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Anticipating Endoplasmic Reticulum Stress: A Novel Early Response before Pathogenesis-Related Gene Induction

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When it is attacked by a pathogen, a plant produces a range of defense-related proteins. Many of these are synthesized by the rough endoplasmic reticulum (RER) to be secreted from the cell or deposited in vacuoles. Genes encoding endoplasmic reticulum (ER)–resident chaperones, such as the lumenal binding protein (BiP), are also induced under these conditions. Here, we show that BiP induction occurs systemically throughout the plant. Furthermore, this induction occurs rapidly and precedes expression of genes encoding pathogenesis-related (PR) proteins. The underlying signal transduction pathway was shown to be independent of the signaling molecule salicylic acid and the unfolded protein response pathway. In addition, BiP induction was independent of *PR* gene induction. Overproduction of BiP alone was not sufficient to cause induction of *PR* gene expression; however, limiting the amount of BiP in the ER lumen via superimposed ER stress inhibited the induction of *PR* gene expression. We propose that the induction of BiP expression during plant–pathogen interactions is required as an early response to support PR protein synthesis on the RER and that a novel signal transduction pathway exists to trigger this rapid response.

INTRODUCTION

During infection of a plant, bacterial and fungal plant pathogens often produce and secrete a large variety of hydrolytic enzymes that break down the plant cell wall. This helps the pathogen to penetrate the plant tissue and also causes the release of cell wall degradation products, which are taken up as nutrients by the pathogen. Plants have developed defense responses that use such products as elicitors for the production of defense-related proteins to combat the pathogen and prevent further pathogen attack (reviewed in Benhamou, 1996). Important measures that play a role in the plant defense response are cell wall strengthening, synthesis of antimicrobial compounds, such as phytoalexins, and production of a wide variety of pathogenesis-related (PR) proteins. Among the PR proteins, glucanase and chitinase are thought to protect plants by hydrolyzing the pathogen's cell wall as well as by releasing elicitors to further stimulate plant defense responses (Mauch et al., 1988; Lamb et al., 1989; Sela-Buurlage et al., 1993). Direct evidence for the role of PR proteins in plant defense has been obtained by overproduction of individual PR proteins in transgenic plants. This has led to enhanced pathogen resistance in the cases of PR-1 (Alexander et al., 1993) and chitinase (Broglie et al., 1991).

It has been well established that the defense mechanism is not only restricted to the infected tissue (Ross, 1961a) but also may occur in distal uninfected leaves (Ross, 1961b). This process has been termed systemic acquired resistance (SAR; Ryals et al., 1992). The onset of SAR is characterized by induction of the expression of a distinct set of defense genes, the *SAR* genes. *SAR* genes primarily encode acidic PR proteins and are used as molecular markers for the pathogenesis-induced defense mechanism (Ward et al., 1991). Salicylic acid (SA) has been implicated in SAR because exogenous SA induces SAR and the expression of *PR* genes in the absence of pathogens (Ward et al., 1991). Furthermore, transgenic plants that overexpress the enzyme salicylate hydroxylase, which converts SA to an inactive form (so-called NahG plants), are unable to induce systemic *SAR* gene expression (Gaffney et al., 1993; Delaney et al., 1994).

Although SA is an important compound in the initiation of the plant defense mechanism, several pathogens induce the plant defense response in an SA-independent manner. Among these are bacterial pathogens such as *Erwinia carotovora* and *Pseudomonas fluorescens*, which secrete plant cell wall–degrading enzymes (CDEs), including pectate lyases, polygalacturonases, pectin lyases, cellulases, and proteases

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(reviewed in Pérombelon and Salmond, 1995). Furthermore, plants treated with the CDEs pectinase and cellulase are able to induce a defense response both locally and systemically (Vidal et al., 1997, 1998) by a signal transduction cascade that is independent of SA (Vidal et al., 1997).

A common feature of PR proteins and cell wall–strengthening proteins is that they are all manufactured and delivered by the secretory pathway. This means that these proteins are synthesized by the rough endoplasmic reticulum (RER), regardless of their final destination, which can be either the extracellular matrix or the vacuole. Under normal circumstances, the secretory activity in leaves is very low, and amounts of endoplasmic reticulum (ER) chaperones, such as the lumenal binding protein (BiP), are low (Denecke et al., 1991). However, it has been shown that expression of the ER lumenal proteins endoplasmin, BiP, protein disulfide isomerase, and calreticulin is induced during plant–pathogen interactions (Walther-Larsen et al., 1993; Denecke et al., 1995).

In this study, we tested whether elevated expression of ER chaperones is the result of a feedback mechanism using the well-known unfolded protein response (UPR; Shamu, 1997) or whether plants use a more rapid signal transduction pathway to anticipate the need for increased levels of ER chaperones required for PR protein synthesis.

RESULTS

ER Chaperone Expression Is Induced Systemically and Occurs before *PR* Gene Induction

We have shown that local treatment of tobacco plants with CDEs mimics bacterial pathogen attack and leads to the rapid systemic induction of β-1,3-glucanase expression as well as that of other *PR* genes (Vidal et al., 1997). We now have repeated these experiments and monitored expression of the ER chaperone BiP in addition to that of β-1,3-glucanase, which has been shown to be the most rapidly expressed PR protein in this experimental system (Vidal et al., 1997). We used a commercial preparation of fungal CDEs rather than custom-made *E. carotovora*–derived hydrolases to increase reproducibility. RNA was extracted from the CDE-treated (local) and distal untreated (systemic) leaves from the same plants for subsequent RNA gel blot analysis.

Accumulation of β-1,3-glucanase transcripts was detectable after 4 hr of incubation and reached a maximum after 8 hr (Figure 1A), as described previously for infection with *E. carotovora* or treatment with *E. carotovora* hydrolases (Vidal et al., 1997). Thus, our experimental system mimics the local infection of a bacterial pathogen. In comparison to those of β-1,3-glucanase, BiP transcripts accumulated more rapidly, reaching a maximum after just 2 hr of incubation. This induction was observed locally as well as systemically with the same timing and intensity. The systemic signal involved, therefore, must be transported very rapidly throughout the plant. Whereas BiP induction was transient, β-1,3-glucanase mRNA amounts continued to be high after prolonged incubation times (24 to 48 hr). In comparison to the pattern of BiP induction, similar patterns were obtained for expression of protein disulfide isomerase and calreticulin (data not shown), demonstrating that other reticuloplasmins were induced locally and systemically as well.

The timing of CDE-mediated BiP protein synthesis was determined by protein gel blot analysis (Figure 1B). A significant increase in BiP protein levels was observed, with a clear delay compared with the timing of mRNA accumulation (4 hr after CDE application), after which BiP amounts continued to rise. As observed in plants overexpressing BiP (Leborgne-Castel et al., 1999), this increase was much lower compared with the marked rise in mRNA levels. However, the BiP expression profile in local and systemic leaves was very similar.

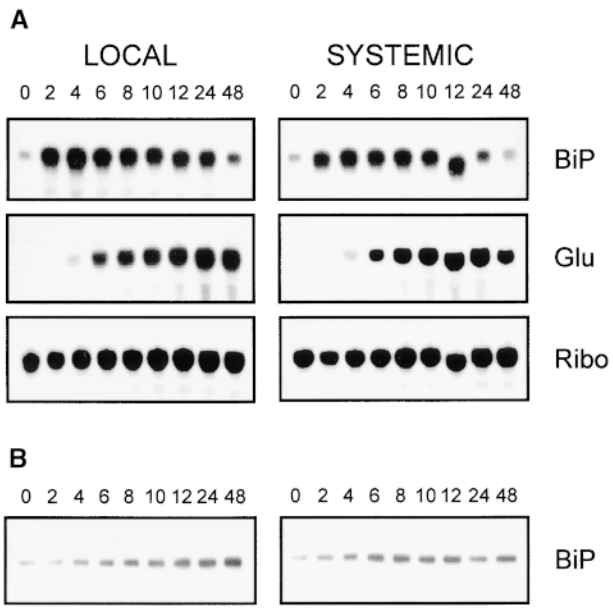

Figure 1. Local and Systemic Expression of BiP and β-1,3-Glucanase.

Tobacco leaves were analyzed in response to treatment with CDEs. The local response was determined by treatment of one leaf from each tobacco plant with CDEs, followed by collecting these leaves after different time points (numbers above the lanes are time points in hours). Untreated leaves were collected from the same plant to measure the systemic response.

(A) Twenty plants per time point were used for extraction of RNA. Samples of local and systemic leaves were run on the same gel and exposed for the same time period. An rRNA probe (Ribo) was used as a control for loading differences. Note that BiP induction is transient and occurs before β-1,3-glucanase induction. Glu, β-1,3-glucanase probe.

(B) BiP protein amounts in the same local and systemic leaves were detected by using protein gel blot analysis with anti-BiP antibodies.

CDE-Mediated BiP Gene Expression in SA-Insensitive Mutants

Because SA was shown to induce ER chaperone expression (Denecke et al., 1995), we wanted to test whether SA plays a role in the induction of BiP expression during treatment of leaves with CDEs. We used an SA-insensitive mutant of Arabidopsis (*sai1*), in which *PR-1* gene expression in the presence of SA was not induced (Shah et al., 1997). Wild-type plants and *sail* mutants were treated with CDEs and incubated for 6 hr. As a negative control, leaves were mock infected with water for the same time period. Total RNA was extracted, and RNA gel blot analysis was conducted using a probe for BiP. As a positive control, a probe against a hevein-like protein gene (*hel*) was used because it was shown to be the strongest CDE-induced PR protein in Arabidopsis (C. Norman and S. Vidal, unpublished results), and an equivalent of the basic β-1,3-glucanase of tobacco appears to be lacking in Arabidopsis. Figure 2 shows that wild-type Arabidopsis plants exhibit a specific CDE-induced accumulation of BiP transcripts (compared with the mock infection), as observed in tobacco plants. In the Arabidopsis *sail* mutant, BiP gene expression exhibits exactly the same profile as that seen in wild-type Arabidopsis plants. The induction of *hel* expression by CDE treatment indicated that a proper defense response had occurred in both cases, as expected from previous experiments showing that CDE-mediated *PR* gene induction is SA independent (Vidal et al., 1997). A control experiment confirmed that SA treatment no longer induced *PR-1* in *sai1* mutants (data not shown).

The results demonstrate either that BiP induction occurs upstream of *sail* in an SA-dependent signal transduction pathway or that the SA-dependent signal transduction pathway is not involved in CDE-mediated induction of BiP gene expression.

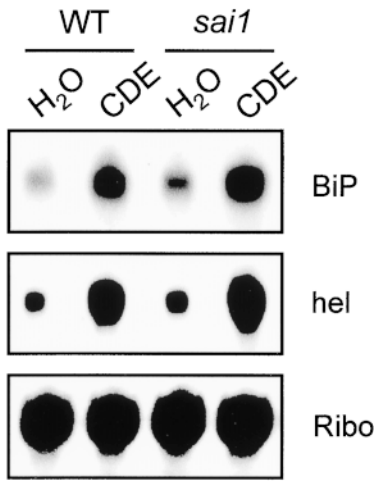

Figure 2. RNA Gel Blot Analysis of BiP and *hel* Transcripts.

Arabidopsis ecotype Nössen wild-type (WT) plants and SA nonresponsive (*sai1*) mutants were tested after treatment with CDEs. Plants were mock infected with water or treated with CDEs for 6 hr, after which total mRNA was extracted from the treated leaves. An rRNA probe (Ribo) was used as an internal control for gel-loading differences.

Both Local and Systemic Induction of BiP and β-1,3-Glucanase Gene Expression by CDEs Is SA Independent

Although *sai1* mutants are insensitive to SA, it has been shown that they still are able to accumulate SA when infected by a pathogen (Shah et al., 1997). To further test the possible involvement of SA in the induction of BiP by CDEs, we used transgenic tobacco NahG plants overexpressing salicylate hydroxylase. It has been clearly established that such plants are unable to accumulate SA due to its enzymatic conversion to an inactive form (Gaffney et al., 1993) and even convert SA applied to the leaves (Vidal et al., 1997). After 6 and 24 hr of CDE treatment, local and systemic leaves were harvested from untransformed tobacco plants (wild type) and NahG plants and used for RNA extraction. RNA gel blot analysis was performed using BiP and β-1,3-glucanase probes.

Our results clearly demonstrate that the presence of the *nahG* gene product has no influence on CDE-mediated induction of BiP gene expression (Figure 3). This strongly suggests that in this experimental system, SA is not involved in the signal transduction mechanism for either the local or the systemic induction of BiP gene expression by CDEs. As expected, β-1,3-glucanase was expressed locally and systemically in both wild-type and NahG plants when treated with CDE, as shown previously (Vidal et al., 1997). In addition, NahG plants treated with 5 mM SA (24 hr) did not show elevated *PR-1* gene expression (data not shown), confirming the functionality of the nahG gene product.

Systemic Induction of ER Chaperone Gene Expression Is Independent of the UPR

A unique feature of the plant ER is its continuity throughout the entire plant by way of numerous plasmodesmata (reviewed in Ghoshroy et al., 1997). We wanted to determine whether this continuity could enable a locally triggered UPR to develop into a systemic UPR in cells that do not experience ER stress. Leaves of tobacco plants were locally treated with the drug tunicamycin, which inhibits *N*-glycosylation of proteins in the ER, resulting in the accumulation of malfolded proteins and the UPR (Kozutsumi et al., 1988; Shamu, 1997). As a negative control, tobacco leaves were mock infected with water to examine the possible induction of reticuloplasmin gene expression upon wounding; as a positive control, treatment with CDEs was conducted. Total

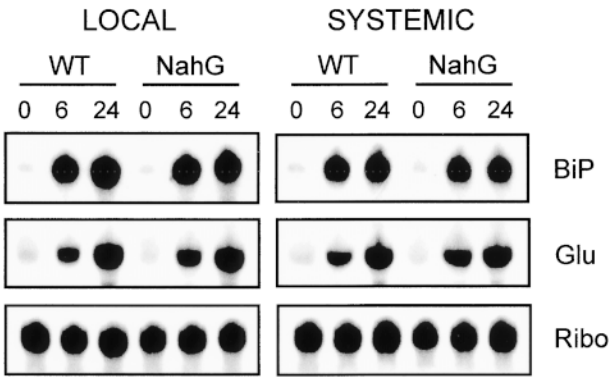

Figure 3. Comparison of Wild-Type and NahG Tobacco Plants.

Local and systemic expression of BiP and β-1,3-glucanase (Glu) was monitored after CDE treatment. Leaves were inoculated with CDEs for 6 and 24 hr, after which RNA was extracted from the treated (local) and nontreated leaf (systemic). Samples of local and systemic leaves were run on the same gel and exposed for the same time period. An rRNA probe (Ribo) was used as a control for loading differences. WT, wild type.

RNA was extracted from the local and systemic leaves after 3 and 8 hr of incubation.

Figure 4 shows that treatment with CDEs gave rise to a local and systemic induction of BiP and β-1,3-glucanase gene expression, with BiP expression being the faster response, as also seen in Figure 1. In contrast, when tobacco leaves were treated with tunicamycin, the induction of BiP mRNA amounts was restricted to the local leaves. The results show that the UPR cannot constitute a systemic signal to induce BiP expression in plants. In addition, tunicamycin treatment did not induce the β-1,3-glucanase gene, demonstrating that the UPR is not involved in the production of PR proteins either. The activity of tunicamycin was monitored by detecting the presence of deglycosylated calreticulin (Denecke et al., 1995), which is of a lower molecular weight (data not shown).

BiP gene expression during the mock infection shows a slight induction 3 hr after treatment of local and systemic leaves. This induction was transient and could be due to wounding, but the BiP mRNA level returned to its basal steady state level after 8 hr (Figure 4). β-1,3-Glucanase mRNA levels did not increase at all upon mock infection, confirming that the *PR* gene is not affected by wounding alone (Vidal et al., 1997).

Overexpression of BiP Is Not Sufficient to Trigger the Induction of β-1,3-Glucanase Expression

The more rapid induction of BiP expression compared with that of β-1,3-glucanase could suggest that BiP is an early element of the signal transduction cascade leading to the induction of *PR* gene expression. To test this possibility, we used transgenic plants that overproduce BiP under the control of the strong constitutive cauliflower mosaic virus 35S promoter. These plants exhibit increased steady state BiP protein levels exceeding those observed after treatment with CDEs, as seen in Figure 1B (Leborgne-Castel et al., 1999). If BiP is part of the signal transduction cascade leading to expression of the target gene β-1,3-glucanase, BiP overexpression alone should trigger β-1,3-glucanase gene expression.

RNA was extracted from untreated (0 hr) and CDE-treated (6 hr) plants, after which the amounts of BiP and β-1,3-glucanase transcripts were studied by RNA gel blot analysis. Figure 5 shows that BiP overproduction alone does not lead to the induction of β-1,3-glucanase expression (cf. lanes 0, Glu probe). Otherwise, β-1,3-glucanase was induced after 6 hr of CDE treatment in both the wild-type and BiP-overproducing plants.

The data clearly demonstrate that high BiP levels do not replace the signal that leads to induction of β-1,3-glucanase gene expression during CDE treatment. Thus, the signal transduction pathways leading to either BiP or β-1,3-glucanase genes are likely to be distinct or branched.

BiP Overexpression Leads to Downregulation of the CDE-Mediated Induction of BiP Gene Expression

BiP overexpression was shown to inhibit the UPR (Leborgne-Castel et al., 1999). Therefore, we tested whether BiP overexpression would affect the response of endogenous BiP genes to treatment with CDEs. Leaves of wild-type and BiP-overproducing plants were treated with CDEs, and RNA was extracted from local leaves at 1-hr intervals during a time

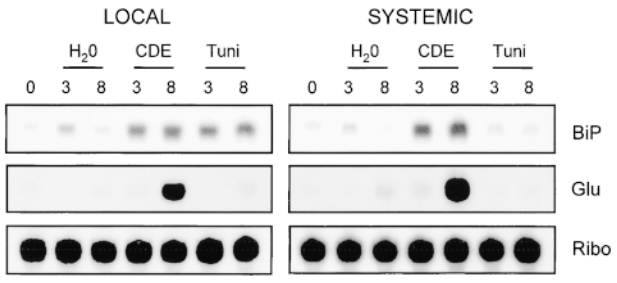

Figure 4. Effect of Tunicamycin on Local and Systemic Induction of BiP and β-1,3-Glucanase Expression.

Tobacco leaves were inoculated with water, CDEs, or 20 μg/mL tunicamycin (Tuni). Total RNA was extracted from the treated leaf (local) and a leaf from the opposite side of the stem (systemic) after 3 and 8 hr of treatment. Samples of local and systemic leaves were run on the same gel and exposed for the same time period. Equal loading of the RNA was assessed by hybridizing the filters with an rRNA probe (Ribo). Note that tunicamycin fails to cause BiP induction in systemic leaves. Glu, β-1,3-glucanase probe.

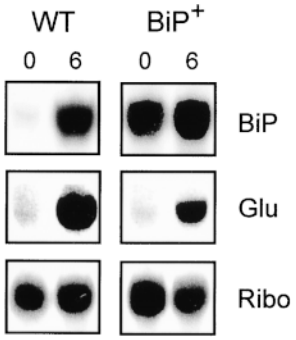

Figure 5. Expression of BiP and β-1,3-Glucanase in Untransformed Tobacco and BiP-Overproducing Plants.

RNA was extracted from untreated leaves (0) and treated leaves 6 hr after CDE treatment. The amount of RNA in the different samples was assessed by hybridization with an rRNA probe (Ribo). BiP$^+$, BiP overproducing plants; Glu, β-1,3-glucanase probe; WT, untransformed tobacco.

course of 1 to 6 hr. RNA gel blot analysis was performed by hybridization with a probe generated with the entire *BLP4* coding region (BiP, recognizing all endogenous and introduced BiP mRNA) or a *BLP4* 3′ end probe (Denecke et al., 1991; Leborgne-Castel et al., 1999), which only recognizes endogenous *BLP4* mRNA.

Figure 6 shows that the induction of BiP transcription in wild-type plants occurred after just 1 hr of CDE treatment and reached a plateau after 2 hr. BiP overproducing plants had high steady state amounts of BiP transcripts remaining at the same level during the 6 hr of CDE treatment. The use of the *BLP4* 3′ end probe that recognizes only endogenous *BLP4* gene transcripts revealed that high levels of BiP reduce CDE-mediated BiP induction. This also has been observed for the UPR (Leborgne-Castel et al., 1999), but the fact that the initial induction of BiP expression (i.e., after 1 hr) remains unaffected in BiP-overproducing plants suggests that a signal distinct from the UPR is involved as the initial trigger.

The UPR Is Additive to the CDE Response and Inhibits the Expression of β-1,3-Glucanase

To test the involvement of the UPR directly, we determined whether the UPR and the CDE response are additive. For this purpose, we prepared tobacco protoplasts, which are known to have an increased level of β-1,3-glucanase (Denecke et al., 1995). This is not surprising because protoplasts are prepared using CDEs. These protoplasts were treated with tunicamycin, to superimpose the UPR onto the CDE response. Figure 7 shows that a very strong additional induction of BiP transcription is triggered by tunicamycin treatment, indicating that CDE and UPR stimuli are additive. This suggests that these two induction mechanisms are fundamentally different.

We postulated that the rapid BiP induction is required to allow cells to cope with increased PR protein synthesis by the ER. Tunicamycin treatment leads to the recruitment of BiP to malfolded protein complexes, limiting the availability of free BiP molecules and causing ER stress (Leborgne-Castel et al., 1999). If our hypothesis is correct, the UPR thus should have an inhibitory effect on *PR* gene expression. Figure 7 shows that β-1,3-glucanase expression is indeed inhibited by tunicamycin, whereas BiP is induced. This confirms our hypothesis. In addition, the opposite behavior of BiP and β-1,3-glucanase to ER stress shows that the two genes are regulated by very distinct signal transduction pathways.

DISCUSSION

Rapid Local and Systemic BiP Induction Occurs Independently of SA

The induction of BiP expression occurs before that of β-1,3-glucanase, which is the most rapidly induced *PR* gene under this type of stress (Vidal et al., 1997). Rapid BiP induction was observed in tobacco as well in Arabidopsis and is likely to be a fundamental and conserved process in plants experiencing pathogen stress. Interestingly, BiP mRNA levels accumulate locally and systemically with identical timing,

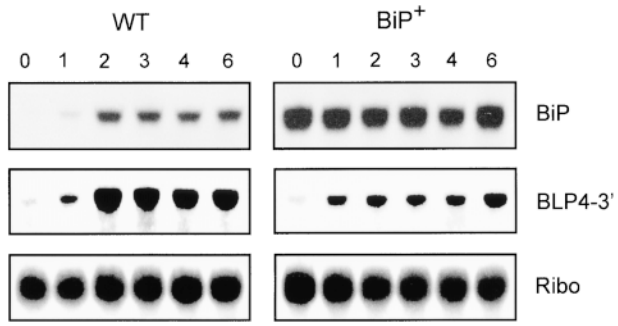

Figure 6. Effect of BiP Overproduction on the Induction of Endogenous BiP Gene Expression during CDE Treatment.

Time course (0 to 6 hr) of endogenous BiP gene expression (3′ end of *BLP4*) in wild-type tobacco (WT) and BiP-overproducing (BiP$^+$) plants during CDE treatment. RNA gel blot analysis was performed by hybridization with probes against the inserted *BLP4* construct (BiP) and the specific 3′ end of *BLP4* (BLP4-3′), which is absent in the 35S–*BLP4* construction (Leborgne-Castel et al., 1999). Samples of local and systemic leaves were run on the same gel and exposed for the same time period. Equal loading of the RNA was confirmed by hybridizing the filters with an rRNA probe (Ribo). Note that in BiP overproducers, no further induction of *BLP4* occurs after 1 hr of treatment.

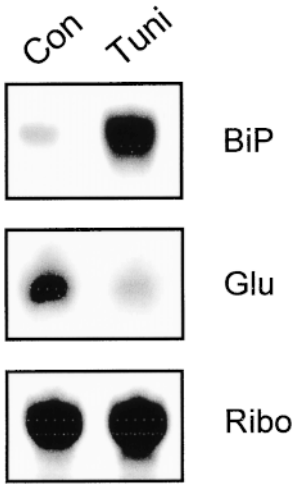

Figure 7. Effect of ER Stress on BiP and β-1,3-Glucanase Expression.

RNA gel blots of total mRNA isolated from wild-type tobacco protoplasts incubated in the presence (Tuni) or absence (Con) of tunicamycin (20 μg/mL) for 2.5 hr. RNA was extracted, and blots were probed for BiP and β-1,3-glucanase (Glu). An rRNA probe (Ribo) was used as a control for loading differences. Note the opposite effect of tunicamycin on BiP and β-1,3-glucanase.

suggesting the action of a rapidly transported signaling molecule through the plant.

We have established previously that application of CDEs leads to the induction of *PR* gene expression via a rapid signal transduction pathway that is independent of SA (Vidal et al., 1997). With the help of SA-insensitive mutants or SA-modifying plants, we could show that CDE-mediated induction of BiP gene expression is also SA independent. A possible candidate for a signaling molecule could be nitric oxide because it induces *PR* gene expression independently of SA (Van Camp et al., 1998). Alternatively, ethylene and jasmonates have been shown to induce a distinct set of mainly basic *PR* genes (reviewed in Boller, 1991; Hyodo, 1991; Creelman et al., 1992; Farmer et al., 1992). Because ethylene and methyl jasmonate are both volatile compounds, diffusion from the site of synthesis may occur, and the gaseous forms could act as the long-distance signal. Further work is required to test whether any of these compounds can induce BiP gene expression. It also will be interesting to study further the transient induction of BiP during mechanical wounding (Figure 4) and to draw parallels with the insect-induced BiP expression reported for alfalfa (Kalinski et al., 1995).

The fact that CDE treatment induces both BiP and β-1,3-glucanase in an SA-independent fashion does not mean that both target genes are induced in the same fashion. In contrast, the timing of the induction strongly suggests that BiP is induced via a faster signal transduction pathway than β-1,3-glucanase. In addition, the UPR inhibits β-1,3-glucanase expression, whereas the BiP gene is further induced. This shows that the two genes are regulated in a different way. Further experiments to unravel signal transduction pathways should be based on a comparison of BiP and β-1,3-glucanase to clarify this point.

BiP Induction in Anticipation of ER Stress: A Novel Process That Is Distinct from the UPR

An important goal of our work was to determine whether BiP induction occurs as a response to increased PR protein synthesis on the RER or whether plants anticipate the need for increased BiP levels via a more rapid mechanism. The fact that BiP expression is induced before β-1,3-glucanase expression does not exclude the possibility that other, unidentified PR proteins may be synthesized before BiP. However, most, if not all, abundant PR proteins have been discovered, and any unidentified PR proteins probably would not be abundant enough to impose significant ER stress. For this reason, we postulated that a feedback mechanism, such as the UPR (Shamu, 1997), is unlikely to be responsible for the rapid induction of BiP expression.

To substantiate this hypothesis, we tested whether a typical UPR, triggered by treatment with tunicamycin, would lead to systemic induction of BiP expression. The fact that this is not the case demonstrates that the UPR cannot be the systemic signal itself. However, it is possible that a systemic signal is transported through the plant and triggers the UPR locally. Two further results suggest that this also is not the case. First, constitutive overexpression of BiP, which is known to inhibit the UPR very efficiently (Leborgne-Castel et al., 1999), only partially inhibits CDE-mediated BiP induction. Second, the fact that CDE- and UPR-triggered BiP inductions are additive shows that the underlying signal transduction pathways must be fundamentally different.

Evidence that BiP is not merely an element of the CDE-mediated signal transduction pathway leading to the β-1,3-glucanase gene arose from the observation that overproduction of BiP alone does not replace the CDE signal and does not lead to elevated β-1,3-glucanase transcript levels. This suggests either that two different signal transduction pathways exist or that a common pathway contains two branches, one leading to BiP and one leading to the *PR* genes, including β-1,3-glucanase.

Regardless of which model is correct, the reason BiP is expressed before the *PR* genes must be explained. The most likely explanation is that induction of BiP expression is required to accommodate translation of large numbers of *PR* transcripts on the RER membrane. The low secretory activity of leaves and the low BiP amounts observed under normal physiological conditions (Denecke et al., 1991) could be insufficient to support the increased biosynthetic activity of the RER in translating *PR* transcripts. In a stress situation, the plant thus would anticipate the need for more ER chap-

erones required for the synthesis, folding, and transport of defense-related proteins. The fact that other ER residents also are induced (Denecke et al., 1995; E.P.W.M. Jelitto-Van Dooren and J. Denecke, unpublished results) suggests that the entire endoplasmic reticulum proliferates before *PR* gene induction.

To test whether BiP is required for *PR* gene expression, we superimposed the UPR on CDE stress. Trapping BiP in complexes with malfolded proteins and limiting its availability for translocation and folding of polypeptides correlate with an inhibition of *PR* gene expression, as shown by the reduction in the level of β-1,3-glucanase transcripts after treatment with tunicamycin. This correlation does not prove a causal relationship, but it has been shown that tunicamycin stress does, indeed, limit the availability of BiP (Leborgne-Castel et al., 1999). We propose that BiP gene induction is required before increased PR protein synthesis, and induction of *PR* genes is only possible when BiP protein levels have significantly increased. The relevance of the BiP protein levels also is supported by the first experiment (Figure 1), which demonstrated that β-1,3-glucanase gene induction occurs only when BiP protein amounts are rising above the basal level and not when BiP mRNA amounts start to increase.

Interesting parallels can be drawn between our results and findings with barley aleurone layers. During germination of barley seeds, the embryo synthesizes gibberellins, which trigger the production of secreted hydrolases on the RER in aleurone cells (Jones, 1985). Gibberellin also induces BiP levels (Jones and Bush, 1991) before gibberellin-induced hydrolase production (Denecke et al., 1995). This response thus may be similar to the rapid induction of BiP during plant–pathogen interactions in anticipation of increased protein synthesis on the RER. The underlying signal transduction mechanism must be distinct from the UPR, which is clearly a feedback mechanism (Shamu, 1997).

We propose that induction of BiP gene expression during plant–pathogen interactions is a novel, early response required to allow efficient PR protein synthesis. BiP thus can be used as a target gene for the study of pathogen-related signal transduction pathways.

METHODS

Plant Material and Growth Culture Conditions

Tobacco (*Nicotiana tabacum* cv Petit Havana) plants (Maliga et al., 1973) were grown axenically in Murashige and Skoog medium (Murashige and Skoog, 1962), plus 2% sucrose, in a temperature-controlled room at 25°C with a 16-hr-light and 8-hr-dark regime and a light irradiance of 200 μmol^{-2} sec^{-1}. We used untransformed plants as well as a transgenic line producing the lumenal binding protein (BiP) under the control of the cauliflower mosaic virus 35S promoter (Leborgne-Castel et al., 1999) or NahG-producing plants (a gift from J. Draper, University of Wales, Aberystwyth). Wild-type (*Arabidopsis thaliana* ecotype Nössen) and mutant (salicylic acid [SA] insensitive [*sai1*]; Shah et al., 1997) plants were grown under the same conditions.

Treatment of Plants with Cell Wall–Degrading Enzymes

Leaves were treated by pressing a micropipette tip to the surface of the leaf while it was supported by a metal spatula and subsequently injecting 1 μL of the cell wall–degrading enzyme (CDE) solution used for the routine preparation of tobacco protoplasts (Denecke and Vitale, 1995). This solution contains 0.4% cellulase (Onozuka R10; Yakult Honsha Co., Ltd., Osaka, Japan) and 0.2% Macerozyme (Onozuka R10; Yakult Honsha Co., Ltd.). Leaves with a 1- to 2-cm^2 surface area were inoculated 10 to 20 times evenly over the leaf surface. After different incubation times, the inoculated leaves were used to measure local effects, whereas untreated leaves on the opposite side of the stem were used to monitor systemic effects. For each sample, 20 independent plants were treated, and local or systemic leaves were pooled.

SA Treatment

Plants were sprayed from all sides with 5 mM SA containing 0.5% Tween 20 to ensure that SA contacted both sides of each leaf of the plant. Typically, 10 mL of the SA solution was sprayed onto each plant. Plants were then transferred from a 16-hr-day and 8-hr-night regime to a constant light regime.

Preparation of Leaf Protoplasts and Tunicamycin Treatment

Tobacco leaf protoplasts were prepared as previously described (Denecke and Vitale, 1995), except that electroporation buffer was replaced by the following medium: B5 salts, 250 mg/L NH_3NO_4, 500 mg/L $CaCl_2(H_2O)_2$, 500 mg/L 2-(*N*-morpholino)ethanesulfonic acid, and 136.9 g/L sucrose; all chemicals were from Sigma Chemical Co. Washed protoplasts were incubated with or without tunicamycin (20 mg/L) for 2.5 hr.

RNA Gel Blot Analysis

RNA was extracted as described by Jones (1985). Frozen leaves were ground in liquid nitrogen and transferred to NTES buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1% SDS). Tobacco leaf protoplasts were prepared as described above (Denecke and Vitale, 1995) and transferred directly to NTES buffer. RNA then was extracted using 1 volume of phenol–chloroform. Ethanol precipitation was performed overnight at −20°C, followed by LiCl precipitation (1 volume of 3 M LiCl) for 2 hr on ice. The pellet was dried at room temperature and resuspended in water treated with diethyl pyrocarbonate.

Fifteen micrograms of total RNA was denatured with formamide and formaldehyde and loaded on a 1.5% agarose gel. RNAs were blotted onto Hybond-N membranes (Amersham Corp.), as described by the manufacturer. Probes were labeled by random primed DNA synthesis by using the Klenow fragment of DNA polymerase I. Hybridization was performed as previously described (Denecke et al., 1995). Probes for tobacco BiP and *BLP4* 3′ (Denecke et al., 1991), tobacco β-1,3-glucanase (Castresana et al., 1990), tobacco *PR-1a*

(Cornelissen et al., 1986), Arabidopsis BiP, and Arabidopsis *PR-1a* were prepared as described previously (Denecke et al., 1995; Vidal et al., 1997). The Arabidopsis *hel* gene probe was generated by using primers corresponding to bases 131 to 155 and 571 to 595 of the cDNA sequence (Potter et al., 1993). We used the 28S RNA from asparagus as an rRNA probe (kindly provided by J. Draper).

Protein Gel Blots

Leaves were collected and rapidly frozen in liquid nitrogen before grinding with a mortar and pestle. This allowed us to extract RNA or protein from the same samples. A portion of the powdered material was extracted using extraction buffer (50 mM Tris-HCl, pH 7.5, and 2 mM EDTA) and 5 sec of sonication. Soluble proteins were recovered in the supernatant obtained after 10 min of centrifugation in a microcentrifuge at maximum speed at 4°C. Protein concentrations were determined using a Bio-Rad protein assay reagent and equalized to 0.1 μg/mL. Ten microliters was loaded on 12% acrylamide-SDS gels. Proteins were electroblotted onto a nitrocellulose membrane and then blocked for 1 hr with phosphate-buffered saline (PBS) containing 0.5% (v/v) Tween 20 supplemented with 5% (w/v) milk powder. The filter then was incubated for 1 hr in blocking buffer with primary antibody diluted 1:5000 for anti-BiP antibodies (Denecke et al., 1991). The blot was washed once for 15 min and then three times for 5 min each with PBS containing 0.5% Tween 20. The secondary antibody was goat anti–rabbit antibody conjugated to horseradish peroxidase, which was used at a dilution of 1:5000 in PBS, 0.5% Tween 20, and 5% milk powder. The filter was incubated with the secondary antibody for 1 hr and washed as described previously. Antigen–antibody complexes were detected using enhanced chemiluminescence (Amersham Corp.), and the images were recorded on film.

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