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Overexpression of BiP in Tobacco Alleviates Endoplasmic Reticulum Stress

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To study the role of the lumenal binding protein (BiP) in the transport and secretion of proteins, we have produced plants with altered BiP levels. Transgenic plants overexpressing BiP showed dramatically increased BiP mRNA levels but only a modest increase in BiP protein levels. The presence of degradation products in BiP overproducers suggests a regulatory mechanism that increases protein turnover when BiP is abundant. Antisense inhibition of BiP synthesis was not successful, demonstrating that even a minor reduction in the basal BiP level is deleterious to cell viability. Overexpression of BiP leads to downregulation of the basal transcript levels of endogenous BiP genes and greatly reduces the unfolded protein response. The data confirm that BiP transcription is regulated via a feedback mechanism that involves monitoring of BiP protein levels. To test BiP activity in vivo, we designed a functional assay, using the secretory protein α-amylase and a cytosolic enzyme as a control for cell viability. During tunicamycin treatment, an overall reduction of α-amylase synthesis was observed when compared with the cytosolic marker. We show that the tunicamycin effect is due to the depletion of BiP in the endoplasmic reticulum because coexpressed BiP alone is able to restore efficient α-amylase synthesis. This is a novel assay to monitor BiP activity in promoting secretory protein synthesis in vivo.

INTRODUCTION

The lumen of the endoplasmic reticulum (ER) is a specialized environment that promotes the synthesis of a subset of secretory proteins destined for the vacuolar compartment or the extracellular matrix (Vitale et al., 1993). Among the family of ER-resident proteins identified in plants, the lumenal binding protein (BiP) is one of the best characterized (Denecke 1996). The role of BiP in the ER is to transiently bind to unfolded proteins and to prevent intramolecular and intermolecular interactions that can result in permanent misfolding or aggregation (Gething et al., 1986; Hurtley et al., 1989; Gething and Sambrook, 1992; Hendershot et al., 1996). In vitro studies using synthetic peptides have demonstrated that BiP has an affinity for polypeptides with a high degree of hydrophobicity (Blond-Elguindi et al., 1993), but only a few model proteins have been used for interaction studies. It also has been proposed that BiP retains unfolded proteins in the ER as part of a quality control mechanism and to allow more time for proper folding (Hammond and Helenius, 1995). These properties define BiP as a molecular chaperone. An additional role for BiP in protein translocation has been established recently (Vogel et al., 1990; Brodsky et al., 1995; Lyman and Schekman, 1997). In particular, BiP seals off the translocation pore at the lumenal end when the pore is not in use by a ribosome (Hamman et al., 1998).

Overexpression of BiP in mammalian cells has been shown to reduce the rate of protein secretion (Dorner et al., 1992), but other reports have shown only an effect on cell viability during ER stress, with no effect on the transport rate of secretory proteins (Morris et al., 1997). In yeast, lower BiP levels caused reduced secretion, whereas overexpression of BiP did not result in any noticeable effect on the secretion rate (Robinson et al., 1996). The introduction of dominant negative BiP ATPase mutants able to bind to unfolded proteins but unable to release them caused inhibition of immunoglobulin folding (Hendershot et al., 1996) and illustrates the importance of the ATPase activity. It is possible that ATP-driven release of ligands from BiP involves partial unfolding, possibly to allow the protein to refold correctly, but this remains to be shown.

BiP is present under normal growth conditions, but transcription of its gene can be induced by a variety of stresses that lead to the accumulation of malfolded proteins in the ER,

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a situation that triggers the unfolded protein response (UPR; Shamu, 1997). The accumulation of unfolded proteins is thought to reduce the number of free BiP molecules, leading to the induction of BiP transcription (Kohno et al., 1993). The signal is transduced from the ER lumen to the nucleus by a transmembrane kinase, which is essential for the UPR (Cox et al., 1993; Mori et al., 1993). This transmembrane kinase is a bifunctional enzyme that operates in a very unusual fashion. When autophosphorylated, the site-specific endoribonuclease at the C terminus is activated and causes alternative splicing of the transcript encoding the transcription factor Hac1p, which controls BiP transcription. The splicing event allows the synthesis of the transcription factor, which does not occur from the unspliced message (Cox and Walter, 1996; Sidrauski and Walter, 1997; Sidrauski et al., 1998). It is not clear how the receptor kinase monitors BiP levels in the ER lumen, but overexpression of an ATPase-defective BiP mutant has been shown to induce endogenous BiP levels. This demonstrates that the ATPase domain of BiP is involved in the monitoring process (Morris et al., 1997).

Evidence for the function of BiP in plants has been acquired by genetic complementation studies (Denecke et al., 1991) and biochemical studies, using the model protein phaseolin (Pedrazzini et al., 1994). In particular, phaseolin folding intermediates or unassembled subunits of phaseolin were found in association with BiP, in contrast to assembled phaseolin (Vitale et al., 1995; Pedrazzini et al., 1997). Other evidence arose from the *floury2* mutant of maize that produces a mutant storage protein with a defective signal peptide processing site (Coleman et al., 1995). This causes anchoring of the protein to the ER membrane and results in severe ER stress accompanied by drastically increased expression of BiP and other ER chaperones (Boston et al., 1995; Coleman et al., 1997; Gillikin et al., 1997). In tobacco, as in maize and soybean, BiP is encoded by a multigene family (Denecke et al., 1991; Fontes et al., 1991; Kalinski et al., 1995), a situation that seems to be unique to plants. Nevertheless, there are some exceptions, such as spinach (Anderson et al., 1994) and Arabidopsis (Koizumi, 1996), for which the presence of a BiP gene family has not been demonstrated.

Although there is strong evidence that BiP binds to malfolded proteins, there is no evidence that this binding has a beneficial effect on the ligand. In other words, there are still no model systems available to test the activity of BiP on secretory protein synthesis in vivo. This study presents a novel in vivo system to monitor such activity.

RESULTS

Stable Overproduction of BiP in Tobacco Plants

To alter the expression levels of BiP in plants, we generated two chimeric genes: a wild-type BiP coding region under the transcriptional control of the cauliflower mosaic virus (CaMV) 35S promoter (pDE800) and a similar construct in which the BiP coding region was present in the reverse orientation, generating an antisense transcript (pNL100). More than 60 independent transgenic plants at the same developmental stage were analyzed for each construct. These plants were screened by using protein gel blot analysis with polyclonal anti-BiP antibodies raised against the C-terminal portion of BiP to prevent cross-reaction with other heat shock proteins in the Hsp70 family (Denecke et al., 1991).

Figure 1A shows the results of an analysis using leaf extracts from plants overproducing wild-type BiP. Compared with the untransformed plants, the majority of the transgenic plants contain an increased level of BiP protein. The best overexpressers showed a four- to fivefold increase in BiP protein level. Moreover, we observed the presence of lower molecular weight bands, which are likely to represent BiP

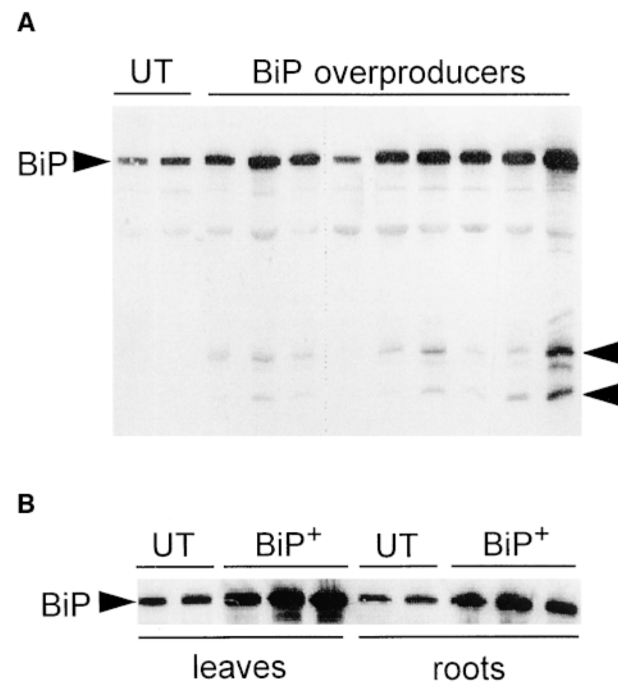

Figure 1. Screening of Tobacco Plants Transformed with BiP Construct Allowing Overexpression.

(A) Protein gel blot using anti-BiP polyclonal antibodies. Extracts were prepared from leaves of untransformed (UT) plants as controls and from transgenic plants overexpressing BiP. Arrowheads at right indicate BiP degradation products.

(B) Protein gel blot of leaf and root extracts (as indicated) using BiP antibodies. Untransformed plants (UT) and BiP overproducers (BiP$^+$) were compared. Fivefold lower amounts of protein were loaded for the root extracts to allow better comparison of the signals. The position of BiP is indicated by an arrowhead.

degradation products, and the detection of such bands correlated with the overexpression of BiP.

To determine whether overproduction of BiP was restricted to leaves in which the secretory activity and endogenous BiP levels were low (Denecke et al., 1991, 1995), we also tested extracts from other tissues. Young roots with a fivefold higher basal level of BiP for the same amount of total protein were analyzed. For this purpose, we diluted the root extracts fivefold to compare them directly with leaf extracts. Successful overproducers also showed elevated, although less pronounced, BiP protein levels in the roots (Figure 1B). None of the 60 independent transgenic plants harboring the sense construct showed any detectable phenotypic alteration.

Antisense Inhibition of BiP Synthesis Has a Deleterious Effect on Cell Viability

It has been shown that BiP is an essential gene for viability of *Saccharomyces cerevisiae* (Rose et al., 1989). In mammalian cells, a minimum basal level of expression is also required for cell viability (Li et al., 1992). Our antisense approach is based on the assumption that plants with reduced BiP levels (instead of being completely devoid of BiP) would be viable and might provide useful tools to study BiP function under stress conditions. Thus, transgenic plants were generated with pNL100, which carries the antisense gene. Due to the very high degree of sequence similarity (Denecke et al., 1991), the antisense transcript forms hybrids with the transcripts of all BiP family members. Although some plants with apparently lower BiP steady state levels could be identified among the primary transformants (Figure 2), this reduction in BiP levels was less obvious in the first generation (Figure 3). In contrast, seedlings from the first generation of BiP-overexpressing plants also had high levels of BiP, showing that the overexpression of BiP is stable (Figure 3). In particular, plants of the second generation that were homozygous for the transgenes showed even higher expression levels than did the primary transformants (A.J. Crofts, unpublished results).

There were no clear phenotypes visible with the mature BiP antisense plants, but we observed that freshly cut plants required more time to form roots than did cuttings from untransformed plants or BiP overproducers (Table 1). In addition, we conducted transformation of tobacco BY2 suspension cultures (Gomord et al., 1998), because we thought that lower BiP levels may be less deleterious to cell division in undifferentiated calli than to the regeneration of entire plants. Perhaps in these cells, a different subset of transgenic lines, with higher levels of the antisense transcript, would be tolerated. However, the number of resistant calli was drastically reduced for the antisense construct, and none of the proliferating calli showed significantly decreased BiP levels (data not shown). The results indicate that even minor reductions in BiP levels can be lethal to tobacco plants.

Discrepancy between BiP mRNA Levels and Protein Levels

The relatively poor overexpression of BiP at the protein level, as well as the presence of degradation products, led us to compare BiP mRNA levels with BiP protein levels. To ensure homogeneity of the cellular material for protein and mRNA extraction, we ground leaves from untransformed or BiP-overexpressing plants in liquid nitrogen. The homogeneous powder was split in two for either isolation of mRNA or extraction of proteins to permit a meaningful comparison.

RNA gel blots were hybridized using a BLP4 cDNA probe detecting both endogenous and introduced BiP transcripts. Quantification using a PhosphorImager revealed a 100- to 150-fold increase in the mRNA levels in the best overexpressing BiP plants compared with the wild-type plants (Figure 4). However, protein gel blots revealed only a modest fivefold increase of BiP protein, thus demonstrating a discrepancy between mRNA and protein levels.

Overexpression of BiP Downregulates the Endogenous BiP mRNA Level and Inhibits Its Induction by Tunicamycin

To examine the endogenous level of BiP in the overexpressing plants, we took advantage of the fact that BiP is encoded by a multigene family in tobacco (Denecke et al., 1991). Therefore, it was possible to use another member (BLP2) of the BiP gene family as a probe by using a region that does not cross-hybridize with BLP4. Rather than leaves, protoplasts were prepared from untransformed and BiP-overexpressing plants to allow the generation of two identical cell populations. One of these was subjected to ER stress by treatment for 2.5 hr with 20 μg/mL tunicamycin, which prevents glycosylation, promotes malfolding, and is generally used to trigger the UPR (Shamu, 1997). Figure 5 shows that the basal BLP2 mRNA level is significantly lower in protoplasts from the BiP overproducers (cf. first and third lanes). In addition, the tunicamycin-mediated induction observed in the untransformed cells (second lane) is barely noticeable in

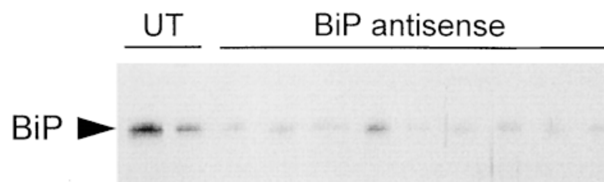

Figure 2. Analysis of BiP Antisense Plants.

Shown is a protein gel blot of leaf extracts from untransformed plants (UT) and transgenic plants transformed with the BiP antisense construct using anti-BiP antibodies. The position of BiP is indicated by an arrowhead.

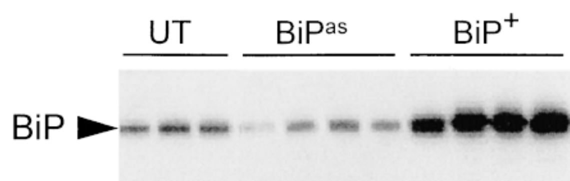

Figure 3. Screening of Seedlings from the First Generation of Transgenic Plants.

Leaves from 3-week-old seedlings were analyzed using anti-BiP antibodies. The results with untransformed plants (UT), progeny of BiP antisense plants (BiP[as]), and progeny of BiP-overexpressing plants (BiP[+]) are shown. The position of BiP is indicated by an arrowhead.

the transformed cells overproducing BiP (fourth lane). Thus, artificially increasing the BiP protein levels leads to a downregulation of the unfolded protein response, as observed in other systems (Dorner et al., 1992; Kohno et al., 1993).

Hybridization with a calreticulin-specific probe showed a similar reduction in the basal calreticulin mRNA levels (Figure 5). This shows that the regulatory effect of BiP overexpression is not restricted to BiP itself but extends to other ER-resident chaperones.

Minor Levels of BiP Antisense mRNA Can Cause a Stress Response

The failure to generate plants containing significantly lower BiP levels by using an antisense inhibition strategy prompted us to test expression of endogenous and introduced BiP mRNAs. We could distinguish endogenous BiP mRNA levels from the introduced antisense mRNA because the 3′ untranslated end was not included in the antisense construct. A probe detecting both sense and antisense transcripts did not show a significantly higher signal than did a probe specific for the endogenous sense transcript (Figure 6). This shows that only very low levels of antisense transcripts are present in the cells. Measurement of endogenous BiP mRNA levels revealed that antisense plants showed slightly increased basal BiP mRNA levels. This second observation suggests that cells compensate for the inhibitory effect of minor amounts of BiP antisense mRNA by inducing the endogenous genes, probably via the UPR. The fact that antisense plants show a less pronounced induction of BiP transcripts during tunicamycin treatment is consistent with the fact that the UPR already appears to be partially induced before the addition of tunicamycin.

A Functional Assay for Protein Synthesis by the ER in Vivo

BLP4 has been used in genetic complementation of the yeast KAR2 mutant (Denecke et al., 1991), and overexpression of BLP4 in this study leads to all of the expected effects on the regulation of BiP, showing that our chimeric gene encodes a biologically active BiP. To test further the activity of BiP in vivo, we have established a model system based on the comparison of protein biosynthesis in the cytosol and on the rough ER by using transient expression.

A plasmid was constructed (pNL200; Figure 7A) containing two genes, one encoding the secreted barley α-amylase (Rogers, 1985) and the other encoding the cytosolic marker β-glucuronidase (GUS; Jefferson et al., 1987). α-Amylase was used to measure secretory protein biosynthesis, and GUS was used to control for transfection efficiency and overall cell viability. We compared cells under normal culture conditions with cells subjected to ER stress by treatment with tunicamycin. Figure 7B shows that tunicamycin does not affect cell viability during the course of the experiment, as monitored with the internal marker GUS, confirming previous results (Denecke et al., 1990). In contrast, total α-amylase activity in the cell suspension was greatly reduced.

Because α-amylase is not glycosylated, tunicamycin should not have a direct effect on this protein. In a later experiment, we used a mixture of two plasmids, containing either the GUS or the α-amylase coding region under control of the CaMV 35S promoter, and obtained similar results (see further). This confirmed that the tunicamycin effect is protein specific and not dependent on the promoter used. Therefore, we postulated that during tunicamycin stress, α-amylase synthesis, translocation, or folding is compromised.

Positive Effect of Overexpression of BiP on the Synthesis of α-Amylase under Stress

One possible explanation for the tunicamycin effect could be that BiP is recruited by other malfolded proteins and is not available in sufficient quantities to promote optimal α-amylase synthesis, translocation, and folding. To test this hypothesis, we coexpressed BiP to determine whether increased

Table 1. Root Formation of Shoot Cuttings[a]

Plants	5 Days[b] (%)	14 Days[b] (%)
Control	73.4	96.2
Sense	61.6	79.0
Antisense	4.5	55.5

[a] The shoots of 60 independent plants for either untransformed plants or BiP sense or BiP antisense plants were transferred to new medium, and root formation was monitored after 5 or 14 days of incubation.

[b] The results are given as the percentage of the total number of shoots that have formed roots successfully. Note the low percentage of rooting shoots after 5 days in the case of the antisense transformants.

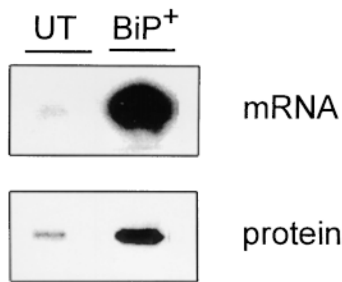

Figure 4. BiP mRNA and Protein Levels in BiP-Overproducing Tobacco Plants.

Total RNA was extracted from the leaves of untransformed (UT) and BiP-overproducing (BiP+) plants and analyzed by RNA gel blotting, as indicated (mRNA). Protein extracts were prepared from a portion of the frozen ground leaf material used for the preparation of RNA to ensure that identical biological material was used. The extracts were analyzed by protein gel blotting with anti-BiP antibodies (protein). Note that the difference in mRNA levels between untransformed and BiP-overproducing plants is more substantial than is the difference in protein levels.

BiP levels would alleviate the ER stress and restore efficient α-amylase production.

The protoplasts were coelectroporated with pNL200 and plasmids carrying (1) a gene encoding the bulk flow secretory marker phosphinothricin acetyltransferase (ssPAT; Denecke et al., 1990), (2) a BiP overexpression construct (pDE800), or (3) a BiP antisense construct (pNL100). The BiP isoform used in these experiments was the one that complemented the yeast KAR2 mutant (Denecke et al., 1991), whereas ssPAT is a neutral secretory protein used for control purposes. The protoplasts were incubated for 20 hr with and without tunicamycin, and the activities of α-amylase and GUS were measured.

Experiments were conducted in such a manner that similar internal marker activities (GUS) were obtained in each experiment. The α-amylase activity of the total extract was then corrected with the final GUS activities, and Figure 8A shows the ratio of α-amylase activity to GUS activity. If PAT is coexpressed, tunicamycin leads to a reduction of α-amylase activities, as shown in Figure 7. BiP coexpression alone leads to slightly lower α-amylase activities compared with PAT coexpression, but no further reduction of α-amylase activity was seen during tunicamycin treatment. Coexpression of the antisense construct was indistinguishable from PAT coexpression. Figure 8B shows the percentage of α-amylase activity that remains after tunicamycin treatment and illustrates clearly that BiP overexpression protects the cells from tunicamycin stress.

Next, we wanted to determine whether protoplasts prepared from the stably transformed plants exhibiting fivefold-increased BiP levels also would prevent the negative effect of tunicamycin on transient α-amylase production. In addition, we wanted to test whether the ER stress–mediated reduction of α-amylase activity was due to lower synthesis or due to incomplete folding. Therefore, we monitored α-amylase protein levels in addition to the enzymatic activities. This would reveal whether tunicamycin treatment leads to a pool of unfolded and inactive α-amylase, which would still be detected by protein gel blot analysis. Figure 9 clearly shows that ER stress was indeed due to lower protein levels and not incomplete folding because the reduction in protein level (Figure 9A) is comparable with the reduction in activity (Figure 9B). However, when BiP was overproduced to approximately fivefold higher protein levels, no tunicamycin effect was observed, as seen by the similar levels of α-amylase protein (Figure 9A) and activity (Figure 9B) in the treated and untreated cells. This confirms the result from the transient expression experiments, suggesting that BiP levels are the limiting factor during ER stress.

Figure 9A also illustrates that α-amylase is not a glycoprotein, because the tunicamycin treatment did not result in the formation of a lower molecular weight form of the protein. This confirms that tunicamycin cannot influence the enzyme

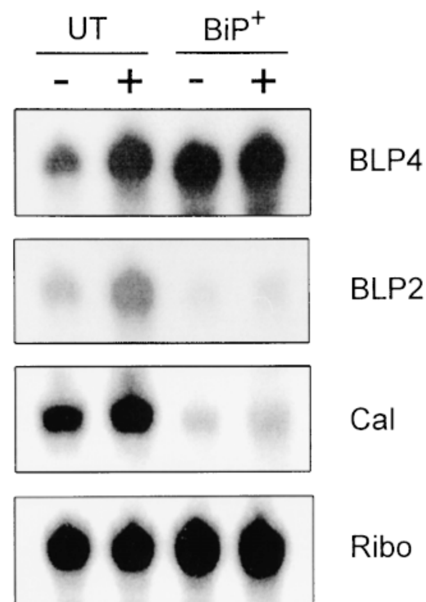

Figure 5. Downregulation of Endogenous BiP and Other Chaperones and Cancellation of Tunicamycin Induction in the BiP-Overexpressing Plants.

Shown are RNA gel blots of total mRNA isolated from protoplasts incubated in the presence (+) or absence (−) of tunicamycin (20 μg/mL) for 2.5 hr. Protoplasts were isolated from untransformed plants (UT) or BiP overproducers (BiP+). Blots were probed with a BLP4 probe detecting both endogenous and introduced BLP4 transcripts, a BLP2 probe specific for the endogenous BLP2 transcripts, a calreticulin probe (Cal), or an rRNA probe (Ribo) to control for loading differences.

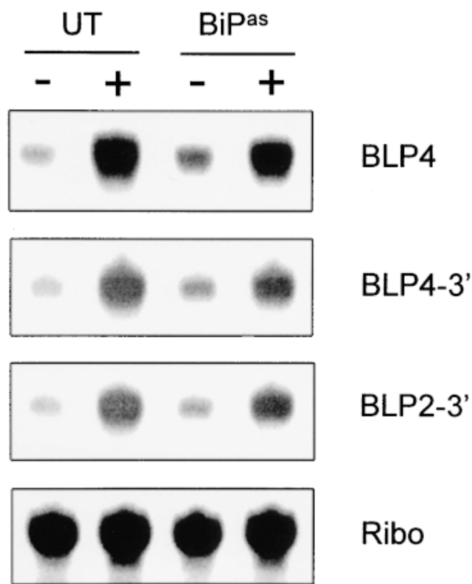

Figure 6. Increased Endogenous BiP mRNA Levels in BiP Antisense Tobacco Plants.

Untransformed (UT) and antisense (BiP^{as}) tobacco protoplasts were incubated for 2.5 hr in the presence (+) or absence (−) of tunicamycin (20 μg/mL), after which total RNA was extracted. Blots were probed with either the total BLP4 cDNA (BLP4) detecting both endogenous BLP4 and introduced antisense BLP4 transcripts or the 3′ ends of BLP4 (BLP4-3′) or BLP2 (BLP2-3′), respectively, to detect endogenous BiP transcripts only. An rRNA probe (Ribo) was used to control for loading differences.

directly. In contrast, tunicamycin treatment resulted in the synthesis of a nonglycosylated form of calreticulin, as observed previously (Denecke et al., 1995). This demonstrates that tunicamycin was inhibiting glycosylation in this experiment. Interestingly, neither tunicamycin treatment nor BiP overproduction affected the secretion rate of α-amylase. Figure 9B illustrates that the secretion index (ratio of the extracellular/intracellular protein level; Denecke et al., 1990) of the enzyme remained essentially unaltered in all conditions.

DISCUSSION

Effects of BiP Overproduction on Downregulation of BiP Levels in Transgenic Plants

Stable overexpression of BiP protein in tobacco plants resulted in a marked discrepancy between BiP mRNA and protein levels. mRNA levels were increased by as much as 100- to 150-fold, whereas protein levels were only increased by fivefold. This indicates either a negative regulation at the translational level or a higher BiP turnover when BiP is too abundant. The large amount of degradation products that correlated with overexpression of BiP would support the latter possibility. Indeed, similar degradation products were observed previously when expressing the same tobacco BiP isoform in yeast (Denecke et al., 1991) and when Harmsen et al. (1996) overexpressed KAR2 in yeast. BiP behaves very differently than other ER proteins, such as calreticulin, that can be overexpressed 50- to 100-fold in transgenic plants without a discrepancy between protein and mRNA levels (A.J. Crofts and J. Denecke, unpublished results). Therefore, it is possible that the level of BiP is more critical than that of calreticulin. We have shown that >50% of the cellular BiP is bound to calreticulin, whereas calreticulin is present in excess with only a minor portion in complexes with BiP (Crofts et al., 1998). The fact that higher BiP levels lead to the further recruitment of BiP into complexes with calreticulin (Crofts et al., 1998) prompted us to propose that BiP is stored in complexes with calreticulin to provide a buffer for free BiP in the ER lumen (Crofts and Denecke, 1998). Our results presented here suggest that the

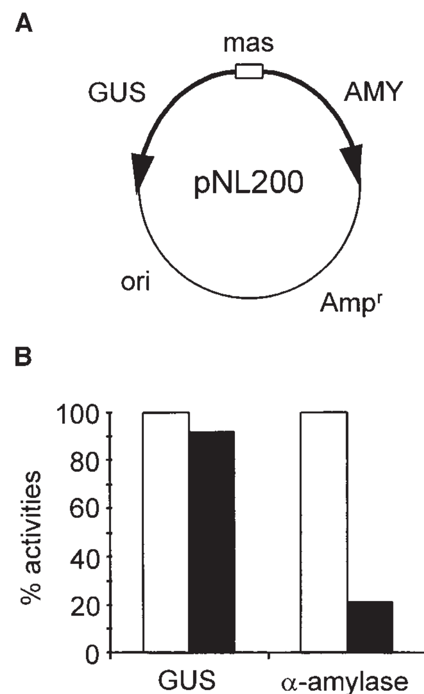

Figure 7. Effect of Tunicamycin on GUS and α-Amylase Activities.

(A) Schematic representation of the chimeric genes and the vector pNL200. Amp^r, ampicillin resistance; AMY, α-amylase gene; GUS, β-glucuronidase gene; mas, dual mannopine synthase promoter; ori, *Escherichia coli* origin of replication.

(B) Cytoplasmic GUS and total α-amylase activities after electroporation of tobacco protoplasts and incubation for 20 hr in the presence (black bars) or absence (white bars) of tunicamycin (20 μg/mL). The activities are shown as percentages of the activities without tunicamycin (set to 100% in each case).

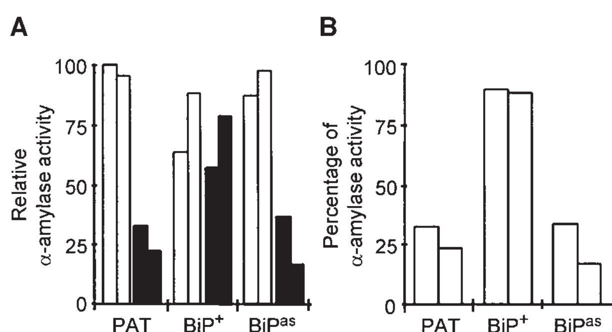

Figure 8. Effect of Altered BiP Levels on the Activity of α-Amylase.

(A) Coelectroporation experiments using pNL200 in the presence of plasmids carrying (1) a gene encoding phosphinothricin acetyltransferase (PAT), (2) a BiP overexpression construct (BiP^{+}), or (3) a BiP antisense construct (BiP^{as}). Cells were incubated for 20 hr in the presence (white bars) or absence (black bars) of tunicamycin (20 μg/mL), after which α-amylase and GUS activities were measured. The ratio of α-amylase to GUS activities is shown for two independent experiments.

(B) Data from **(A)** presented as a percentage of α-amylase activity that remains after tunicamycin treatment (20 μg/mL) in the different coelectroporation experiments. Note the almost full recovery to 100% in cells cotransfected with the BiP overexpression construct.

BiP concentration is closely monitored by the plant cell and that excess BiP is disposed of by an as yet unknown pathway.

The deleterious effect observed in the BiP antisense plants corresponds well with the fact that the deletion of the yeast KAR2–BiP gene generated a recessive lethal mutation in yeast, showing that this chaperone is required for cell viability (Rose et al., 1989). It is possible that the need for the regeneration of plants or cell division in undifferentiated calli resulted in a selection for low-expressing antisense transformants. In addition, our antisense plants showed higher basal BiP mRNA levels when compared with the untransformed plants. This suggests that the cells compensate for the inhibitory effect of the antisense mRNA by inducing the UPR. Similar observations were made in CHO cell lines expressing BiP antisense transcripts (Li et al., 1992). BiP also has been shown to be important for the biosynthesis of yeast cell wall material (Simons et al., 1998). This process also may require BiP in plants. Failure to synthesize cell walls efficiently would certainly interfere with successful regeneration of plants. We did not pursue this problem further and concentrated on the plants exhibiting higher BiP levels.

The UPR Is Dependent on BiP Protein Levels

BiP overexpression leads to downregulation of the endogenous BiP mRNA levels and a reduction of the UPR. This indicates that the plant contains a regulatory mechanism that monitors the concentration of BiP in the ER. When sufficient BiP is present in the ER, the cell avoids unnecessary BiP gene transcription. Furthermore, the increased BiP levels appear to be sufficiently high to assist protein folding during ER stress, hence the reduced UPR. These results correspond to BiP expression studies performed with yeast and mammalian cells in which overexpression of a functional BiP protein mitigates the UPR in a similar way (Dorner et al., 1992; Kohno et al., 1993). The UPR signal transduction pathway involving a receptor kinase and a nuclear transcription factor has been elucidated in yeast (reviewed in Shamu, 1997), and it is most likely that the free BiP concentration is the critical factor in the ER lumen. However, the exact mechanism by which this information is sensed and transduced from the lumen of the ER across the ER membrane remains to be established in any eukaryotic system. Our transgenic plants exhibiting stable BiP overproduction could be instrumental in this analysis.

An in Vivo System to Monitor BiP Activity

With the goal of acquiring more information on the role of BiP in vivo, we needed a system in which BiP constructs could be tested quickly. Transient expression in tobacco protoplasts has proven to be an ideal method to study protein synthesis and secretion quantitatively (Denecke and Vitale, 1995). In addition, higher expression levels can be obtained in electroporated protoplasts compared with transgenic plants due to the presence of many plasmid copies. We have developed a functional in vivo assay using a plasmid containing both a secretory marker protein and an internal cytoplasmic control. The α-amylase from barley aleurone was chosen as a secretory marker because it is a very stable enzyme (Rogers, 1985) and easy to detect. The enzymatic activity can be used easily as an indication of correct synthesis and folding of this protein. The presence of the internal control GUS in the same plasmid is a useful tool to avoid differences in activity due to variations in independent transfections and to assess cell viability under stress conditions.

The synthesis of α-amylase is clearly sensitive to tunicamycin, whereas GUS synthesis is barely affected by this treatment. This demonstrates that we are monitoring a specific effect on a secretory marker and not a general effect on cell viability. It has been observed before that inhibition of N-glycosylation by tunicamycin treatment reduces the stability and the secretion rate of some glycoproteins (Faye and Chrispeels, 1989). However, in our case, it cannot be a direct effect of the drug because α-amylase is not glycosylated. In addition, we showed that the tunicamycin-mediated reduction of α-amylase activity is comparable with the reduction in protein levels. Either the synthesis or the translocation must be compromised. A recently developed in vitro translation–translocation assay that uses plant microsomes (Lupattelli et al., 1997) will be useful to test this directly.

The effect on α-amylase could be explained by the

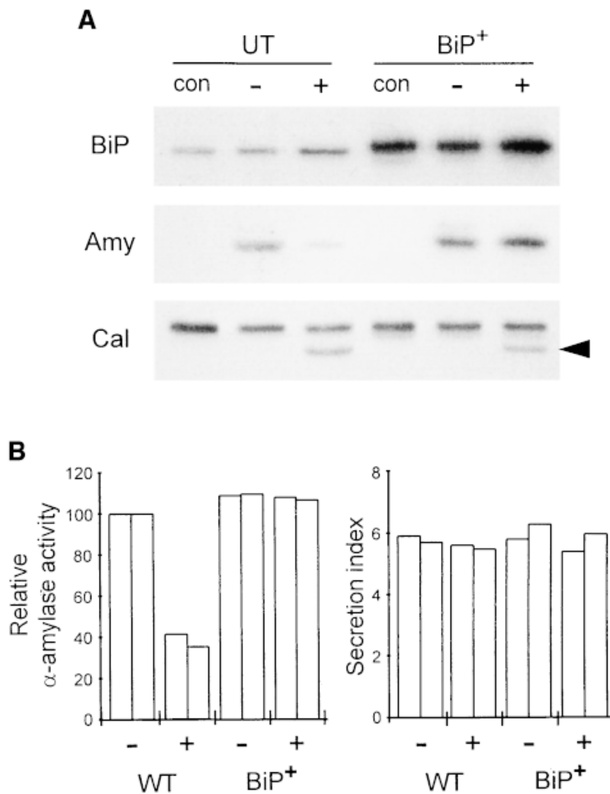

Figure 9. Effect of Altered BiP Levels on the α-Amylase Protein Level.

(A) Electroporation experiments using an equimolar mixture of pDE4 (CaMV 35S–GUS) and pAmy (CaMV 35S–α-amylase) with protoplasts prepared from untransformed plants (UT) and from a BiP overproducer (BiP^+). Cells were incubated for 20 hr in the presence (+) or absence (−) of tunicamycin (20 μg/mL), after which GUS activities were measured. Equal GUS activities were loaded for protein gel blot analysis. The blots were probed with anti-BiP antibodies (BiP), anti–α-amylase antibodies (Amy), and anti-calreticulin antibodies (Cal). As a negative control for the protein gel blots, protoplasts electroporated without plasmids were used (con). Note that a reduction in α-amylase protein level in the presence of tunicamycin occurs only in protoplasts from untransformed plants. The arrowhead indicates a nonglycosylated lower molecular weight form of calreticulin that was synthesized during tunicamycin treatment.

(B) At left, the four transfected cell suspensions from **(A)** but showing the ratio of α-amylase to GUS enzymatic activities of two independent measurements. − and + indicate the absence or presence of tunicamycin (20μg/mL). Note the reduction of α-amylase activity in the presence of tunicamycin in protoplasts from untransformed plants and compare with Figure 7B. The two mock-transfected control suspensions of **(A)** were used as negative controls in the enzymatic reactions. At right, the secretion index (ratio of extracellular to intracellular α-amylase activities) is shown for the same four cell suspensions. A secretion index of 6 means that sixfold higher levels of the enzyme are present in the medium compared with the cells. Note that the secretion index is comparable in all cases. WT, wild type. BiP^+, BiP overproducer.

requirement of an unstable glycoprotein for optimal α-amylase synthesis. Alternatively, ER stress could lead to depletion of BiP in the ER due to its binding to the accumulating malfolded proteins (D'Amico et al., 1992). BiP would then be limiting for optimal α-amylase synthesis and translocation. To distinguish between these two possibilities, we increased the level of BiP by cotransfection and showed that this leads to a recovery of α-amylase synthesis during tunicamycin treatment. Increased BiP levels would neither prevent tunicamycin action nor restore the glycans on underglycosylated proteins. Therefore, the results support the model in which BiP is the limiting factor. In yeast, the role of BiP in protein translocation is well established (Vogel et al., 1990; Brodsky et al., 1995), and a recent report showed that BiP regulates the binding of precursor polypeptide to a translocon complex (Lyman and Schekman, 1997). In mammalian cells, BiP was shown to bind to translocation pores at the lumenal side. Our data on the effect of BiP on α-amylase synthesis correspond well with these observations.

With transgenic plants, we could show that a fivefold higher BiP level is sufficient to abolish the negative effect of ER stress on α-amylase synthesis. However, we could not observe any effect of BiP overproduction on the α-amylase secretion rate. This is in contrast to observations from Dorner et al. (1992) but confirms another, more recent report (Morris et al., 1997). Tunicamycin treatment did not affect the transport of the enzyme, confirming previous experiments with tunicamycin (Denecke et al., 1990).

In summary, ER stress can lead to a situation in which BiP becomes limiting for optimal protein synthesis on the rough ER. This can be monitored by measuring a reduction in α-amylase synthesis. Reconstituting α-amylase synthesis by coexpression of BiP provides a rapid and novel assay for monitoring BiP activity in vivo. This assay can now be used to analyze the BiP coding region for important functional domains, providing an invaluable tool to aid in our understanding of the mechanism of chaperone action. In addition, microsomes from BiP overproducers can now be used for in vitro translation–translocation assays to test the effect of the lumenal BiP concentration on protein synthesis and translocation on the rough ER.

METHODS

Plasmid Constructs

All DNA manipulations were performed according to established procedures. The MC1061 strain of *Escherichia coli* (Casadaban and Cohen, 1980) was used for the amplification of all plasmids.

Plasmids for Transient Expression

The plasmid pDE203 containing the dual mannopine synthase (*mas*) promoter driving the chloramphenicol acetyltransferase (*CAT*) gene

and the β-glucuronidase (*GUS*) gene is identical to pDE222 (Denecke et al., 1992), except for the presence of the *CAT* coding region rather than the *bar* coding region. pDE203 was digested with ClaI, filled in by using the Klenow fragment of DNA polymerase I, and digested with HindIII. The α-amylase coding region was inserted as a blunted NcoI–HindIII fragment, resulting in pNL200 (Figure 7).

The lumenal binding protein (BiP) coding region of isoform BLP4 (Denecke et al., 1991) was amplified by polymerase chain reaction, creating an NcoI site overlapping with the translation initiation codon and a BamHI site just after the stop codon. This fragment was inserted between the cauliflower mosaic virus (CaMV) 35S promoter and the 3′ untranslated end of the nopaline synthase (*nos*) gene present on pDE4 (Denecke et al., 1990), resulting in pDE800. To obtain a BiP antisense construct, pDE800 was digested with NcoI and BamHI, releasing the BiP sequence; the vector was dephosphorylated by using calf intestine alkaline phosphatase; and both vector and fragment were filled in. After gel purification, the two parts were ligated again. The plasmid containing the BiP coding region in the antisense orientation was named pNL100.

Plasmids for Stable Expression

Chimeric genes containing the CaMV 35S promoter and the coding region of BiP, in sense and antisense orientations, were ligated into the *Agrobacterium tumefaciens* transformation vector pDE1001 (Denecke et al., 1992).

Plant Material and Growth Culture Conditions

Plants (*Nicotiana tabacum* cv Petit Havana; Maliga et al., 1973) were grown in Murashige and Skoog medium (Murashige and Skoog, 1962) and 2% sucrose in a controlled room at 25°C with a 16-hr daylength at the light irradiance of 200 μE m^{-2} sec^{-1}. Tobacco BY2 cultures were maintained and transformed as described previously (Nagata et al., 1992).

Plant Transformations

The pT plasmids were mobilized into the Agrobacterium rifampicin-resistant strain C58 (pGV2260) (Debleare et al., 1985) by using the *E. coli* helper strain HB101 (pRK2013). Transformed plants were obtained by agrobacterial infection of leaf pieces with the respective strains. Transformants were selected on Murashige and Skoog medium with 3% sucrose containing 100 μg/mL kanamycin and 250 μg/mL cefotaxime.

Protein Gel Blotting

Fully expanded leaves were collected and quickly frozen in liquid nitrogen. Frozen samples were then ground with a mortar and pestle. Protein concentrations were determined using Bio-Rad protein assay reagent.

Proteins in SDS–polyacrylamide gels were transferred onto a nitrocellulose membrane and then blocked with PBS, 0.5% Tween 20, and 5% milk powder for 1 hr. The filter was then incubated in blocking buffer with primary antibody at a dilution of 1:5000 for anti-BiP (Denecke et al., 1991) and anti-calreticulin antibodies (Denecke et al., 1995). Antibodies to barley α-amylase were used at a dilution of 1:10,000. After 1 hr, a 15-min wash and three 5-min washes were performed with 1 × PBS and 0.5% Tween 20. The secondary antibody used was anti–rabbit antibody conjugated to horseradish peroxidase at a dilution of 1:5000 in 1 × PBS, 0.5% Tween 20, and 5% milk powder. The filter was incubated with the secondary antibody for 1 hr. Washes were for 15 min, with four washes of 5 min with 1 × PBS and 0.5% Tween 20, followed by a final wash with 1 × PBS. Detection of antigen–antibody complexes was performed by using the enhanced chemiluminescence system (Amersham), and the images were recorded on film.

RNA Gel Blots

Protoplast RNA was extracted as described by Jones (1985). Protoplast samples were frozen in liquid nitrogen and thawed in NTES buffer (0.1 M NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, and 1% SDS). Extraction was with an equal volume of phenol–chloroform. RNA was selectively precipitated with 2 M Li acetate at 4°C. The pellet was washed with 70% ethanol and resuspended in diethyl pyrocarbonate–treated water. Gel blots of total RNA denatured in formamide and formaldehyde were prepared. RNA was blotted onto Hybond-N membrane (Amersham), as described by the manufacturer. A BLP4 BiP-specific probe containing the full-length cDNA, partial length BLP2, and full-length calreticulin cDNA were labeled using random primer DNA synthesis with the Klenow fragment. Hybridization was performed as described previously (Denecke et al., 1995). As a riboprobe, we used the 28S RNA from asparagus, kindly provided by J. Draper (University of Wales, Aberystwyth, UK). Quantification was performed using a PhosphorImager SI and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Transient Expression Experiments

Tobacco leaf protoplasts (from transformed or untransformed plants) were prepared, and electroporation experiments were performed as previously described (Denecke and Vitale, 1995), with minor modifications to the electroporation conditions. The conditions used in these experiments were 910 μF and 130 V. These optimal conditions were established with the expression of the α-amylase gene in tobacco protoplasts. For each experiment, 2.5 × 10^6 protoplasts were used with 20 to 40 μg of DNA. After 24 or 48 hr, the protoplasts were analyzed by enzymatic assay or by protein gel blotting. Tunicamycin was used at a concentration of 20 μg/mL. Harvesting of cells and culture medium was performed as described previously (Denecke and Vitale, 1995).

Enzymatic Assays

α-Amylase activity was measured with a kit (Megazyme, Dublin, Ireland). The reaction was performed in a microtiter plate at 45°C with 30 μL of extract and 30 μL of substrate. The reaction was stopped by the addition of 150 μL of stop buffer. The absorbance was measured at a wavelength of 405 nm, using a microtiter plate reader against a blank containing stop buffer alone. Each experiment was performed twice with three replicates.

GUS activity in protoplasts was measured with a colorimetric assay. Protoplasts were floated at 80*g* for 5 min, and the underlying solution was removed. The cells were then diluted in 10 mL of 250 mM NaCl and pelleted at 80*g* for 5 min. The supernatant was removed,

and the pellet was resuspended in extraction buffer (50 mM phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100, and 10 mM β-mercaptoethanol) and sonicated. The reaction was performed at 37°C as follows. Five hundred microliters of 2 × reaction buffer (50 mM phosphate buffer, pH 7.0, 0.1% Triton X-100, 2 mM α-*p*-nitrophenylglycerine, and 10 mM β-mercaptoethanol) was added to 490 μL of dilution buffer (50 mM phosphate buffer, pH 7.0, 0.1% Triton X-100, 10 mM β-mercaptoethanol) and 10 μL of supernatant. The reaction was stopped by the addition of 400 μL of 2.5 M 2-amino-2-methyl propanediol. The absorbance was measured at 415 nm against a blank, which was done as the test reaction except for the presence of stop buffer from the beginning of the incubation.

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