The Plant Cell, Vol. 6, 1703–1712, December 1994

RESEARCH ARTICLE

Function of Oxidative Cross-Linking of Cell Wall Structural Proteins in Plant Disease Resistance

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Elicitation of soybean cells causes a rapid insolubilization of two cell wall structural proteins, p33 and p100. Likewise, a short elicitation of 30 min rendered cell walls more refractory to enzyme digestion as assayed by the yield of protoplasts released. This effect could be ascribed to protein cross-linking because of its insensitivity to inhibitors of transcription (actinomycin D) and translation (cycloheximide) and its induction by exogenous H_2O_2. Moreover, the induced loss of protoplasts could be prevented by preincubation with DTT, which also blocks peroxidase-mediated oxidative cross-linking. The operation of protein insolubilization in plant defense was also demonstrated by its occurrence in the incompatible interaction but not in the compatible interaction between soybean and *Pseudomonas syringae* pv *glycinea*. Likewise, protein insolubilization was observed in bean during non-host hypersensitive resistance to the tobacco pathogen *P. s.* pv *tabaci* mediated by the hypersensitive resistance and pathogenicity (*Hrp*) gene cluster. Our data strongly suggest that rapid protein insolubilization leads to a strengthened cell wall, and this mechanism functions as a rapid defense in the initial stages of the hypersensitive response prior to deployment of transcription-dependent defenses.

INTRODUCTION

Plants exhibit natural resistance to disease, and attempted infection by an avirulent pathogen or non-pathogen induces a number of defenses, including phytoalexin synthesis, wall toughening, and deployment of lytic enzymes, such as chitinase and other antimicrobial proteins (Lamb et al., 1989; Bowles, 1990; Dixon and Lamb, 1990). Such defenses can also be induced by the direct or indirect products of pathogen avirulence genes, as well as other microbial elicitors, and endogenous plant elicitors (Ayers et al., 1976; Darvill and Albersheim, 1984; Templeton and Lamb, 1988; Hahn et al., 1989). Deployment of many of these inducible defense mechanisms involves the activation of batteries of defense genes as part of a massive switch in the pattern of plant gene expression (Dixon and Lamb, 1990; Lindsay et al., 1993). In cell suspension cultures, stimulation of the transcription of genes encoding chitinase and enzymes of phytoalexin biosynthesis can be observed within 10 min of the addition of fungal elicitors (Hedrick et al., 1988; Templeton and Lamb, 1988). However, although defense gene transcription is set in motion rapidly following perception of pathogen elicitor signals, effective deployment of transcription-dependent defenses necessarily takes several hours for cumulative transcription and translation, followed by cellular targeting of antimicrobial proteins and biosynthesis of protective natural products.

During a study of early events at the surface of elicitor-treated soybean and bean cells, we observed in the cell wall an oxidative insolubilization of two structural proteins previously designated as p33 and p100. N-terminal sequence analysis has shown that PRP2 is a member of a proline-rich protein family (Bradley et al., 1992); its N-terminal sequence is identical to that predicted from the soybean PRP2 family (Hong et al., 1989, 1990). p100 represents a polydisperse protein family that reacts with MAC 265, a monoclonal antibody that detects a glycoprotein family found in the intercellular spaces of legume roots and nodules (Bradley et al., 1988). Furthermore, physicochemical properties including molecular weight and polydisperse distribution suggest that the p100 family is a family of hydroxyproline-rich glycoproteins, as described by Cassab and Varner (1988).

Disappearance of these tyrosine-rich cell wall proteins is driven by H_2O_2, which rapidly accumulates from an oxidative burst at the cell surface. This insolubilization is initiated within 2 to 5 min and is completed within 20 to 30 min as assayed by the loss of SDS-extractable forms of p33 and p100 in a protein gel blot immunoreacting with either a polyclonal antiserum generated by immunization with p33 or with the monoclonal MAC 265 (Bradley et al., 1992). Interestingly, immunofluorescence observations have shown that abundant immunoreactive proteins remain in situ in the cell wall or in the intercellular

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Table 1. Time Course of the Release of Protoplasts from Control and *P. m. glycinea*–Elicited Cells[a]

Time (Hr)	Control (%)	*P. m. glycinea* Elicited (%)
0.5	0 to 5 ± 3	0 to 5 ± 4
1.0	45 ± 5	10 ± 10
2.0	65 ± 8	12 ± 10
5.0	80 ± 4	60 ± 7
18.0	95 ± 2	95 ± 3

[a] Percentages were obtained from three replicate experiments ± SD.

fluid and cannot be solubilized following wounding, elicitor treatment, or developmental cross-linking. The absence of low molecular weight partial degradation products and the frequent detection of dimers and tetramers of p33 in the protein gel blot provide further evidence for cross-linking of these proteins (Bradley et al., 1992).

The rapidity of the cross-linking of these abundant cell wall structural proteins suggests that this might represent a rapid defense mechanism to toughen the cell wall as a barrier to pathogen ingress prior to the deployment of transcription-dependent defenses (Bradley et al., 1992). To test this hypothesis, we have investigated cell wall protein cross-linking in genetically defined plant–pathogen interactions and examined its impact on the ability of microbial protoplasting enzymes to release protoplasts from suspension-cultured cells as a bioassay of the resistance of the cell wall to microbial digestion.

RESULTS

Elicitors Rapidly Reduce the Digestibility of Soybean Cell Walls

Microbial pathogens often gain ingress by enzymatic digestion of host cell walls, and microbial hydrolytic enzymes are routinely used for cell wall digestion in the generation of plant cell protoplasts (Gamborg et al., 1981). We therefore developed an assay based on protoplast release from suspension-cultured soybean cells to determine whether elicitor-induced oxidative insolubilization of cell wall structural proteins makes the cell wall more refractory to microbial digestion. Protoplasting enzymes comprising a mixture of cellulase and pectinase rapidly digested soybean cell walls to give essentially complete release of protoplasts (75 to 85%) within 5 hr (Table 1). For sensitive assay of changes in cell wall digestibility, protoplasting conditions were selected that gave ~50 to 60% release of protoplasts during a 2-hr incubation of control cells with the microbial enzyme mixture. Cell wall digestibility was measured by determining the ratio of protoplasts to intact cells. Protoplasts were distinguished by a spherical shape and lack of fluorescence following calcofluor white staining of cell wall material.

Glucan elicitor isolated from the mycelial cell walls of the soybean pathogen *Phytophthora megasperma* f sp *glycinea* causes rapid insolubilization of p33 and p100, with complete insolubilization after 30 min (Bradley et al., 1992). *P. m. glycinea* elicitor likewise caused a drastic reduction in wall digestibility of the soybean cells, such that 2 hr after elicitation the cells had become almost completely refractory to rapid protoplast release by the microbial protoplasting enzymes (Figure 1). Thus, the ratio of protoplasts to intact cells typically released by a 2-hr incubation with the protoplasting enzymes was reduced ~10-fold from ~1.5 in control cells to less than 0.15 if cells were exposed to *P. m. glycinea* glucan elicitor for 2 hr prior to the start of the standard 2-hr protoplasting incubation. As illustrated in Figure 1, reduction in the yield of protoplasts was manifest by extensive retention of calcofluor white staining after the protoplasting incubation of elicited cells compared with the preponderance of calcofluor white–negative, spherical protoplasts in parallel incubations with control cell suspensions.

The severe reduction in the yield of protoplasts reflects the retardation of protoplast release and not their subsequent lysis, because reduction in protoplast numbers was accompanied by a reciprocal increase in the numbers of intact cells remaining after the enzyme incubation, and prolonged, overnight exposure to the protoplasting enzyme mixture gave a similar yield of protoplasts from control cells or elicitor-treated cells (Table 1). Therefore, elicitation affects the rate of protoplast release, and fungal elicitor and glutathione do not affect the viability, which was estimated by Evans blue and fluorescein diacetate staining, of protoplasts previously isolated from unelicited control cells (Table 2).

P. m. glycinea elicitor caused a very rapid reduction in wall digestibility such that there was over a 50% reduction in the protoplast/cell ratio in this assay when cells were exposed to the elicitor for only 30 min prior to the initiation of the protoplasting incubation (Table 3). Glutathione, which also induces rapid insolubilization of p33 and p100, likewise caused a dramatic reduction in soybean cell wall digestibility. H_2O_2, which is the metabolic intermediate driving elicitor-induced oxidative insolubilization of p33 and p100, also caused a substantial reduction in the digestibility of cell walls (Table 3).

Table 2. Effect of Elicitors on the Viability of Protoplasts Released from Unelicited Control Cells

Treatment	Viable Protoplasts[a]	
	Evans Blue (%)	Fluorescein Diacetate (%)
Control	73.5 ± 4.5	75.2 ± 5.2
P. m. glycinea–elicited protoplasts	74.7 ± 2.0	70.4 ± 4.0
Glutathione-elicited protoplasts	72.0 ± 2.0	73.5 ± 5.5

[a] Percentages were the mean of four replicates ± SD.

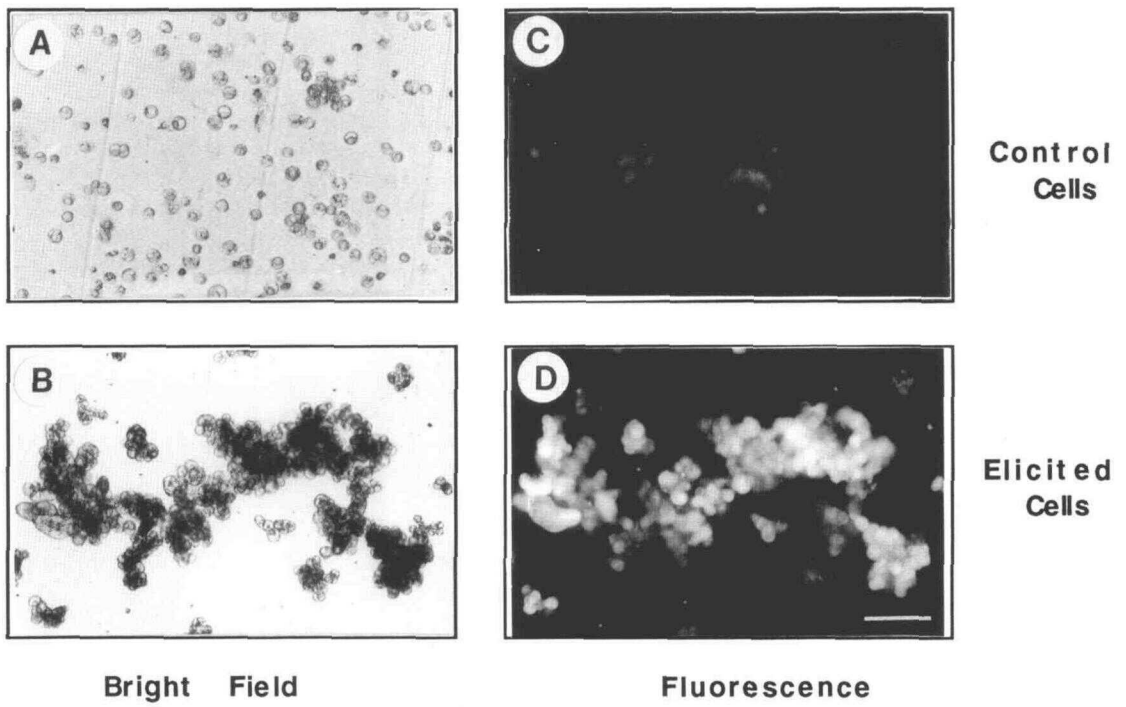

Figure 1. Effects of Elicitation on the Release of Soybean Protoplasts.

(A) Protoplast suspension isolated from control cells.
(B) Protoplast suspension isolated from elicited cells treated with *P. m. glycinea* glucan elicitor (60 μg of glucose equivalents per milliliter).
(C) Calcofluor white staining of the protoplast suspension isolated from control cells.
(D) Calcofluor white staining of the protoplast suspension isolated from elicited cells. Bar = 200 μm.

Relationship between Oxidative Insolubilization and Loss of Digestibility of Cell Wall

The reduction of soybean cell wall digestibility indicated that *P. m. glycinea* elicitor induces rapid modifications of the cell wall. The kinetics of the response and the ability of exogenous H_2O_2 to mimic the effect of fungal elicitor are consistent with the involvement of oxidative insolubilization of p33 and p100; therefore, we next performed a series of experiments to investigate further the relationship between wall protein insolubilization and reduced wall digestibility.

The rapidity of the elicitor-induced insolubilization of the major cell wall structural proteins p33 and p100 suggests that this may represent a rapid defense response to modify the cell wall prior to the deployment of transcription-dependent defenses. This model predicts that the elicitor-induced reduction of soybean cell wall digestibility should not require transcription or translation. Figure 2 shows that the *P. m. glycinea* elicitor–induced oxidative insolubilization, as monitored in protein gel blots by loss of SDS-extractable p100 from the cell wall, was not blocked by either the RNA synthesis inhibitor actinomycin D or the 80S ribosomal protein synthesis inhibitor cycloheximide. Similar results were obtained with p33 (data not shown). Likewise, induction of the loss of cell wall digestibility by the *P. m. glycinea* elicitor was not blocked by either actinomycin D or cycloheximide at concentrations that effectively inhibit total cellular RNA and protein synthesis, respectively (Table 4). Internal control experiments established that actinomycin D blocks chalcone synthase (CHS) mRNA induction, and cycloheximide reduced phenylalanine ammonia-lyase activity in cell cultures, indicating the effectiveness of these treatments. Moreover, this loss of wall digestibility was not inhibited by α-aminooxy-β-phenylpropionic acid (AOPP), a potent and specific inhibitor of phenylalanine ammonia-lyase (Amrhein and Godeke, 1977; Noé et al., 1980), indicating that the underlying wall modifications did not require the de novo synthesis of phenolic products from phenylalanine.

Although oxidative insolubilization of p33 and p100 is a substrate-controlled process driven by the accumulation of H_2O_2 from an elicitor-induced oxidative burst, the reaction in the cell wall is mediated by peroxidase (L.F. Brisson, unpublished data), and the peroxidase inhibitor DTT blocked elicitor-induced insolubilization of p33 and p100. DTT inhibition of the insolubilization of p100 induced by elicitor is illustrated in Figure 3A. Similar results were obtained with the blot immunoreacting with p33 (data not shown). Moreover, DTT caused a dramatic protection against elicitor-induced reduction in the loss of protoplast release, completely blocking the response (Table 4).

Table 3. Elicitor-Induced Reduction in the Wall Digestibility of Cultured Soybean Suspension Cells

Treatment	Time (Hr)	Replicates (Number)	Protoplasts/ Cells (Mean ± SD)
Control		16	1.36 ± 0.4
P. m. glycinea elicitor (60 μg/mL)	0.5	4	0.45 ± 0.07
	1.0	4	0.34 ± 0.03
	2.0	16	0.14 + 0.10
	3.0	4	0.27 ± 0.03
Glutathione (reduced, 50 μM)	2.0	4	0.28 ± 0.07
H_2O_2 (1 mM)	2.0	7	0.55 ± 0.40

This effect of DTT was selective because DTT potentiated the *P. m. glycinea* elicitor induction of CHS transcripts when used as a marker for transcription-dependent defense responses (Figure 3B).

Oxidative Insolubilization in Genetically Defined Plant–Pathogen Interactions

Treatment of soybean suspension-cultured cells with the *P. m. glycinea* elicitor is a convenient experimental system for biochemical analysis. A major question is whether the oxidative insolubilization also operates as a response to attempted infection of intact plant tissue, especially in relation to the genetically controlled race/cultivar-specific interactions that underlie many agriculturally important plant diseases. Because of the low concentration of p100 in leaf tissues (L.F. Brisson and C.J. Lamb, unpublished data), we monitored insolubilization of p33 in soybean leaves inoculated with the soybean pathogen *Pseudomonas syringae* pv *glycinea*. *P. s. glycinea* race 4 is virulent on all soybean cultivars and causes extensive spreading chlorosis characteristic of the bacterial blight disease (Staskawicz et al., 1987). Introduction of plasmid pavrD34 carrying the *avrD* avirulence gene makes race 4 incompatible on specific soybean genotypes, such as cultivar Harosoy, which contain the corresponding resistance gene *Rpg4*, and instead of disease, a localized hypersensitive resistance response (HR) develops at the site of bacterial inoculation (Keen and Buzzell, 1991). We therefore used this genetically defined system to examine pathogen induction of oxidative insolubilization in an isogenic setting and the hypersensitive resistance pathogenicity–dependent (*Hrp*) insolubilization induced in bean in response to a non-host pathogen. *P. s. glycinea* race 4 carrying the *avrC* avirulence gene was used as a virulent control because the Harosoy genotype lacks the corresponding resistance gene (Keen and Buzzell, 1991).

Figure 4A shows differences observed after inoculation between the compatible and incompatible interactions. During the incompatible interaction, no SDS-extractable immunoreactive p33 could be detected in tissue excised from the site of bacterial inoculation, whereas immunoreactive p33 was readily extractable from the cell walls isolated from healthy tissue in the vicinity of the HR (Figure 4A) and in mock-inoculated tissue samples in the same leaf (Figure 4). In contrast to the insolubilization of cell wall p33 in the incompatible interaction with avirulent *P. s. glycinea* race 4 (*avrD*), p33 remained readily extractable from the cell walls of equivalent tissues that had been inoculated with the virulent strain *P. s. glycinea* race 4 (*avrC*) and showed the spreading chlorosis characteristic of a compatible interaction.

In the incompatible interaction between Harosoy and avirulent *P. s. glycinea* race 4 (*avrD*), substantial hypersensitive necrosis was first observed ~30 hr after inoculation. Figure 4B shows that complete insolubilization of p33 was observed as early as 24 hr after inoculation, at the stage when visible hypersensitive necrosis is just becoming apparent at some inoculation sites. In contrast, p33 could be readily extracted from the cell walls of tissues infected with virulent *P. s. glycinea* race 4 (*avrC*) up to at least 72 hr after inoculation (Figure 4C).

Inoculation of a pathogen on a non-host plant species can also induce the HR that is mediated by the *Hrp* operon; therefore, we also examined the operation of the insolubilization response in such non-host resistance. Figure 5A shows that p35, the bean homolog of p33, remained in a soluble form in leaves inoculated with the bean pathogen *P. s.* pv *phaseolicola*, whereas inoculation with the tobacco pathogen *P. s.* pv *tabaci* induced complete insolubilization of p35, which is associated with a non-host HR. In contrast, inoculation with *P. s. tabaci* carrying an insertion mutation in the *Hrp* gene cluster, which blocks *Hrp*-mediated HR induction, failed to induce p35 insolubilization.

The accumulation of CHS transcripts as a marker for transcription-dependent defense responses parallels the insolubilization response with no accumulation of CHS mRNA in tissue infected with *P. s. phaseolicola*, as compared with strong induction associated with the HR to *P. s. tabaci* (Figure 5B). In the same infection experiment with parallel RNA isolation and RNA gel blot analysis, only weak accumulation of CHS transcripts was observed in tissue inoculated with *P. s. tabaci*

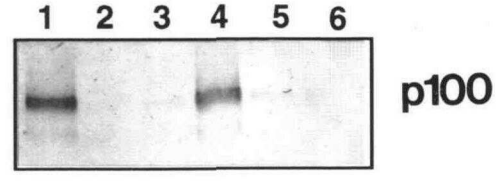

Figure 2. Effects of Metabolic Inhibitors on the Insolubilization of p100 Induced by Fungal Elicitor.

Protein gel blot analysis showing the presence of SDS-extractable p100 immunoreacting with MAC 265 in walls of control cells (lanes 1 and 4) and not in cells elicited with *P. m. glycinea* elicitor (lanes 2, 3, 5, and 6) in the presence of actinomycin D (lane 3), cycloheximide (lane 5), or AOPP (lane 6).

Table 4. Effects of Metabolic Inhibitors on the Reduced Protoplastability Induced by *P. m. glycinea* Fungal Elicitor

Treatment	Replicates (Number)	Protoplasts/ Cells (Mean ± SD)
Control	4	2.14 ± 0.40
P. m. glycinea elicitor	4	0.04 ± 0.02
+ Cycloheximide (30 μg/mL)	4	0.06 ± 0.02
+ Actinomycin D (20 μg/mL)	4	0.15 ± 0.05
+ AOPP (20 μg/mL)	4	0.54 ± 0.23
+ DTT (10 mM)	4	2.96 ± 0.77

Protoplasts were distinguished from cells by their spherical shape and lack of staining with calcofluor white, which strongly stains the walls of intact cells (see Figure 1) after 2 hr of elicitation treatment.

carrying *Hrp* knockout mutations that are unable to elicit a nonhost HR (Jacobek and Lindgren, 1993).

DISCUSSION

Many microbial pathogens obtain ingress by deployment of a battery of enzymes to degrade external barriers, such as the cuticle and plant cell wall (Deschamps, 1989; Joseleau and Ruel, 1989). Change in the digestibility of the wall represents a convenient model for the assay of stress-induced modifications that affect plant cell structural integrity in relation to pathogen ingress. Local wounding of the lower leaves of young potato or tomato plants leads to a dramatic change in the cells of upper undamaged leaves manifest by extensive lysis of protoplasts during their isolation from these organs (Walker-Simmons et al., 1984). This effect was ascribed to a wound-induced weakening of the plasma membrane because newly recovered protoplasts released from leaves of wounded plants were extremely fragile and readily lysed by low-speed centrifugation.

The effect of fungal elicitor on soybean cell wall digestibility appears to involve a different mechanism because the reduction in protoplast numbers is accompanied by a reciprocal increase in the number of intact cells remaining after incubation with the protoplasting enzymes, and elicitor treatment of protoplasts isolated from control cells does not decrease viability. Moreover, prolonged exposure of elicitor-treated cells with the protoplasting enzymes eventually releases protoplasts in a 95% yield, indicating that elicitation affects the rate of protoplast release rather than their intrinsic stability. Hence, we concluded that the elicitor induces changes in the plant cell wall that slow wall digestion by the microbial enzymes and hence retard protoplast release.

Several lines of evidence point to the involvement of oxidative insolubilization in the elicitor-induced modifications of the cell wall that retard protoplast release. Thus, protoplast release is inhibited by the *P. m. glycinea* elicitor and glutathione, both of which stimulate rapid insolubilization of p33 and p100. Moreover H_2O_2, which has been implicated as the rate-determining step driving oxidative insolubilization of these proteins (Bradley et al., 1992), likewise reduces the digestibility of the soybean cell walls. Both protein cross-linking and reduction in the yield of protoplasts are rapid processes and do not appear to require gene transcription or protein synthesis after introduction of the elicitation stimulus.

The reduction in the yield of protoplasts is complete within 2 hr of *P. m. glycinea* elicitor treatment, and there is a greater than 70% reduction in protoplast release within 30 min. Whereas sufficient insolubilization of p33 and p100 occurs within 30 min of *P. m. glycinea* elicitor treatment (Bradley et al., 1992) to prevent SDS extraction, it might be expected that additional cross-linking would be required for effective blocking of protoplast release by enzyme digestion of the cell wall with cellulase and pectinase. Hence, the kinetics for inhibition of protoplast release are consistent with a central role for oxidative cross-linking in the elicitor-induced modifications of the cell wall that reduce its digestibility by the microbial enzymes. A previous study noted a slow decrease in protoplast release from tomato cells following treatment with fungal elicitors, which was ascribed to the accumulation and cross-linking in the cell wall of phenolic products synthesized

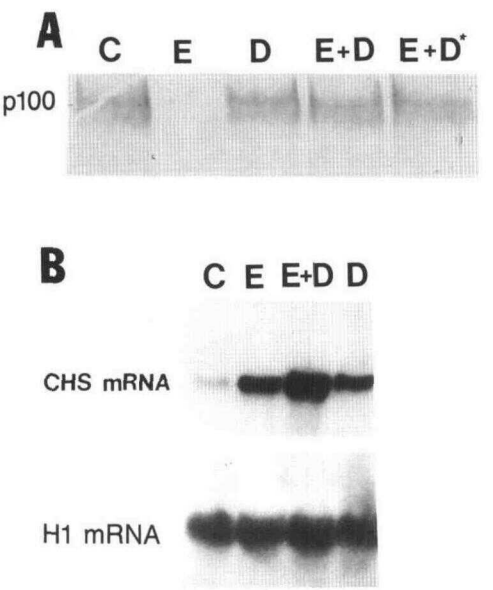

Figure 3. Effect of DTT on Defense Responses Induced by Fungal Elicitor.

(A) Protein gel blot showing inhibition of protein cross-linking by DTT. Soybean cells were untreated (C) or elicited (E) with *P. m. glycinea* elicitor (60 μg of glucose equivalents per mL). D denotes pretreatment of cells with 10 mM DTT for 15 min. Cells were collected 1 hr (E; E+D) or 2 hr (E+D*) after the elicitation treatment.

(B) Effects of DTT on the accumulation of *CHS* and *H1* transcripts. Cells were treated as described in **(A)** and collected 6 hr after the elicitation treatment.

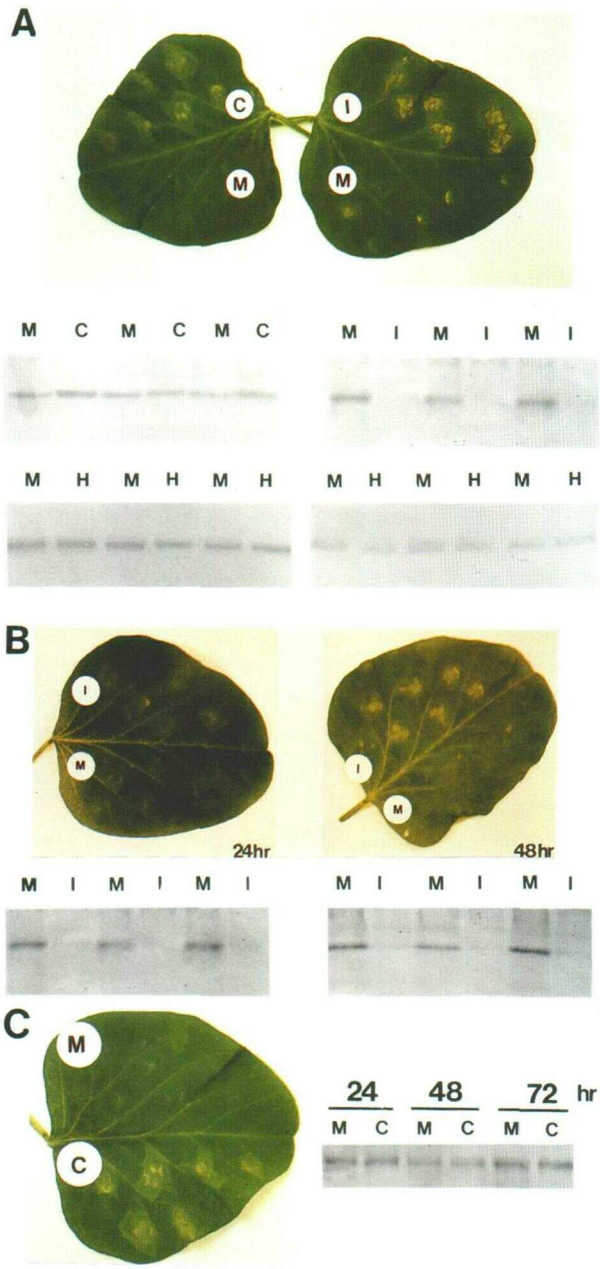

Figure 4. Response of Soybean Cultivar Harosoy Leaves to Inoculation with a Virulent and Avirulent Race of *P. s. glycinea.*

SDS-extractable p33 was analyzed by protein gel blotting of cell walls isolated from bacterially inoculated tissue (areas labeled on the leaves) compared with equivalent mock-inoculated tissue in the other half of the same leaf.

(A) Comparison of incompatible (I) and compatible (C) interactions 48 hr after inoculation. Shown is a protein gel blot of p33 in the noninoculated area (H) and in those areas inoculated with water (M) or with the compatible (C) *P. s. glycinea* race 4 (*avrC*) or incompatible (I) *P. s. glycinea* race 4 (*avrD*) pathogen.

(B) Cross-linking of p33 in the incompatible interaction with *P. s. glycinea* race 4 (*avrD*) at the onset of the visible HR. Inoculated leaves were analyzed at 24 and 48 hr after inoculation. Abbreviations are as given in **(A)**.

(C) Soluble p33 extracted at various time points (24, 48, and 72 hr) during the compatible interaction with *P. s. glycinea* race 4 (*avrC*). Abbreviations are as given in **(A)**.

de novo (Beimen et al., 1992). In our investigation, the rapidity of the inhibition of protoplast release in *P. m. glycinea* elicitor–treated soybean cells and its insensitivity to the inhibitor of phenolic synthesis AOPP (Amrhein and Godeke, 1977; Noé et al., 1980) indicate that the underlying cell wall modifications are not dependent on the de novo synthesis of phenolics from phenylalanine.

The selective inhibition of elicitor-induced p33 and p100 insolubilization by DTT demonstrates a specific link between oxidative cross-linking and modification of the cell wall to become refractory to the protoplasting enzymes. Thus, DTT, which inhibits peroxidase-mediated cell wall protein cross-linking (Fry, 1986), enhances rather than inhibits the elicitor induction of CHS transcript accumulation, which served as a marker for transcription-dependent defenses. The increased yield of protoplasts isolated from control cells in the presence of DTT could reflect inhibition of background cross-linking induced by endogenous elicitors released during the protoplasting reaction (Davis and Hahlbrock, 1987). This conclusion is consistent with the increased yield of rice protoplasts isolated in the presence of exogenous superoxide dismutase and catalase (Ishii, 1987, 1988). These observations indicate that accumulation of reactive oxygen intermediates such as H_2O_2 has a pronounced effect on protoplast release. The selective effects of DTT on the battery of elicitor-induced defense responses in soybean cells confirm the role of peroxidase-mediated oxidative cross-linking in the rapid increase in the resistance of the cell wall to digestion by protoplasting enzymes. While we cannot rule out contributions from the oxidative cross-linking of other preexisting cell wall polymers, such as pectins carrying feruloyl or other phenolic residues (Fry, 1986), p33 and p100 are abundant structural proteins, and their cross-linking might be expected to effect a major change in cell wall digestibility by protoplasting enzymes.

The selective insolubilization of p33 in a genetically defined incompatible interaction between leaves of soybean cultivar Harosoy and *P. s. glycinea* race 4 carrying the *avrD* avirulence gene implicates the oxidative cross-linking response in the expression of hypersensitive resistance. Thus, the cross-linking response was essentially complete, as determined by the insolubilization of p33, by the onset of visible hypersensitive lesions in the incompatible interaction specified by *avrD* and *Rpg4.* In contrast, p33 and p100 remained in a soluble, SDS-extractable form throughout the time course of disease development in the isogenic compatible interaction with *P. s. glycinea* race 4 (*avrC*). Likewise, oxidative cross-linking is not observed in bean leaves in a compatible interaction with the bean pathogen *P. s. phaseolicola*, but complete insolubilization of p35 was

observed in an *Hrp*-dependent reaction following inoculation with the tobacco pathogen *P. s. tabaci*, for which bean is not a host. The protoplast release assay cannot be applied to infected leaf tissue samples because the protoplasting enzymes and the osmotica needed to protect protoplasts from lysis would interfere with infection processes. Taken together with the observation that peroxidase-mediated oxidative cross-linking makes the walls of suspension-cultured cells more refractory to enzyme digestion, the timing and selectivity of p33 insolubilization in leaf tissue infected with avirulent but not virulent *P. s. glycinea* in an otherwise isogenic setting indicate that this response indeed functions as a defense mechanism early in the expression of hypersensitive resistance.

Clear differences in the temporal and/or spatial patterns of defense gene activation between incompatible and compatible interactions have been observed in many studies, and early activation of defense genes in an incompatible interaction is closely correlated with expression of the HR (Lamb et al., 1989; Dixon and Harrison, 1990). This has been confirmed in an isogenic setting by the demonstration that induction of genes encoding two phenylpropanoid biosynthetic enzymes, phenylalanine ammonia-lyase and cinnamyl alcohol dehydrogenase, is strictly dependent on a dominant allele at the Arabidopsis *RPM1* disease resistance locus in specific interactions with *P. syringae* (Kiedrowski et al., 1992). The current data indicate that induction of defense genes is also tightly correlated with the HR associated with resistance to non-pathogens. Previous studies noted that in bean, the pathogen *P. s. phaseolicola* and the non-pathogen *P. s. tabaci* both induce defense gene transcripts following leaf inoculation (Jacobek and Lindgren, 1993; Meier et al., 1993). However, when transcript accumulation is directly compared in the same infection experiment with parallel RNA extraction and RNA gel blot analysis, it is clear that the pathogen evokes only a very weak response during disease development compared with marked induction of CHS by the non-pathogen correlated with HR induction. Moreover, *P. s. tabaci* with mutations in the *Hrp* gene cluster do not induce an HR, and likewise CHS is induced only to a low level when compared with induction by wild-type *P. s. tabaci*. The picture that emerges is of a tight correlation between expression of the HR and activation of the oxidative cross-linking response and defense gene transcription in a gene-for-gene incompatible plant.

As part of the battery of inducible defenses, we propose that rapid oxidative cross-linking enhances the effectiveness of the plant cell wall as a barrier to slow pathogen ingress and spread prior to the deployment of the transcription-dependent defenses, such as phytoalexins, lytic enzymes, and other antimicrobial proteins. Moreover, rapid oxidative cross-linking of the cell wall will also serve to trap pathogens in cells destined to undergo hypersensitive cell death, thereby enhancing the effectiveness of host cell suicide in pathogen restriction. Analyses of mutants that spontaneously develop hypersensitive lesions in the absence of pathogen attack indicate that the death of challenged cells during an incompatible interaction likely reflects activation of a cell death program (Greenberg et al., 1994). Recent data indicate that in animals programmed cell death is mediated by reactive oxygen intermediates (Hockenbery et al., 1993). Oxidative cross-linking of the cell wall is driven by the rapid accumulation of H_2O_2 from a potential oxidative burst at the plant cell surface; this is a characteristic response of plant cells to microbial elicitors or challenge with an avirulent pathogen (Apostol et al., 1989; Sutherland, 1991) and is reminiscent of phagocyte activation in the mammalian immune system (Baggilioni and Wyman, 1990). Hence, oxidative cross-linking of the cell wall may be an integral component of a programmed hypersensitive response orchestrated by the accumulation of H_2O_2 from the elicitor- or pathogen-induced oxidative burst.

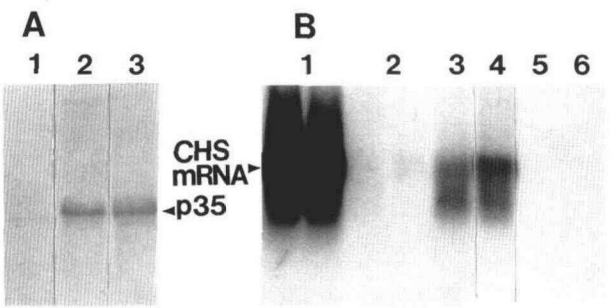

Figure 5. Defense Induction in Non-Host Resistance in Bean Leaves.

(A) Protein gel blot analysis of p35 insolubilization.

(B) RNA gel blot analysis of CHS transcript accumulation.

Defense induction was analyzed in tissue 24 hr after inoculation with (lanes 1) *P. s. tabaci* (non-pathogen, induces *Hrp*-mediated HR); (lanes 2) *P. s. phaseolicola*, a disease-causing virulent pathogen; (lanes 3) *P. s. tabaci* strain *Pst::H1* with a defective *Hrp* gene cluster (no induction of the HR); (lane 4) *P. s. tabaci* strain *Pst1::H12* with a defective *Hrp* gene cluster (no induction of the HR); (lane 5) H_2O; and (lane 6) uninoculated tissue.

METHODS

Plant Material

Suspension cultures of soybean (*Glycine max*) cultivar Harosoy were maintained as described by Norman et al. (1986). Cells were elicited 6 days after subculture by the addition of glutathione (1 mM), H_2O_2 (1 mM), fungal elicitor (60 μg of glucose equivalents per mL). The fungal elicitor preparation was the high molecular weight fraction that was heat released from washed mycelial walls of the soybean pathogen *Phytophthora megasperma* f sp *glycinea* (Ayers et al., 1976). For inhibition experiments, soybean cells were preincubated for 30 min with 30 μg/mL cycloheximide or 20 μg/mL actinomycin D and for 15 min with 0.1 mM α-aminooxy-β-phenylpropionic acid (AOPP), 10 mM DTT prior to the addition of elicitors.

Soybean (cv Harosoy) and bean (cv Immuna) plants were grown to the primary leaf stage (2 to 3 weeks) in a greenhouse and transferred 2 days before bacterial inoculation to growth chambers maintained at 21 to 22°C and 90% relative humidity with a 16-hr day. *Pseudomonas syringae* pv *glycinea* race 4 with plasmids carrying avirulence gene

avrC or *avrD* was kindly supplied by N. Keen (University of California, Riverside). *P. s.* pv *phaseolicola* and *P. s.* pv *tabaci* were kindly provided by P. Lindgren (North Carolina State University, Raleigh). Bacteria used for the inoculation were grown at 28°C in King's B media (King et al., 1954) supplemented with 50 μg/mL kanamycin. Small areas of expanding primary leaves were inoculated by infiltration with a suspension of 10^8 bacterial cells per mL using a 1-mL hypodermic syringe without a needle (Keen et al., 1990). One side of each leaf was inoculated at six different places with ~20 μL of bacterial suspension per wound, and the other side was mock-inoculated with 20 μL of sterile water (six wounds per half leaf). Leaf tissue was collected with a cork borer at the six inoculation sites and in the immediate vicinity and rapidly frozen in liquid N_2. Samples collected from six sites were pooled and collected at the times indicated, and four plants were analyzed separately at each time point.

Wall Digestibility Assay

Protoplasts were isolated from soybean cell suspension cultures at the stage of their optimal elicitor responsiveness, 6 days after transfer, when the conductivity of the medium had fallen below 2.6 mhos (Lawton and Lamb, 1987). Control and elicited cells were collected by centrifugation at 100*g*, and 1 g of packed cells was washed twice in 10 mL of Murashige and Skoog (1962) medium, pH 5.8, containing 0.4 M mannitol. Cells were incubated with the protoplasting enzymes cellulysin (1% [w/v]; Calbiochem) and pectolyase Y23 (0.1% [w/v]; Karlan, Tokyo, Japan) for various times (0.5, 1, 2, 5, and 24 hr) in the dark at 20°C with constant shaking. The suspension was then centrifuged, and the number of protoplasts and cells was determined using bright-field or fluorescence microscopy (Nikon Diaphot TMD, Santa Rosa, CA). Protoplasts were distinguished from intact cells by their spherical shape and lack of calcofluor white staining (Gamborg et al., 1981). The number of protoplasts released was monitored in relation to the number of cells (protoplast/cell ratio) in the absence and presence of elicitors. Viability of isolated protoplasts was estimated by the Evans blue stain exclusion procedure (Graff and Okong-Ogola, 1971) and by fluorescein diacetate staining (Kartha, 1981). Samples were counted in a hemocytometer, and the protoplast/cell ratio, determined by observing four replicates from a single enzyme treatment, provided an index of wall digestibility. Each treatment was repeated at least four times, and each experiment was internally controlled by reference to the wall digestibility of equivalent unelicited control cell suspensions taken through the procedure in parallel. Mean values were compared by Student's *t* test.

Protein Gel Blots

Proteins from cell cultures and from the leaves of soybean or bean plants were analyzed by protein gel blotting using anti-p33 sera, a polyclonal antiserum generated by immunization with purified p33 (Salk Institute numbers 4916 and 4917), and anti-p100 sera, kindly provided as MAC 265 by N. Brewin (John Innes Institute, Norfolk, U.K.). Protein extraction, separation, and analysis were performed as described by Bradley et al. (1992). Total protein concentration in the extract was assayed using the biuret reaction (Smith et al., 1985).

RNA Gel Blots

Suspension-cultured cells were collected by aspiration and snap-frozen in liquid N_2. Infected leaf tissues were excised and snap-frozen in liquid N_2. Total RNA was isolated from frozen cells using the guanadinium thiocyanate method (Mehdy and Lamb, 1987), and RNA was fractionated by electrophoresis in 1% agarose gels containing 6.5% formaldehyde and transferred to a nylon membrane (Nytran; Schleicher & Schuell; 0.45 μm) using a pressure blotter (Stratagene). Blots were hybridized for 12 hr at 65°C with ^{32}P-labeled cDNAs of the bean chalcone synthase 15 (CHS15) gene or the constitutive H1 transcript (Lawton and Lamb, 1987). Hybridization was performed in 0.5 M phosphate buffer, pH 7.2, containing 1% BSA, 7% SDS, and 1 mM EDTA (Church and Gilbert, 1984). Filters were washed in several changes of 1 × or 0.1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) containing 1% SDS at room temperature and exposed to x-ray film.

ACKNOWLEDGMENTS

We thank Wolfgang Barz for helpful discussions; Michael Hahn for the fungal elicitor; and Noel Keen, Peter Lindgren, and Brian Staskawicz for bacterial strains and advice on soybean infection. This research was supported by grants to C.J.L. from the Samuel Roberts Noble Foundation and the National Science Foundation (Grant No. IBN-9104551). L.F.B. thanks the Natural Sciences and Engineering Research Council of Canada for a research fellowship, Eileen Maher and Jonathan Arias for helpful discussions, and Israel Zelitch for his help in the preparation of the manuscript. R.T. thanks the Deutscher Akademischer Austauschdientst (NATO program) and Deutsche Forschungsgemeinschaft for fellowships. We thank Dan Sherman for RNA gel blot analysis of non-host resistance.

Received June 29, 1994; accepted October 5, 1994.

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