant systems (18, 22). These results have led to the conclusion that, like mammals and yeast BiP, plant BiP is most likely regulated through an unfolded protein response pathway. This idea is supported by the observation that, as in mammalian cells, overexpression of BiP in tobacco leaf protoplasts attenuates ER stress caused by tunicamycin and prevents activation of the unfolded protein response pathway (23, 24). However, in some plant species, specific stress conditions and developmental events alter BiP mRNA and protein levels to different extents, suggesting that post-transcriptional mechanisms are also involved in the regulation of BiP synthesis in plants (13, 25).

Alternatively or additionally, these discrepancies between the level of BiP mRNA and protein may reflect differential expression and regulation of the different BiP gene families, since the genome of several plant species is represented by multiple BiP genes (25–27). Both alternatives support the argument that multiple, complex regulatory mechanisms control BiP gene expression in plants.

In soybean, three distinct BiP cDNAs have been isolated from a leaf library (25), and one has been identified in a seed cDNA expression library (28). In this paper, we used two-dimensional gel electrophoresis and reverse transcript (RT)-PCR assays to examine the potential BiP regulatory mechanisms in plants. We compared the mechanisms controlling BiP up-regulation by tunicamycin treatment and water stress as well as the phosphorylation state of the induced BiP forms.

**EXPERIMENTAL PROCEDURES**

**Plant Material and Greenhouse Experiments—**Soybean plants (Glycine max cv. Cristalina) were germinated in 5-liter pots containing a mixture of soil, sand, and dung (3:1:1) and grown in standardized greenhouse conditions. Plant tissues were harvested, immediately frozen in liquid nitrogen, and stored at −80 °C.

Water stress condition was induced in 40-day-old plants by withholding watering for 8 days before harvesting of leaves. Half of the leaves were used to measure the relative water content (29), and the other half was frozen in liquid nitrogen.

**Cell Culture and Induction of Soybean BiP—**Cotyledons cells from the soybean variety IAC-12 were cultured as described previously (30). Tunicamycin was added to cultures at 4 days after passage by dilution of a 5 mg/ml stock in Me2SO into normal growth medium to 10 μg/ml and incubated for the intervals indicated in the figure legends. For water stress, the cells were washed and then cultured with normal growth medium containing 10% (w/v) PEG-8000 (polyethylene glycol), which corresponds to a water potential of −1.4 megapascals for the indicated intervals.

For two-dimensional gel electrophoresis, SDS-PAGE, and RT-PCR assays, the cells were filtered under vacuum and washed with an isotonic solution (0.25 M NaCl) to remove any adhered medium and PEG. The cells were then frozen in liquid nitrogen before protein and RNA extractions.

**Whole Cell Protein Extraction, Two-dimensional Gel Electrophoresis, Immunoblotting, and Dephosphorylation Assays—**Total protein was extracted from an acetone dry powder as described (31) with some modifications. Protein extracts from an acetone dry powder, as described (31) with some modifications. Protein extracts (1 ml) were preheated by incubating with 100 μl of 50% (v/v) protein A-Sepharose (Amersham Pharmacia Biotech) at a ratio of 1 g of tissue/mL of extraction buffer. After incubation with the protein extract for 10 min, cell debris was removed by centrifugation at 13,000 × g for 15 min.

Immunoprecipitations were performed as described (37) with some modifications. Protein extracts (1 ml) were preheated by incubating with 100 μl of 50% (v/v) protein A-Sepharose (Amersham Pharmacia Biotech) at a ratio of 1 g of tissue/mL of extraction buffer. After incubation with the protein extract for 10 min, cell debris was removed by centrifugation at 13,000 × g for 15 min.

Immunoprecipitation Assays—All of the procedures were conducted at 4 °C. Protein extracts were prepared by homogenization of 1 g of leaves with 20 ml of Tris-Cl, 0.5% (v/v) Triton X-100, 1 m M PMSF, and 1 unit/ml apyrase or 2 mM ATP at a ratio of 1 g of tissue/mL of extraction buffer. After incubation with the protein extract for 10 min, cell debris was removed by centrifugation at 13,000 × g for 15 min.

Immunoprecipitations were performed as described (37) with some modifications. Protein extracts (1 ml) were preheated by incubating with 100 μl of 50% (v/v) protein A-Sepharose (Amersham Pharmacia Biotech) at a ratio of 1 g of tissue/mL of extraction buffer. After incubation with the protein extract for 10 min, cell debris was removed by centrifugation at 13,000 × g for 15 min.
Regulation and Phosphorylation of the Soybean BiP Forms

RESULTS

The Phosphorylation State of Water Stress-induced BiP Forms Is Distinct from That of Tunicamycin-induced BiP Forms—Previously we showed that soybean BiP is induced by water stress (28). In water-stressed leaves, BiP synthesis is up-regulated to the same extent as in nutritionally stressed leaves and in pathogen-infected leaves. However, the synthesis of spinach BiP, which is a product of a single gene, is unaffected by water stress (13). In fact, in spinach, water stress resulted in disappearance of the BiP mRNA, although the level of protein remained unaltered. To characterize the water stress regulation of the soybean BiP family, we examined the BiP isoforms in control (relative water content 85%) and in water-stressed (relative water content 50%) soybean leaves by immunoblotting assays of two-dimensional gels (Fig. 1). The pH range of the BiP species detected in water-stressed leaves was more acidic than that of the BiP isoforms from control leaves (compare Fig. 1, A and B). To identify whether water stress resulted in the appearance of phosphorylated BiP isoforms or caused the induction of more acidic, distinct BiP species, the leaf protein extract was treated with phosphatase before electrophoresis (Fig. 1C). Dephosphorylation of BiP caused a shift in the isoelectric focusing migrations of the water-stressed-induced forms (pI 5.4–5.8) toward a less acidic pI cluster (pI 6.1–6.25) and a less acidic, more basic pI cluster (pI 6.2–6.5).  

Fig. 1. Induction and phosphorylation of soybean BiP forms in response to water deficit. Whole cell protein extracts from control (A) and water-stressed soybean leaves (B and C) without (−) and with (+) alkaline phosphatase (AP) treatment were displayed in two-dimensional gels, transferred to nitrocellulose, and probed with the anti-BiP serum. The directions of migration for the isoelectric focusing gels and SDS gels are indicated by the arrows at the top of the panels. The pH gradient (pI) is shown at the bottom, and prestained molecular markers are shown on the left. Arrows indicate the position of individual BiP polypeptides, and bars indicate clusters of BiP species. 

For microsomal membrane isolation, normal and water-stressed leaves were homogenized with 25 mM Tris-HCl (pH 7.0), 250 mM sucrose, 2.5 mM dithiothreitol, 10 mM MgSO4, 0.5% (w/v) gelatin, and 0.5 mM PMSF (40). The homogenate was filtered and centrifuged at 80,000×g for 15 min at 4 °C. Microsomal preparations were isolated by density gradient centrifugation (41–43).

Fig. 2. Differential phosphorylation state of tunicamycin- and water stress-induced BiP forms. Whole cell protein extracts from control (A and B), tunicamycin-treated (C and D), and PEG-treated (E and F) soybean cell cultures were resolved by two-dimensional isoelectric focusing/SDS-PAGE with (−) and without (+) alkaline phosphatase (AP) treatment. After electrophoresis, the proteins were immunoblotted using an anti-soybean BiP serum. Prestained molecular markers are on the left of the membrane. Ranges of isoelectric points are shown at the top of the gels as an acidic cluster (Ac, pI 5.4–5.7), intermediate cluster (In, pI 5.85–6.2), and a less acidic, more basic pI cluster (Bs, pI 6.2–6.5).
were resolved in several isoelectric states ranging from pI 5.8 to 6.5 (compare Fig. 2, A and C, Bs and In). In contrast, treatment of the cells with PEG promoted the induction of a subset of BiP forms, which were resolved in a more acidic isoelectric focusing position cluster, pI 5.4–5.7 (Fig. 2E, Ac).

The relative differences in the isoelectric focusing migrations between the water stress- and tunicamycin-induced forms prompted us to examine whether their phosphorylation state changed following stress conditions. In untreated control cells (Fig. 2A), BiP occurred in several isoelectric states that resolved as an acidic cluster (3–4 forms, pI 5.8–6.1, In) and a more basic species, pI 6.5 (Bs). The acidic cluster represents different phosphorylated forms of the same protein because dephosphorylation assays caused their co-migration as a less acidic single species (compare BiP forms under In in Fig. 2, A and B). In contrast, phosphatase treatment of the samples did not cause a shift in the isoelectric point of the more basic species (Fig. 2B, Bs). Together, these results indicated that soybean BiP exists as a pool of phosphorylated and nonphosphorylated forms in untreated soybean cells. Because treatment of the cell protein extracts with alkaline phosphatase before electrophoresis did not alter their relative migration and amounts in two-dimensional gels, the tunicamycin-induced BiP forms are nonphosphorylated (Fig. 2, C and D). In contrast, osmotic stress resulted in the appearance of phosphorylated BiP forms, as judged by the complete conversion of the acid induced forms (Fig. 2E, Ac) to less acidic forms upon alkaline phosphatase treatment (Fig. 2F, In). The acid-induced cluster (pI 5.4–5.7, Ac) migrated at a position consistent with phosphorylated forms of the less acidic cluster (pI 6.0–6.3, In). These results demonstrated that, unlike tunicamycin, water stress induces BiP phosphorylation in cultured cells.

**BiP Isoforms from Water-stressed Leaves Exhibit Protein Binding Activity**—The differential post-translational modification of water stress- and tunicamycin-induced soybean BiP forms prompted us to investigate whether the phosphorylated water stress-induced species were functional. An inherent property of molecular chaperones is their capacity to associate with protein substrates in an ATP-dependent manner. To analyze the protein binding activity of water stress-induced BiP isoforms, we took advantage of water stress-induced proteins as targets for co-immunoprecipitation assays. In water-stressed leaves, we detected the induction of a 28-kDa polypeptide (Fig. 3, A and B, lanes 3), which was not detected in normal leaves (lanes 1) and insect-attacked leaves (lanes 2). Antibodies prepared against a purified BiP fraction from water-stressed leaves, here referred as anti-WSBiP, cross-reacted with the 28-kDa polypeptide (Fig. 3B, lane 3), whereas the anti-soybean BiP serum prepared against the BiP carboxyl terminus (28), here referred as anti-BiP, was specific to BiP and failed to recognize the water stress-induced 28-kDa polypeptide (lane 5). The 28-kDa protein was not a BiP degradation product, because antibodies prepared against BiP purified from soybean (44) or maize (18) seeds cross-reacted with all soybean BiP isoforms from water-stressed leaves but did not recognize the 28-kDa water stress-induced polypeptide (data not shown). This result indicated that the 28-kDa polypeptide did not share conserved epitopes with BiP. More likely, it was a contaminant of the BiP-purified fraction used to raise the anti-WSBiP antibody. In view of this observation, the cross-reactivity of the anti-WSBiP antibody raised the possibility that the 28-kDa contaminant polypeptide had apparently co-purified with BiP from water-stressed leaves as a result of a previous association between these proteins.

Specific interaction between BiP and the water stress-induced 28-kDa polypeptide was confirmed by immunoprecipitation of water-stressed leaf protein extracts with anti-BiP followed by Western blotting with anti-WSBiP (Fig. 3C). Although anti-BiP serum did not cross-react with the 28-kDa polypeptide, this water stress-induced polypeptide was co-immunoprecipitated by the BiP-specific antibody in ATP-depleted conditions (lane 3). The precipitation of the 28-kDa polypeptide by anti-BiP serum was not due to nonspecific interactions, because antibodies to an unrelated protein failed to precipitate the water stress-induced protein (data not shown). Inclusion of ATP in the immunoprecipitation assays prevented BiP-28-kDa polypeptide association (lanes 1 and 2). This result is in agreement with previous data showing that the addition of ATP to protein extracts causes the disruption of BiP-substrates complexes (1, 2, 16, 37). In addition, the 28-kDa water stress-induced protein was found to be strongly enriched in microsomal vesicles derived from the endomembrane system (Fig. 3D, lane 2). The subcellular localization of the *in vitro* BiP substrate further indicates that association of BiP from water-stressed leaves and the 28-kDa-induced polypeptide may be biologically relevant.
expression was monitored by Northern blotting with BiP cDNA as a probe. Soybean-cultured cells were treated with either PEG-8000 or tunicamycin (TUN) for the indicated number of hours. After incubation, soluble protein and total RNA were isolated from the treated cells. In A and D, total protein was fractionated by SDS-PAGE and immunoblotted with an anti-soybean BiP serum. In B and E, BiP expression was monitored by Northern blotting with BiP cDNA as a probe. C and F correspond to the same membranes as in B and E, respectively, reprobed with an 18 S rDNA probe.

The kinetics of BiP induction by water stress in soybean-cultured cells differs from the delayed kinetics of BiP induction by tunicamycin—in the previous experiment, we demonstrated that tunicamycin treatment induced all BiP species, whereas osmotic stress only promoted the accumulation of a subset of soybean BiP isoforms in cell cultures. This observation is consistent with different mechanisms controlling BiP expression under abiotic stresses. As a first step in characterizing the mechanisms by which water stress regulates BiP gene expression, we performed a time course experiment to compare the kinetics of BiP induction by water stress and tunicamycin. Levels of BiP protein and RNA were examined at various times after supplementation of the normal growth medium with PEG or tunicamycin. Although both treatments promoted BiP induction, the kinetics were different (Fig. 4). PEG treatment resulted in increased BiP protein and RNA levels as early as 30 min after the treatment, with maximal accumulation occurring after 2 h (Fig. 4, A and B). In contrast to the rapid induction of BiP by PEG, the induction of BiP by tunicamycin occurred with delayed response kinetics. A slight increase in BiP protein was first detected 6 h post-treatment and increased gradually until saturation after 24–36 h (Fig. 4D). BiP mRNA induction was initially detected by 2 h post-treatment and reached full induction by 6–12 h but declined with the accumulation of the protein as the glycosylation block stress persisted (Fig. 4E). As RNA loading controls, the membranes were reprobed with a 32P-labeled ribosomal cDNA, and the induction level was normalized to the 18 S rRNA signal (Fig. 4, C and F).

Differential regulation of the BiP gene family in response to water stress—The level of BiP induction by PEG was significantly lower than its induction by tunicamycin (Fig. 4). As a possible explanation for this difference in BiP induction, we analyzed the individual contribution of the members of the soybean BiP gene family to the general pattern of BiP mRNA up-regulation by PEG and tunicamycin treatments through RT-PCR with gene-specific primers. The gene-specific primers were designed to take advantage of the most divergent sequences of the known BiP cDNAs (GenBank™ accessions U08382, U08383, U08384, and AF031241), which differ most in their 5' and the 3'-untranslated sequences. Consequently, primer sets were designed to amplify small fragments from either 3' or 5' ends of the genes (Table I). The set of forward and reverse primers were 100% complementary to the annealing sequences of their cognate cDNA, whereas they were a minimum of four mismatches to the other cDNAs.

The specificity of the primers was further confirmed in control PCR reactions performed with soybean genomic DNA, soyBiPA- and soyBiPD-specific DNA sequences as templates (data not shown). Our results indicate that under the conditions used for the PCR analyses, the A and D primers are gene-specific and do not cross-amplify a non-cognate BiPA or BiPD gene sequence. The sets of B and C primers are capable of discriminating between sequences present in the soybean genome and BiP A- or BiP D-specific sequences. Because soyBiPB and soyBiPC DNA are no longer available, the specificity of B and C primers was confirmed further by sequencing the amplified fragments from leaf cDNA. For the RT-PCR assays, the integrity and amount of the cDNA prepared from soybean-cultured cells were routinely assessed with actin-specific primers. The actin primers were designed to amplify a 580-bp fragment from genomic DNA and a 440-bp fragment from actin cDNA such that the presence of contaminating genomic DNA could be easily assessed in our cDNA preparations.

All RT-PCR reactions were repeated with different numbers of cycles to ensure a quantitative linear amplification (data not shown). In addition, the integrity and amount of the cDNA from different treatments were routinely assessed with actin-specific primers (Fig. 5, Actin). Because the four sets of gene-specific primers were effective to detect increased amounts of the amplified fragment from cDNA prepared from RNA of tunicamycin-treated cells (BiPA, BiPC, BiPD, BiPB, lanes T), all soybean BiP mRNAs were up-regulated by tunicamycin. In contrast, osmotic stress did not induce the soyBiPB, soyBiPC, and soyBiPD expression (BiPC, BiPD, BiPB, lanes B) but caused the up-regulation of soyBiPA transcripts (BiPA, lane P). These results suggested that the soybean BiP gene family is differentially regulated by abiotic stresses through different signaling pathways. In addition, they may explain the lower level of BiP induction by PEG compared with its induction by tunicamycin (Fig. 4).

To test further the hypothesis that water stress induces the synthesis of BiP by a pathway distinct from the unfolded response pathway, we analyzed whether the simultaneous treatment of the cell with both PEG and tunicamycin promoted a synergistic effect on BiP induction. Analysis of the induction of soyBiPA-specific transcripts by RT-PCR demonstrated that the combination of PEG and tunicamycin treatments had an additive effect on BiP mRNA levels (Fig. 5, BiPA, lane PT). Fig. 6 shows the induction of BiP protein accumulation by tunicamycin (lanes 3 and 4), PEG (lanes 5 and 6), and a combination of both treatments (lanes 7 and 8) in soybean suspension cell cultures. When cells were treated with both PEG and tunicamycin, the two stimuli appeared to act synergistically to increase BiP accumulation. Collectively, these results may indicate that induction of soybean BiP in response to water stress occurs by a nonlinear or distinct pathway from that regulating the BiP response to the accumulation of unfolded proteins in the ER.

Discussion
Regulation of plant BiP gene expression has been examined primarily by the detection of BiP RNA and protein levels under stress conditions and at different developmental stages of the plant organs (reviewed in Ref. 1). In general, developmental events that are associated with high secretory activity of the cells and exposure of cells to agents that result in accumulation of unfolded proteins in the ER cause induction of plant BiP (2, 16). However, discrepancies between the level of BiP mRNA...
Regulation and Phosphorylation of the Soybean BiP Forms

The numbering scheme was taken considering the first nucleotide of the BiP cDNA sequence in the GenBank™ as the nucleotide +1. F and R following the name of the primers refer to forward and reverse, respectively. ND, not determined.

| Primer | Sequence | % identity |
|--------|----------|------------|
| BiPAF  | CGAGCTCTAGAGATGTTGCTTT | 100 |
| BiPAR  | TACGTAGACGGCGTATGTCCTC | 100 |
| BiPBF  | TGAGCTCTAGTTATGCGGATCTTG | 45.8 |
| BiPBR  | CGATGCGGCTCAGAGAATGTTG | 40 |
| BiPCF  | CGAGCCGACTCTCAACTTAACCC | 22.7 |
| BiPCR  | CAACATGGGCATCTCTGTAAAAACCCG | 92 |
| BiPDF  | ATCTGGAGGACCGCTCTGTGCGGTGG | 62.9 |
| BiPDR  | CTTGAAGAATCTGCGCTTAAACTAAG | 44 |

FIG. 5. Differential regulation of the soybean BiP gene family in response to stress. RT-PCR assays were performed using cDNA prepared from poly(A)+ RNA of control (C), tunicamycin-treated (T), PEG-treated (P), or PEG plus tunicamycin-treated (PT) soybean cells and the BiP gene-specific primers (BiPA, BiPC, BiPB, and BiPD). Actin mRNA was used as an internal standard. M corresponds to DNA standard markers, whose sizes are shown on the left in bp.

FIG. 6. Synergistic effect on BiP induction by the combination of PEG and tunicamycin treatments. Soybean-cultured cells were treated with either tunicamycin alone (TUN), PEG-8000 alone, or with PEG in the presence of tunicamycin (PEG + TUN) for 12 and 24 h. After incubation, total protein was fractionated by SDS-PAGE and immunoblotted with an anti-soybean BiP serum.

and protein have been observed in spinach and soybean plants under specific stress conditions or developmental stages (13, 25). These observations suggest that post-transcriptional mechanisms and/or differential expression of plant BiP gene family are involved in regulation of BiP synthesis in plants. Both alternatives are consistent with the existence of multiple, complex regulatory mechanisms controlling plant BiP gene expression. In support of this hypothesis, we showed that the soybean BiP gene family exhibits differential regulation in response to abiotic stresses. Three lines of evidence indicated that stimulation of BiP expression by water stress occurs through a pathway distinct from the UPR signaling cascade. First, although all BiP forms were up-regulated by tunicamycin, only a subset of the BiP forms were induced by osmotic stress in cell cultures. Similarly, the mRNA levels of all four soyBiP genes were controlled by tunicamycin, but only the soyBiPA RNA was up-regulated by osmotic stress. Water stress represents the first example of a stimulus that differentially up-regulates BiP genes of the same organism. The absence of soyBiPC, soyBiPB, and soyBiPD induction in PEG-treated cells suggests that the UPR and water-stress regulated pathways are independently controlled. Second, the rapid induction of BiP by PEG was distinct from the delayed tunicamycin-induction kinetics. The difference in the kinetics of BiP induction suggests that different components from the UPR pathway are involved in the signaling pathway that regulates BiP expression under osmotic stress. Finally, treatment of soybean suspension cell cultures with both PEG and tunicamycin promoted a synergistic effect on the level of BiP induction. These data indicate that the BiP inductions by osmotic stress and a glycosylation block are additive and support the notion that the regulation of BiP expression by these abiotic stresses functions in distinct signal transduction pathways.

We have also demonstrated that the soybean BiP forms induced by water stress are post-translationally phosphorylated. This result was unexpected because tunicamycin-induced BiP or BiP bound to nascent proteins is unmodified in animal cells, and it is generally accepted that the nonphosphorylated form is the biologically active BiP species in the folding pathway (10–12). In fact, in tunicamycin-treated soybean cell cultures the induced BiP forms were also found to lack phosphorylation. Thus, the modification of plant BiP protein in response to water stress differs from the usual pattern of post-translational modifications of eukaryotic BiPs.

Our data do not allow us to reconcile the apparent contradiction between dephosphorylation in response to some ER stresses (like tunicamycin) and phosphorylation in response to water stress. The results are further confounded by the differential induction of BiP gene expression and the apparently uniform phosphorylation of all of the BiP proteins under water deficit. One possibility is that, in addition to its chaperone function, soybean BiP exhibits a regulatory, distinct biological function under osmotic stress. This hypothesis was raised because of the selective up-regulation of soyBiPA mRNA and its rapid induction by osmotic stress, which is in marked contrast with the delayed kinetics of induction along with the coordinated induction of all of the BiP RNAs by tunicamycin. These differences are inconsistent with the water stress response being due to accumulation of denatured proteins in the ER. More likely, induction of soyBiPA mRNA by osmotic stress may represent a primary response to water stress that is activated...
As soon as the stress is sensed and may accommodate a regulatory function.

Alternatively, the soyBiP gene may have evolved independent regulatory mechanisms to coordinate efficiently the demand of BiP chaperone function with the induction of a selected group of secretory proteins by osmotic stress, which are probably involved in the osmotic response mechanism (45). Although the relationship between phosphorylation of soybean BiP and central aspects of its molecular function, such as oligomerization and protein substrate binding, has not yet been fully addressed, we showed that BiP isoforms from water-stressed leaves exhibit protein binding activity and associates with a water stress-induced 28-kDa polypeptide. Although the identity of the water stress-induced polypeptide is unknown at present, we provided two lines of evidence suggesting that the association between BiP and the 28-kDa water stress-induced polypeptide is not an in vitro artifact and may be physiologically relevant. First, the 28-kDa polypeptide was localized in microsomal membranes composed primarily of endomembrane vesicles derived from the ER, Golgi, and tonoplast. As a secretory protein, the 28-kDa polypeptide is expected to be transiently co-localized with BiP as it enters the ER. Second, the complex protein, the 28-kDa polypeptide is not an oligomerization and protein substrate binding, has not yet been identified. In vitro phosphorylation of soybean BiP chaperone function with the induction of a selected group of secretory proteins by osmotic stress, which are evolved independent regulatory mechanisms to coordinate efficiently the degradative and synthesis of BiP isoforms. The identification of water stress-induced secretory proteins encoded by early response genes and their association with BiP would provide further support to this possibility.

We demonstrated that soybean BiP exists in interchangeable phosphorylated and nonphosphorylated forms, and the equilibrium can be shifted to either direction in response to different stimuli. Although water stress-induced phosphorylation of BiP could be a mechanism to confer different biological function or substrate specificity to induced BiP isoforms, the dehydromal-mediated interactions. Therefore, phosphorylation of BiP by osmotic stress cannot be attributed simply to inactivation of induced BiP isoforms. The identification of water stress-induced secretory proteins encoded by early response genes and their association with BiP would provide further support to this possibility.

Although the overall ER stress response in plants is thought to be similar to that of yeast and mammals, our results suggest that the BiP response may differ significantly in plants. We have identified several key differences in the expression and regulation of the ER molecular chaperone soybean BiP under abiotic stress. These differences may be related to the existence of multiple BiP genes in plants and to the unique challenge that stress conditions represent to plants compared with other eukaryotes. Because plants cannot avoid environmental changes, they are constantly subjected to a variety of stress conditions. Acclimation to environmental changes requires responses against cell damages, such as preservation of membrane and protein structures, which enable the plant to tolerate and minimize the deleterious effect of abiotic stress.

Possibly the members of the plant BiP gene family have evolved independent regulatory mechanisms to ensure a high level of expression under a broad range of biotic and abiotic stress conditions to protect the plant against cell damage. In this context, it would be interesting to know whether other soybean ER molecular chaperones follow similar response to osmotic stress.

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