RNAi Silencing of Genes for Elicitation or Biosynthesis of 5-Deoxyisoflavonoids Suppresses Race-Specific Resistance and Hypersensitive Cell Death in Phytophthora sojae Infected Tissues\textsuperscript{1}[OA]

Terrence L. Graham*, Madge Y. Graham, Senthil Subramanian, and Oliver Yu

Department of Plant Pathology and Plant Molecular Biology and Biotechnology Program, Ohio State University, Columbus, Ohio 43210 (T.L.G., M.Y.G.); and Donald Danforth Plant Science Center, St. Louis, Missouri 63132 (S.S., O.Y.)

Isoflavonoids are thought to play an important role in soybean (Glycine max) resistance to Phytophthora sojae. This was addressed by silencing two genes for their biosynthesis and a third gene controlling their elicitation. Silencing of genes for isoflavone synthase (IFS) or chalcone reductase (CHR) was achieved in soybean roots through an Agrobacterium rhizogenes-mediated RNAi approach. Effectiveness of silencing was followed both by quantitative reverse transcriptase-polymerase chain reaction and high-performance liquid chromatography analyses. Silencing either IFS or CHR led to a breakdown of Rps-mediated resistance to race 1 of P. sojae in 'W79' (Rps 1c) or 'W82' (Rps 1k) soybean. Loss of resistance was accompanied by suppression of hypersensitive (HR) cell death in both cultivars and suppression of cell death-associated activation of hydrogen peroxide and peroxidase. The various results suggest that the 5-deoxyisoflavonoids play a critical role in the establishment of cell death and race-specific resistance. The P. sojae cell wall glucan elicitor, a potent elicitor of 5-deoxyisoflavonoids, triggered a cell death response in roots that was also suppressed by silencing either CHR or IFS. Furthermore, silencing of the elicitor-releasing endoglucanase (PR-2) led to a loss of HR cell death and race-specific resistance to P. sojae and also to a loss of isoflavone and cell death responses to cell wall glucan elicitor. Taken together, these results suggest that in situ release of active fragments from a general resistance elicitor (pathogen-associated molecular pattern) is necessary for HR cell death in soybean roots carrying resistance genes at the Rps 1 locus, and that this cell death response is mediated through accumulations of the 5-deoxyisoflavones.

Secondary metabolites play many important roles in plant host-microbe interactions. These include chemotaxis of infective propagules, induction of virulence genes, and resistance against disease (e.g. Dakora and Phillips, 1996). It is the antibiotic activity of secondary metabolites that is thought to play an important role in resistance against pathogens. Such plant antibiotics (Van Etten et al., 1994) can either be preformed (phytoanticipins) or induced by stress or upon pathogen attack (phytoalexins). Usually a given plant species produces only a few metabolically related antibiotics that can have relatively broad spectrum activity against fungal, oomycetic, and bacterial pathogens. Surprisingly, although some elegant biochemical and cellular studies have provided strong correlative evidence for the potential involvement of secondary products in resistance, there is comparatively little genetic evidence supporting their functions (Hammerschmidt, 1999; Essenberg, 2001; Dixon et al., 2002). Some of the most compelling evidence for the importance of phytoalexins comes from the requirement of genes for their degradation for maximum virulence of fungal pathogens (Covert et al., 1996; Wasmann and VanEtten, 1996). The prevailing thinking is that, through their antibiotic effects on the pathogen, the phytoalexins play a lesion-limiting role (e.g. Kuc, 1972; Hammerschmidt, 1999).

One of the most highly characterized systems for the delineation of the roles of secondary products in resistance is the soybean (Glycine max)-Phytophthora sojae association. The pathways for the biosynthesis of the soybean phytoalexins, the glyceollins, have been particularly well elucidated (Ebel, 1986) and it was in this system that one of the first phytoalexin
Silencing of Isoflavonoids Suppresses HR Cell Death

elicitors, the cell wall glucan elicitor (WGE) from *P. sojae* was described (Ayers et al., 1976). High-affinity binding proteins for this elicitor have also been characterized (Cote et al., 1995) and putative receptors cloned (Umemoto et al., 1997; Mithofer et al., 2000). At a physiological level, systematic studies have defined the complex environmental and developmental parameters affecting phytoalexin accumulation and the outcome of the interaction between host and pathogen (Bhattacharyya and Ward, 1986a, 1986b, 1987). A landmark study on *P. sojae* infected soybean hypocotyls demonstrated the critical importance of carefully analyzing the spatial aspects of phytoalexin accumulation in interpreting the role of the phytoalexins (Yoshikawa et al., 1978). This work provided a foundation for later cellular studies in roots (Hahn et al., 1985) and the development of soybean cotyledon protocols for the cellular delineation of the multiplicity and coordination of responses to infection and WGE treatment (Graham and Graham, 1991b, 1996; Graham, 1994).

WGE is considered a classical general resistance elicitor or pathogen-associated molecular pattern (PAMP). Nonetheless, cellular studies on responses to WGE have shown that it is a remarkably global elicitor. In addition to inducing the iso flavonoid phytoalexin, glyceollin, in cells proximal to the point of elicitor treatment, WGE induces large accumulations of conjugates of the iso flavones daidzein and genistein in cells distal to elicitor treatment (Graham and Graham, 1991b). WGE also induces phenolic polymer accumulation in proximal cells (Graham and Graham, 1991a). Finally, WGE induces a series of different pathogen-related (PR) protein genes in proximal and distal cells (Graham et al., 2003). Indeed, in cotyledons WGE induces most aspects of incompatible defense to *P. sojae* except the hypersensitive (HR) response itself. However, intriguing connections of WGE elicitation to HR cell death were later suggested by a phenomenon termed elicitation competency (Graham and Graham, 1994), in which the elicitation of glyceollin by WGE in cotyledon cells only occurred in cells preconditioned by wounding or HR cell death.

We have previously described a high-frequency *Agrobacterium rhizogenes*-mediated transformation protocol for RNAi silencing of soybean roots (Subramanian et al., 2005). This protocol is genotype independent and allows the high-throughput analysis of the effects of silencing genes of interest on root responses to elicitor or infection. Using this protocol, we have previously shown that the silencing of the *iso flavone synthase* (IFS) gene family (IFS1 and IFS2) leads to a nearly complete (>95%) suppression of all iso flavone metabolites in roots, including the aglycones and the glucosyl and malonyl-glucosyl conjugates of daidzein and genistein (Fig. 1; Subramanian et al., 2005). Accompanying this was the accumulation of relatively small amounts of liquiritigenin, the immediate precursor of daidzein. Recently, it was reported that cosuppression of IFS in soybean hairy roots led to the accumulation of *p*-hydroxybenzoic acid and vanillic acid in the soluble fraction (Lozovaya et al., 2007). However, these metabolites did not appear as major peaks in metabolic profiles of our IFS RNAi-silenced tissues.

Here we have extended this work to the silencing of chalcone reductase (CHR) gene family members. CHR is required (Fig. 1) for the formation of isoliquiritigenin, a precursor of daidzein, which is a 5-deoxyiso flavone. As we have for IFS (Subramanian et al., 2005; Fig. 2), we confirmed silencing of the genes for CHR using two assays: quantitative reverse transcriptase (qRT)-PCR to measure transcripts and HPLC analysis to measure metabolites. We assayed the transcript levels of one known CHR gene (Welle et al., 1991) and three of its homologs (Subramanian et al., 2006). Of these four CHR homologs, two (CHR1 and CHR4) were significantly (>95%) silenced (Fig. 2) whereas the other two (CHR2 and CHR3) were not. Nonetheless, all 5-deoxyisoflavonoids (including daidzein) were strongly suppressed (>95%) in the HPLC metabolic profile of silenced roots (Fig. 3). Interestingly, the accumulation of genistein is actually enhanced 3- to 5-fold in these roots. This is not surprising since daidzein and genistein are alternate products of the action of IFS on different precursors (Fig. 1). By suppressing accumulation of daidzein there may be more flux into the alternative metabolite, genistein. These various results are in agreement with our observations on IFS and
CHR RNAi silencing in hairy roots on composite plants (Subramanian et al., 2006).

Silencing of IFS or CHR Leads to a Breakdown of Race-Specific Resistance to \textit{P. sojae}

We next tested the effect of IFS and CHR silencing on root resistance against \textit{P. sojae}. As noted in the methods, all inoculations were made just above the zone of elongation where race-specific resistance responses were most uniformly obtained. In this root zone the strong underlying age-related resistance characteristic of older root tissues does not interfere. As shown in Figure 4, infection and disease development in universally susceptible ‘Williams’ is very rapid and silencing of IFS or CHR had no significant effect on the rate or extent of infection. These compatible lesions showed no necrosis, but rapid spread of water-soaked, rotted tissue. In contrast, infection of vector control roots of ‘W79’ and ‘W82’ (carrying the Rps 1c and Rps 1k loci for race-specific resistance to race 1, respectively) resulted in the expected incompatible reaction and a lack of progression of the disease. Lesions in ‘W79’ and ‘W82’ were characterized by necrotic HR lesions (dark brown to black spots directly under the agar infection plugs) and minimal spread of the pathogen. There was no water-soaked tissue rot as in the compatible infection. In contrast, silencing of either CHR or IFS in ‘W79’ or ‘W82’ led to a greatly enhanced rate and extent of infection and to water-soaked lesions indistinguishable from the compatible interaction. In fact in many samples the rates of infection were as rapid as those seen in the universally susceptible ‘Williams’. These reactions were confirmed by examination of ‘L77-727’ (Rps 1c) and ‘General’ (Rps 1k) and additional isolates of race 1 (data not shown). Thus, silencing of IFS and CHR led to a breakdown of race-specific resistance in roots of lines carrying either Rps 1c or Rps 1k. While daidzein accumulation is suppressed in both IFS- and CHR-silenced
tissues, genistein levels are actually higher in the CHR-silenced tissues. This suggests that the 5-deoxyisoflavone daidzein, but not genistein, plays an important role in race-specific resistance.

Silencing of IFS or CHR Leads to the Expected Loss of Isoflavones and Glyceollin in Tissues Directly under the Agar Infection Plug

In the same experiments shown in Figure 4, for ‘Williams’ and ‘W82’ (Rps 1k) we also harvested a 2-mm long section of root directly under the agar inoculation plug to determine the effects of silencing on infection-induced accumulations of isoflavones and glyceollin. We also analyzed metabolites in the agar plug itself to determine the secretion of metabolites at the site of infection. As shown in Figure 5A, vector control roots showed the expected results, with greater accumulation of glyceollin (Graham et al., 1990) in the incompatible (‘W82’) infection as compared to the compatible infection (‘Williams’). RNAi silencing of IFS and CHR led to the expected suppression of accumulations of both daidzein/genistein or daidzein alone, respectively (Fig. 5A). However, in infected roots, silencing of the isoflavones though strong (80%–95%) was not quite as effective in some cases as seen in uninfected roots, where it was usually >95% (see HPLCs in Fig. 3). This is not surprising given the very strong induction conditions of infection on IFS and CHR, the fact that the target gene is an enzyme, and the fact that the samples were collected after 48 h. Even with 95% to 98% silencing of the CHR and IFS enzymes, some product would be expected to accumulate over this period of time. Figure 5B shows the accumulations of the various isoflavones in the agar plug. This is expressed as the percent of the total (agar plug + section under the agar plug) in the agar plug for each specific isoflavone. Generally, very little (approximately 5%–10%) of the total for each individual isoflavonoid accumulated in the agar plug. However, in ‘W82’ vector controls, nearly 35% of the glyceollin accumulated in the agar plug. In the IFS- and CHR-silenced roots of ‘W82’, but not the vector control, 25% to 30% of the low (remaining) levels of total daidzein were found in the agar plug. In contrast, genistein accumulated to only 2% to 10% in the same agar plugs. Thus, incompatible infection seems to have caused selective secretion of 5-deoxyisoflavonoids via a mechanism that appears to favor glyceollin, but can accommodate daidzein as well. In this regard, it is interesting that vesicular secretion of phytoalexins from producing cells into the extracellular space has been reported for the anthocyanin phytoalexins in sorghum (Snyder and Nicholson, 1990).

Silencing of IFS or CHR Leads to Suppression of Cell Death and Reactive Oxygen Responses Associated with Race-Specific Resistance to P. sojae

Race-specific resistance is accompanied by HR cell death, which is considered the determinative event in
this form of resistance. HR cell death can be monitored by a number of protocols, including vital stains (Evans blue [Baker et al., 1993; Graham, 2005] or Trypan blue [Meier et al., 1993; Ger et al., 2002]), yellow autofluorescence (YAF; Koga et al., 1988; Essenberg et al., 1992; Wäspi et al., 2001; Gray et al., 2002; Graham, 2005), and the accumulations of hydrogen peroxide and peroxidase. The latter response is commonly followed by staining with diaminobenzidine (Hückelhoven et al., 1999; Vanacker et al., 2000) or 3-amino-9-ethylcarbazole (AEC; Ellis and Grant, 2002), which are substrates of peroxidase that are oxidized to colored compounds only in the presence of endogenous hydrogen peroxide. Because of our past use of Evans blue and YAF to follow soybean cell death (Graham, 2005) and the greater contrast we obtained with AEC, we chose to work with these protocols.

As shown in Figure 6, cells at the infection site of vector control ‘Williams’ soybean roots infected with P. sojae (compatible response) showed little to no YAF, indicating the lack of HR cell death. The fading of the GFP compared to uninfected roots (inset) accompanies the onset of root rot and tissue maceration. Transformation of roots with RNAi-silencing constructs for CHR or IFS had no effect on this response. In contrast, the responses of vector control roots of ‘W79’ and ‘W82’ infection (incompatible responses) were both characterized by strong YAF responses, which were restricted to the area directly below the agar plug, characteristic of HR cell death. Silencing of either IFS or CHR led to suppression of YAF and to root rot similar to the compatible infections. Although this root rot in the ‘W79’ and ‘W82’ backgrounds was rapid and led to complete tissue maceration, there was sometimes a very limited yellow autofluorescent response associated with root hairs or a mottled black reaction typical of phenolic polymer accumulation (see sections under IFS RNAi). We do not know the nature of these reactions, but they did not contain the pathogen. These various results with YAF were confirmed by Evans blue vital staining of cells (data not shown), as we have described earlier for lactofen-induced cell death (Graham, 2005).

Thus, silencing of CHR or IFS led to a loss of typical HR cell death as monitored by both YAF and vital staining. A third reaction sometimes used to characterize HR cell death-associated events, the accumulation of hydrogen peroxide and peroxidases, was also examined. From the literature, this response is localized to HR dying cells (Hückelhoven et al., 1999; Vanacker et al., 2000; Ellis and Grant, 2002). As shown in Figure 7, the universally susceptible ‘Williams’ did not show red AEC stain following infection in vector control or IFS/CHR-silenced roots (a typical response is shown in section A). Vector control roots of ‘W79’ and ‘W82’ both showed strong AEC staining beginning approximately 24 h postinoculation. Reactions at 48 h are shown in Figure 7. In some cases, this response was characterized by red spots localized to the area under the agar plug and slightly beyond (section B), while in other cases the staining spread systemically well beyond the point of inoculation (section C). These incompatible infection-related reactions in ‘W79’ and ‘W82’ control roots were suppressed in IFS or CHR RNAi-silenced tissues and root staining was absent, similar to the reaction of ‘Williams’ shown in section A. Thus, as with other host-pathogen interactions, AEC staining reveals activated oxygen species (AOS) reactions associated with race-specific resistance, although in soybean roots the response can be induced in cell populations both at and sometimes beyond the zone of dying cells. This, and the relatively late timing of the responses, suggests that the peroxidase/hydrogen peroxide reactions measured by AEC staining may include reactions after initiation of HR cell death per se. Nonetheless, these various results again suggest an importance of daidzein, but not genistein, in HR cell death and associated reactions.
Silencing of IFS or CHR Leads to Suppression of 5-Deoxyisoflavone and Glyceollin Accumulation and to Suppression of a Cell Death Response Triggered by the WGE from \( \textit{P. sojae} \)

WGE from \( \textit{P. sojae} \) is a very potent elicitor of 5-deoxyisoflavonoids, including daidzein and the daidzein-derived soybean phytoalexin glyceollin (Graham and Graham, 1991b, 1996). The elicitation of these responses by WGE is neither race nor cultivar specific. Indeed, it is an effective elicitor even in universally susceptible ‘Williams’. For these reasons, it has been considered a general resistance elicitor or PAMP. The effects of WGE on root tissues have not been well characterized. Since gene silencing suggested that 5-deoxyisoflavones are critical for cell death in roots undergoing an incompatible infection, we were interested in characterizing the effects of treatment with this elicitor on cell death in control and IFS/CHR-silenced tissues.

Treatment of vector control roots of all soybean lines with WGE led to cell death and tissue collapse (Fig. 8). Thus, unlike \( \textit{P. sojae} \)-induced HR cell death, WGE-induced cell death was not cultivar specific and did not require the presence of an Rps gene in uninfected tissues. This was confirmed in over six separate experiments. However, the severity of the cell death reaction varied somewhat with cultivar, in the order ‘W79’ > ‘Williams’ > ‘W82’. In ‘W82’, cell death was usually localized to a collapsed zone of cells under the point of treatment (Fig. 8A). Yellow autofluorescence was restricted to the same zone of collapse seen under white light and sometimes involved deposition of a black pigmented material (Fig. 8B). In ‘Williams’, cell death was somewhat less restricted. It varied from a slightly more severe localized reaction than seen with ‘W82’, to a very sharply restricted, but complete collapse of root tissues under the point of treatment (Fig. 8C). Accompanying this was an equally sharply defined yellow autofluorescence (Fig. 8D). Finally, in ‘W79’ cell death was sometimes even more severe, involving a longer zone of collapse that occasionally

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**Figure 5.** Isoflavonoid metabolites in tissues directly under the agar inoculation plug in \( \textit{P. sojae} \) infected vector control or CHR or IFS RNAi roots. A, Twenty additional roots from the same experiment in Figure 4 were harvested at 48 h. A 2-mm long cross section of the area directly underneath the agar inoculation plug (UAP) was cut and pooled for analysis by HPLC metabolic profiling. V, C, and I refer to roots transformed with the control vector, CHR RNAi, or IFS RNAi constructs, respectively. The results are presented as totals of the free and conjugated forms of daidzein and genistein and total glyceollins (glyceollin I and II). B, The agar inoculation plugs (AP) from this same experiment were also harvested, pooled, and analyzed by HPLC metabolic profiling. Results are presented as percent of the total (agar plug + root tissue under the agar plug) that was in the agar plug for each metabolite. V, C, and I refer to roots transformed with the control vector, CHR RNAi, or IFS RNAi constructs, respectively. The experiment was repeated once with similar results.

**Figure 6.** Yellow autofluorescence of \( \textit{P. sojae} \) infected vector control or IFS or CHR RNAi roots. Vector control and IFS or CHR RNAi roots were infected with race 1 of \( \textit{P. sojae} \). At 48 h, the roots were excised and the area directly under the agar plug examined by epifluorescence microscopy. Race 1 is compatible (C) on ‘Williams’ (Will) and incompatible (I) on ‘W82’ and ‘W79’. Cell death is indicated by yellow autofluorescence. GFP fluorescence (see inset of uninfected root) was partially quenched in rotten, infected root tissues.
spread along much of the entire root (Fig. 8, E and F). The comparisons of WGE-induced cell death shown in Figure 8 are for roots treated with a 5-μL droplet containing 100 μg/mL of the intact WGE, a concentration that is half saturating for glyceollin accumulation in roots (data not shown). Similar effects were seen at concentrations as low as 30 μg/mL. At yet lower concentrations, cell death was still apparent in isolated cells at the point of treatment. Given the facts that the very high molecular mass intact WGE (Ayers et al., 1976) was used and that there was no wounding to facilitate entry of the elicitor, these are comparatively very low concentrations of WGE.

WGE-induced cell death was suppressed by silencing either CHR or IFS. Silenced roots showed no visible response to elicitor under white light and no YAF (data not shown), suggesting once again that the presence of daidzein, but not genistein, contributes to the cell death response. HPLC metabolic profiling confirmed the expected nearly complete suppression of all isoflavones in IFS-silenced roots and daidzein and other 5-deoxyisoflavonoids in CHR-silenced roots.

It was also of interest to monitor the isoflavones in the various cell death responses in vector control roots. HPLC metabolic profiling revealed some net accumulations of daidzein and glyceollin in the less severe reactions (Fig. 8, A and C). In contrast, in the most severe reactions (Fig. 8E), a dramatic decrease in all isoflavonoids (including the isoflavones and glyceollin) occurred, suggesting a massive degradation of isoflavonoids in these dying tissues. The degradation of most natural products is an oxidative process (Barz and Hoesel, 1979). Whether this degradation contributes to cell death or is simply a consequence of cell death would require further study.

Silencing of the Elicitor-Releasing Endoglucanase, PR-2, Leads to a Loss of Race-Specific Resistance and the Cell Death Response to WGE

To further investigate the role of WGE in cell death and race-specific resistance, we silenced the elicitor-releasing endoglucanase, a PR-2. In soybean, this gene is induced by ethylene (Takeuchi et al., 1990), wounding, or WGE (Graham et al., 2003). It is thought that PR-2 releases active elicitor fragments from WGE, a hypothesis consistent with in vitro enzymatic experiments (Yoshikawa et al., 1981). However, to our knowledge, such a role has never been verified in molecular genetic studies. Moreover, the function of PR-2 during infection of soybean by P. sojae has not been investigated.

As we did with IFS and CHR we used qRT-PCR to determine the effectiveness of our PR-2 RNAi-silencing construct. Since PR-2 is weakly constitutively expressed, but strongly induced by treatment with WGE (Graham et al., 2003), we used WGE-induced roots for extraction of total RNA. Our normal treatments with WGE employ spot treatment with 5 μL of elicitor (e.g. Fig. 8), but to get adequate root tissue for

![Figure 7.](image1.png) Aminoethyl carbazole staining of P. sojae infected vector control or IFS or CHR RNAi roots. Vector control and IFS or CHR RNAi roots were infected with race 1 of P. sojae. At 48 h, the roots were excised and stained with aminoethyl carbazole. The three sections show representative roots for each treatment. The dotted boxes represent the area under the agar inoculation plug. A, ‘Williams’ (susceptible) root. B, ‘W79’ (Rps 1c, incompatible) root. C, ‘W82’ (Rps 1k, incompatible) root. Both IFS or CHR RNAi roots of ‘W79’ and ‘W82’ were similar to A.

![Figure 8.](image2.png) White light and epifluorescence microscopy of WGE treated roots of various cultivars. Vector control roots of ‘W82’ (A and B), ‘Williams’ (C and D), and ‘W79’ (E and F) were treated with 5 μL of WGE (100 μg/mL). The blue color is a cyan dye added to the glucan preparation and used to mark the treatment site. The dye alone had no effects on the tissues or their HPLC metabolic profiles. Pictures were taken at 48 h to show the ultimate tissue necrosis and collapse.
We soaked entire excised PR-2 RNAi roots in 100 µg/mL WGE for 16 h. qRT-PCR revealed that expression of PR-2 in vector control roots of ‘Williams’ and ‘General’ (carrying Rps 1k) was over 10-fold relative to ubiquitin. In PR-2 RNAi roots, even under these very strong induction conditions, silencing was as effective as 70% when normalized to ubiquitin expression (data not shown).

Silencing of PR-2 led to a loss of race-specific resistance to P. sojae in ‘Williams’ isolines carrying Rps 1c or 1k similar to that shown in Figure 4 for IFS or CHR RNAi (data not shown). Moreover, in experiments using spot treatment with 5 µL of WGE, silencing of PR-2 led to a complete loss of activity of the elicitor, including its induction of daidzein and glyceollin, and its induction of cell death in all soybean lines (shown in Fig. 9 for ‘W79’). While vector control roots showed the normal cell death response to WGE (Fig. 9A), PR-2 RNAi transformed roots showed no response to elicitor, either in white light or under fluorescent light (Fig. 9B). Finally, the loss of response of PR-2 RNAi roots to WGE could be restored (biochemically complemented) by applying enzymatically prereleased elicitors from WGE (Fig. 9C). Thus, expression of PR-2 is required for isoflavone accumulation, race-specific resistance, and cell death in soybean roots, consistent with the central role of daidzein and/or glyceollin in the cell death program.

DISCUSSION AND CONCLUSION

The roles of secondary products in plant disease resistance have nearly exclusively been attributed to their antibiotic activity. A notable exception to this is salicylic acid, which is associated with systemic acquired resistance and has been shown to affect the HR cell death response in some plants. In this article, using gene silencing of key enzymes in their biosynthesis, we have shown that soybean 5-deoxyisoflavonoids (daidzein and/or glyceollins) are required for HR cell death and race-specific resistance in roots of soybean lines carrying genes at the Rps 1c and Rps 1k loci for race-specific resistance to P. sojae. Moreover, we show that the WGE from P. sojae (which is the primary pathogen elicitor for daidzein and glyceollin) is sufficient for the induction of cell death. Furthermore, silencing of the WGE elicitor-releasing endoglucanase (PR-2) suppresses isoflavone and cell death responses to WGE as well as HR cell death and race-specific resistance in infected tissues. WGE has long been considered a general resistance elicitor or PAMP. The results presented here suggest that the in situ release of active elicitor fragments from WGE participates in HR cell death. In the sections below, we discuss these findings in the context of (1) possible mechanisms for isoflavone-mediated cell death, (2) previous studies with WGE and other general resistance elicitors (PAMPs), and (3) the relationships of the findings to our current understanding of the nature of soybean Rps genes and the corresponding P. sojae Avr genes.

Possible Mechanisms for Isoflavone-Mediated Cell Death

Whatever the role of WGE, it is clear that the 5-deoxyisoflavones somehow participate in HR cell death. Among the events that commonly contribute to HR cell death are (1) an oxidative burst, (2) an early influx of Ca²⁺, (3) a K⁺/H⁺ exchange reaction that leads to alkalinization of the apoplast, (4) a prolonged membrane depolarization, and (5) a prolonged oxidative state (for review, see Heath, 2000 and refs. therein). What potential roles could soybean 5-deoxyisoflavonoids play in these events? Events 1 and 5 involve AOS. The dramatic loss in isoflavonoids from severely collapsed WGE-treated tissues (Fig. 8) suggests oxidative degradation of the isoflavonoids may be taking place. It is thought that due to their reductive capacity compared to most AOS species, that the isoflavones per se (e.g. free daidzein) may be protective against oxidative stress (e.g. Ruefer and Kulling, 2006). On the other
hand, the redox potential of any given compound is a relative thing, and it is possible that in the process of being oxidized, the isoflavones may generate oxidized intermediates (e.g. peroxidase generated free radicals) that may themselves be toxic in certain compartments, environments, or conditions. The animal literature is full of descriptions of flavonoids that protect cells under certain conditions and contribute to or enhance cell death under others (e.g. Williams et al., 2004). Another possibility is that the more elaborate 5-deoxyisoflavone-derived pterocarps, such as glyceollin contribute to cell death. Indeed, preliminary studies suggest that the glyceollins target both the membrane ATPase that serves as a proton pump (Giannini et al., 1988) and mitochondrial electron transport (Boydston et al., 1983). Given the fact that alkalinization of the apoplast (K\(^+\)/H\(^+\) exchange) and prolonged oxidative stress both are key events in the HR (Heath, 2000), it is possible that such events could contribute to the HR. Finally, the overall light exposure and redox status of the cell also profoundly affects phytoalexin toxicity. For instance, photoactivation of isoflavonoid phytoalexins, such as glyceollin, leads to free radicals that are much more highly toxic to cells (Bakker et al., 1983) and photoactivation also greatly enhances the toxicity of sesquiterpenoid phytoalexins (Sun et al., 1989). Although this latter factor is probably not working in roots, it is quite interesting that light intensity has been correlated with the release of PAMPs (Boydston et al., 1983). The effects of WGE have not been well characterized on roots. Here we report that soybean roots are inherently competent for glyceollin elicitation regardless of wounding or the presence of Rps genes. Moreover, in AOS. The glyceollins induce defense responses in parsley (Petroselinum crispum; see Brunner et al., 2002 and ref.s therein). The Pep-13 motif is conserved among transglutaminases in Phytophthora species, and, like WGE, Pep-13 induces coumarin phytoalexins in parsley. Moreover, while Pep-13 induces many events one would normally attribute to cell death in parsley, including AOS accumulation, Ca\(^{2+}\) influx, etc., it does not induce cell death per se (Blume et al., 2000). However, in potato (Solanum tuberosum; a host of Phytophthora infestans), Pep-13 induces a series of defense reactions including a salicylic acid-dependent HR-like cell death (Halim et al., 2004). Thus it would appear that PAMPs may participate directly in the HR under certain conditions or in the presence of other elicitors or modifiers.

**Relationship of These Studies to Previous Studies with the Wall Glucan Elicitor from P. sojae and Other PAMPs**

The wall glucan elicitor from P. sojae is a very potent elicitor of 5-deoxyisoflavones, including the isoflavone daidzein and the soybean phytoalexin glyceollin (Graham and Graham, 1991b, 1996). Elicitation of these responses by WGE is neither race nor cultivar specific. Indeed, it is an effective elicitor even in universally susceptible ‘Williams’. For these reasons, it has been considered a general resistance elicitor or PAMP. However, from extensive characterization of responses to WGE in cotyledon tissues, it has been known for a number of years that responses to this elicitor are complex and linked to the HR and race-specific resistance (Graham and Graham, 1994, 1999, 2000; Park et al., 2001). One connection between responses to WGE and the HR in cotyledon tissues was earlier termed elicitation competency (Graham and Graham, 1994), a phenomenon in which only wound- or HR-associated cells respond to WGE to produce the phytoalexin, glyceollin. Later genetic studies suggested an additional connection of responses to WGE to race-specific resistance. It was discovered that the presence of genes at several Rps loci (Rps 1k, 3b, and 7) conditioned elicitation competence to WGE (Abbasi et al., 2001). Finally, treatment of elicitation competent cotyledon tissues with WGE causes a rapid alkalinization of the apoplast (T.L. Graham, unpublished data) and treatment of soybean suspension cells with glucan elicitor fragments leads to a Ca\(^{2+}\) influx associated with hydrogen peroxide production (Mithofer et al., 2001). All of these responses are often associated with HR cell death and suggested that WGE might condition some aspects of the cell death program.

The effects of WGE have not been well characterized on roots. Here we report that soybean roots are inherently competent for glyceollin elicitation regardless of wounding or the presence of Rps genes. Moreover, in potato, WGE induces cell death. The glyceollin and cell death responses to WGE are both suppressed by silencing expression of IFS, CHR, or the elicitor-releasing PR-2, providing strong evidence linking cell death to elicitor release and isoflavone accumulation. The silencing of the same three genes lead to suppression of HR cell death in incompatible infected tissues, suggesting that WGE release and induction of isoflavones and cell death participate in the HR itself.

Results similar to the effects described here for WGE are seen with Pep-13, a polypeptide fragment of a cell wall-associated transglutaminase from P. sojae that induces nonhost defense responses in parsley (Petroselinum crispum; see Brunner et al., 2002 and ref.s therein). The Pep-13 motif is conserved among transglutaminases in Phytophthora species, and, like WGE, Pep-13 is considered a general resistance inducer or PAMP (Brunner et al., 2002). In parallel to WGE induction of glyceollin in soybean, Pep-13 induces coumarin phytoalexins in parsley. Moreover, while Pep-13 induces many events one would normally attribute to cell death in parsley, including AOS accumulation, Ca\(^{2+}\) influx, etc., it does not induce cell death per se (Blume et al., 2000). However, in potato (Solanum tuberosum; a host of Phytophthora infestans), Pep-13 induces a series of defense reactions including a salicylic acid-dependent HR-like cell death (Halim et al., 2004). Thus it would appear that PAMPs may participate directly in the HR under certain conditions or in the presence of other elicitors or modifiers.
Relationship of Isoflavone-Mediated Cell Death to Other Studies on the Pathogenicity of *P. sojae* and the Nature of Rps-Mediated Resistance in Soybean

While the results described in this article point to a potentially central role of WGE, PR-2, and the isoflavones in the HR, as pointed out in the results (Fig. 8), WGE induces cell death in roots of all soybean lines regardless of the presence of Rps genes. If we accept these roles in the HR, the fact that WGE-induced cell death does not occur in compatible infected tissues suggests that virulence or pathogenicity functions associated with the pathogen may somehow act to suppress either elicitor release or subsequent function in compatible but not incompatible infections. One plausible mechanism for this is the release of inhibitor proteins by *P. sojae* that inhibit the endoglucanase activity of PR-2 (Ham et al., 1997). These putative functions of virulence genes in suppression of cell death in turn suggest that the interaction of Avr genes from the pathogen with the host Rps genes may counteract this suppression. What do we know about interactions of the soybean Avr/Rps genes that might shed some light?

Two genes involved in avirulence have been identified in the Avr-1b locus of *P. sojae*, which corresponds to resistance conferred by the soybean Rps 1b locus. One of these genes (Avr1b-1) has been sequenced (Shan et al., 2004). Application of a culture filtrate from *Pichia pastoris* (containing expressed Avr1b-1 protein) to soybeans carrying the Rps 1b locus caused a systemic cell death of the entire soybean seedling (Shan et al., 2004). Application of the same Avr1b-1 preparation to soybeans carrying Rps 1k caused a localized necrosis, but had no effect on seedlings of rps lines. It was suggested from these results that Avr1b-1 was an elicitor of HR cell death. In further studies, attempting to link expression of Avr1b-1 to defense gene activation (Valer et al., 2006), it was found that expression of Avr1b-1 was very low in the incompatible interaction at a time (e.g. 3–6 h) when genes for phytoalexin accumulation and PR proteins were already up-regulated. Two hypotheses were put forward to explain these findings. First of all, Avr1b-1 may be active at very low concentrations. Second, another recognition event, occurring before accumulation of Avr1b-1, may complement its action in recognition of the pathogen. Our work, which demonstrates that WGE is in fact an elicitor of cell death, suggests that it may complement Avr-R gene recognition for genes at the Avr 1 and Rps 1 loci. If this is true, it is a very interesting example of a PAMP functioning to trigger cell death that may be conditioned in a race-specific manner by the action of an Avr-R gene interaction.

An interesting question is what potential roles the 5-deoxyisoflavones play in race-specific resistance to other soybean pathogens or in the interactions of *P. sojae* with Rps genes at loci other than Rps 1. While we have not yet studied other soybean pathogens in detail, preliminary gene silencing results suggest that HR resistance to *P. sojae* conferred by some other Rps genes (e.g. Rps 3 and 7) is also mediated by the in situ release of WGE, but may not be mediated by the 5-deoxyisoflavones, suggesting that WGE in combination with other Rps genes can condition different cell death programs. This interesting finding will be described in a future article.

**Working Model for WGE-Induced Cell Death in Elicitor-Treated or Infected Soybean Roots**

The various possible events in WGE-activated cell death discussed in the sections above are schematically outlined in a working model in Figure 10. Further studies are needed to more fully define the nature of the putative interactions between virulence and avirulence gene products from the pathogen and the pathways leading to elicitor release from WGE and subsequent isoflavone-mediated cell death. We also would like to more fully define the relative roles of the various 5-deoxyisoflavones (daidzein and glyceollin) in cell death.

![Figure 10. Working model for regulation of elicited cell death in soybean roots.](image)

Regulation of Cell Death in Soybean Roots

- **WGE**
  - PR-2 RNAi
  - CHR or IFS RNAi
- **Elicitor Active Fragments**
  - PR-2
  - Daidzein/Glyceollin
- **Compatible Interaction**
  - ?
  - Cell Death
- **AOS**

**Silencing of Isoflavonoids Suppresses HR Cell Death**

While this enzyme is constitutively expressed at relatively low levels, elicitor fragments strongly activate further transcription of PR-2 (Graham et al., 2003), amplifying the signaling pathway. Responses to WGE include the induced gene expression of CHR and IFS (M.Y. Graham, unpublished data), daidzein and glyceollin accumulation (Graham and Graham, 1991b), and cell death. Silencing of PR-2 or either IFS or CHR leads to suppression of the induction of daidzein and glyceollin and suppression of cell death in both WGE-treated tissues and incompatible infected tissues. Cell death is hypothesized to require both the accumulation of 5-deoxyisoflavonoids and generation of AOS, which may oxidize the isoflavonoids (e.g. to free radicals) that cause cell death. Although WGE is sufficient to induce cell death in uninfected tissues of all cultivars, in compatible infections we postulate that a virulence factor(s) from *P. sojae* suppresses either elicitor release (e.g. Ham et al., 1997) and/or the cell death process. We further hypothesize that interactions of Avr-1 gene products with their respective Rps 1 gene products, in turn, may act to counteract this suppression of cell death (not shown in the model).
MATERIALS AND METHODS

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any such permissions will be the responsibility of the requestor.

Chemicals

Unless otherwise mentioned, all chemicals were purchased from Sigma Chemical Company. The glucan elicitor was prepared as described below under “Elicitor Treatment of Roots.”

Plant Materials

The soybean ( Glycine max) lines used in this study are near-isogenic lines of ‘Williams’ soybean carrying either no gene for race-specific resistance (‘Williams’) or the Rps 1c and Rps 1k genes for race-specific resistance to Phytophthora sojae (‘W79’ or ‘W82’, respectively). For growth of seedlings, seeds were planted in small plastic flats (5 × 7 × 2 inches, with drainage holes) in Metromix360. The planting medium was first added, leaving three-quarters of an inch of space and the surface evened out. Approximately 150 seeds were spread across the surface of the medium after which the rest of the medium was added (three-quarters of an inch). Plants were grown in a growth chamber under the conditions described previously (Subramanian et al., 2005). The flats are watered thoroughly with one-half-strength Peter’s fertilizer until saturation. The flats were then watered every 2 d with the same nutrients. At day 4, moistened seed coats were removed from all seedlings. Removal of the seed coats is critical to getting large and unblemished cotyledons. Cotyledons are generally ready at day 7, but can also be used at day 8. The cotyledons should be dark green (no yellowing) and firm, but not dense. They should snap in two easily without bending. Normally our seedlings are very uniform and over 90% of the cotyledons are unblemished and at the same stage of maturity. With good seed, we normally get 200 to 300 cotyledons per flat.

Constructs for RNAi Silencing and Transformation of Agrobacterium rhizogenes

The constructs used for silencing IFS and CHR genes have been described elsewhere (Subramanian et al., 2005, 2006). The RNAi construct targeted to the IFS gene (TC214925) was constructed as follows. A 394-bp fragment nearly unique to this contig was amplified by PCR using the primers 5′-TGCCAATCCGGGTATGTTATG-3′ and 5′-AGTGCAAACCTTTACTGCCTCTTC-3′. This fragment was cloned into Ncol-EcoRV digested, Klenow-treated pMH40-ENTR4, a Gateway entry vector, by blunt end cloning (pMH40-ENTR4-925). pMH40-ENTR4 was created by cloning a Nhel-PvuII fragment of pENTR (Invitrogen Corporation) containing the attL1-ccdB-attL2 cassette, into a Sfll (filled in)-SpeI fragment of pMH40 (Jung et al., 2000). A binary vector (pCAMGFP-CvMV:GWi) where a Gateway RNAi (GWi) cassette was driven by a cassava mosaic virus (CvMV) CVP2 promoter was constructed from pCAM-ubiGFP (described in Subramanian et al., 2005). The GiWi cassette was a gift from Dr. Chris Taylor, Donald Danforth Plant Science Center, and contained two attR1-ccdB-attR2 cassettes in opposite orientations separated by intron1 from AtFAD2. The CVP2 promoter was a gift from Dr. Claude Fauquet, Donald Danforth Plant Science Center. A binary vector (pCAMGFP-GmPr2) was constructed by a Gateway LR Clonase reaction between pCAMGFP-CvMV:GWi and pMH40-ENTR4-925.

RNAi binary vector constructs targeting IPS, CHR, or PR2 genes were individually electroporated or cold shock transformed into Agrobacterium rhizogenes K599 cells and transformed cells were selected on Luria-Bertani media agar plates containing kanamycin (50 µg/mL).

Cultures of Transformed A. rhizogenes

A. rhizogenes strain K599 cells, with vector or appropriate RNAi-silencing construct, were grown in 50 mL Erlenmeyer flasks containing 10 mL of Luria-Bertani broth with 50 µg/mL kanamycin. Cultures were started by transferring a small aliquot of bacteria from a frozen 40% glycerol stock to the broth and are then shaken at 160 rpm for 40 h at 25°C. Stationary phase bacterial cells were spun down at low speed for 20 min to obtain a light pink pellet typical of K999. The pellet was drained briefly and gently resuspended (using a 1-mL pipette) to one-half its original volume in 10 mM MgSO4. The OD600 of the cell suspension was adjusted to 0.3 using 10 mM MgSO4. This final suspension was used as soon as possible.

Plant Transformation Protocol

Plant transformation was performed essentially as described elsewhere (Subramanian et al., 2005). Chambers for the cotyledons were made ahead of time and consist of inverted petri plates. A 9-cm diameter circle of sterile Whatman filter paper was placed gradually into a pool of sterile water in a petri dish lid and slowly lowered into place to provide a flat surface. The water was drained from the lid but not shaken out. The petri plate bottom (which is now the top of the chamber) was placed onto the lid to hold the paper and moisture in place.

When the chambers and K999 suspension were both ready, cotyledons were removed from the seedlings by gently twisting them off the seedlings. Cotyledons should be harvested and used in batches of 50 to 60 to avoid too much desiccation during the experiment. The cotyledons were surface sterilized in bulk (50–60 at a time) by washing very briefly (15 s) in 70% ethanol. They were laid out immediately to dry in a single layer, abaxial side up, on sterile filter paper. A sorbent paper, soaking up the ethanol, will lead to some entry of the ethanol into the cotyledon tissues and can change the response. The cotyledons were then surface sterilized once more very briefly with an alcohol swab (moist but not wet with 70% ethanol) immediately before cutting with a sterile razor blade. The swab was squeezed until just moist and the razor can then be wiped immediately before use with the swab to remove excess alcohol. Cutting involved the removal of a circular layer of tissue (approximately 0.4 cm diameter) by making a small, glancing, scalp-like cut near the petiole end. It is important to be near the petiole end (about 0.2 cm away), but to avoid cutting the petiole area per se, since roots emerging from the petiole are more often adventitious. The target of the cutting procedure was to cut horizontally through the major vein. Usually this means a cut that is approximately one-third to one-half of the way through the cotyledon. The vein will be visible after cutting and optimal transformation occurs when the cut is along as much of the vein as possible. The cut cotyledon was placed immediately in the chamber. We put 10 cotyledons per chamber. After all 10 cotyledons were cut, 20 µL of K999 inoculum was pipetted onto each cut surface and the top was put in place. Plates were transferred to the incubation chamber (22°C, 250 µE light on a 12 h photoperiod). The next morning (approximately 18 h later) the plates were sealed with Parafilm. This initial period of more active air exchange increases the efficiency of transformation. At 3 d, the wounded, inoculated cotyledon surfaces were treated with 20 µL 100 µg/mL carbenicillin to kill the A. rhizogenes inoculum. Primary root transformations are complete in 48 to 72 h and treatment with the antibiotics minimizes secondary transformations that can lead to chimeric roots. As described in detail previously (Subramanian et al., 2005), transformed hairy roots were abundant along a callus ridge on the inoculated cotyledons after about 3 weeks. Transformation with the vector control or RNAi constructs was followed by the expression of the GFP marker as noted under “Microscopy.” The transformation frequency was approximately 30% to 50% of the total (hairy and adventitious) roots formed. For infection or elicitor treatment, roots chosen for treatment were also aerial (antigaeotropic), which facilitated treatment of the appropriate root zone.

Infection of Roots

As shown in other soybean tissues, infection of roots by P. sojae leads to different reactions depending on the actual developmental zone inoculated. Much as described at the base of the hypocotyl (Bhattacharyya and Ward, 1986b), we have found that a developmentally age-related resistance occurs at the base of the root. In roots this resistance diminishes as the root tip is approached. In intact roots, zoospore chemotraction and encystment is highly specific to the zone of elongation, just behind the root tip. To achieve the highest consistency we performed point inoculations at this zone. Roots were inoculated with small, 1.5 mm square blocks of agar from young cultures growing on lima bean (Phaseolus lunatus) agar. The agar blocks stayed moist and on the root and led to uniform infections.

Elicitor Treatment of Roots

The intact WGE (Ayers et al., 1976; Graham and Graham, 1991b, 1996; Graham et al., 2003), and enzymatically prereleased elicitor (Yoshikawa et al., 1986b) leads to different reactions depending on the actual developmental zone inoculated. Much as described at the base of the hypocotyl (Bhattacharyya and Ward, 1986b), we have found that a developmentally age-related resistance occurs at the base of the root. In roots this resistance diminishes as the root tip is approached. In intact roots, zoospore chemotraction and encystment is highly specific to the zone of elongation, just behind the root tip. To achieve the highest consistency we performed point inoculations at this zone. Roots were inoculated with small, 1.5 mm square blocks of agar from young cultures growing on lima bean (Phaseolus lunatus) agar. The agar blocks stayed moist and on the root and led to uniform infections.
1981; Abbasi et al., 2003) were prepared as described previously. Unless otherwise noted, WGE was autoclaved at 200 μg/mL for 3 h prior to use to release high molecular mass fragments. For application, elicitors were prepared in sterile 0.15% agar, which was readily pipetteable, but viscous enough to keep the elicitor on the root surface without falling off. Roots were spot treated with elicitors by applying the elicitor as a 5-μL droplet to the same zone as infection (see above).

HPLC and qRT-PCR Analyses

HPLC and quantification of the isoflavonoids were performed as described previously (Graham, 1991). Peak identities were confirmed by retention times against well-established standards and by their UV spectra. qRT-PCRs were carried out as described previously (Subramanian et al., 2006).

Microscopy

White light microscopy was performed on an Olympus SZH dissecting scope with attached digital camera. Fluorescence microscopy was performed on a Nikon Eclipse 80i fluorescence microscope equipped with an EXFO X-Cite 120 fluorescence illumination system, and a RT KE/SE Spot digital imaging system (Diagnostics Instruments). Both GFP and YAF were visualized using the following filter set: a blue excitation filter (450–490 nm), a 500-nm dichroic mirror, and a 515-nm barrier filter.

ACKNOWLEDGMENTS

We would like to acknowledge Dr. Chris Taylor for A. rhizogenes strain K599 and intermediate RNAi vectors, Dr. Claude Faquett for the CvMV promoter, and Dr. Jennifer Zhong for her help in making some of the RNAi constructs. We also wish to thank Ruth Huge for her technical assistance in fluorescence microscopy and for kindly allowing us to use his Nikon epifluorescence scope.

Received February 12, 2007; accepted March 28, 2007; published April 6, 2007.

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