RNA Interference of Soybean Isoflavone Synthase Genes Leads to Silencing in Tissues Distal to the Transformation Site and to Enhanced Susceptibility to *Phytophthora sojae*[1]

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

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

Isoflavones are thought to play diverse roles in plant-microbe interactions and are also potentially important to human nutrition and medicine. Isoflavone synthase (IFS) is a key enzyme for the formation of the isoflavones. Here, we examined the consequences of RNAi silencing of genes for this enzyme in soybean (*Glycine max*). Soybean cotyledon tissues were transformed with *Agrobacterium rhizogenes* carrying an RNAi silencing construct designed to silence expression of both copies of IFS genes. Approximately 50% of emerging roots were transformed with the RNAi construct, and most transformed roots exhibited >95% silencing of isoflavone accumulation. Silencing of IFS was also demonstrated throughout the entire cotyledon (in tissues distal to the transformation site) both by high-performance liquid chromatography analysis of isoflavones and by real-time reverse transcription-PCR. This distal silencing led to a nearly complete suppression of mRNA accumulation for both the IFS1 and IFS2 genes and of isoflavone accumulations induced by wounding or treatment with the cell wall glucan elicitor from *Phytophthora sojae*. Preformed isoflavone conjugates were not reduced in distal tissues, suggesting little turnover of these stored isoflavone pools. Distal silencing was established within just 5 d of transformation and was highly efficient for a 3- to 4-d period, after which it was no longer apparent in most experiments. Silencing of IFS was effective in at least two genotypes and led to enhanced susceptibility to *P. sojae*, disrupting both R gene-mediated resistance in roots and nonrace-specific resistance in cotyledon tissues. The soybean cotyledon system, already a model system for defense signal-response and cell-to-cell signaling, may provide a convenient and effective system for functional analysis of plant genes through gene silencing.

The isoflavones, which are largely restricted to the Papilionoideae (including many of the commercially important legumes such as soybean [*Glycine max*], green beans [*Phaseolus vulgaris*], peas [*Pisum sativum*], and alfalfa [*Medicago sativa*]) play diverse roles in plant-microbe interactions. Isoflavones function as preformed antibiotics (Kramer et al., 1984; Rivera-Vargas et al., 1993) and as precursors for the defense-related coumestan and pterocarpan phytoalexins (Ebel, 1986). They also play roles as signal molecules in the induction of microbial genes involved in infection (Dixon and Sumner, 2003) and symbiosis between soybean and *Bradyrhizobium japonicum* (Pueppke, 1996; Ferguson and Mathesius, 2003). Finally, the isoflavones also have many activities potentially important to human nutrition and medicine, including activities as phytoestrogens, anti-cancer, and anti-oxidant compounds (for review, see Ososki and Kennelly, 2003; Cornwell et al., 2004; Huntley and Ernst, 2004).

In soybean, the major isoflavones include daidzein, genistein, and glycitein. These are the predominant aromatic secondary metabolites in soybean seed and all seedling organs (Graham, 1991a). Other than the presence of relatively high levels of free daidzein in root tissues, the isoflavones in soybean seed and seedling tissues are predominantly present as glucosyl- and malonyl-glucosyl conjugates, the levels of which vary markedly in different developmental stages in various organs (Graham, 1991a). While the isoflavones are normally present at relatively low levels in mature soybean tissues, their accumulation is strongly induced in all tissues in response to pathogen attack or defense elicitors. Even under inducing conditions, the isoflavones often accumulate predominantly in the conjugated forms (Graham and Graham, 1996). The timely release of the active aglycones (e.g. daidzein and genistein) during defense responses is likely under the control of both host (Hsieh and Graham, 2001) and pathogen (Rivera-Vargas et al., 1993) glucosidases. Daidzein is the precursor of both coumestrol and the glyceollin

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* Corresponding author; e-mail graham.1@osu.edu; fax 614–292–4455.

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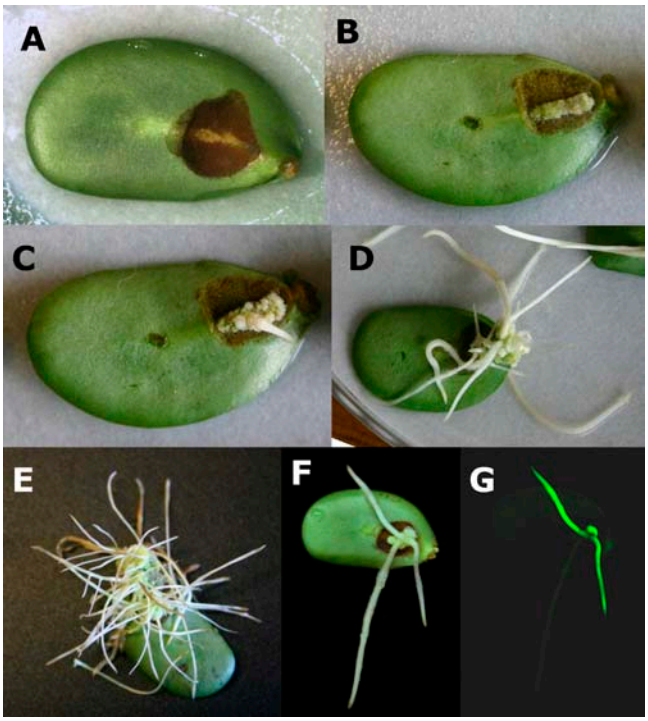

Figure 1. Stages in transformation and hairy root formation on soybean cotyledons. Cotyledon tissues were transformed with *A. rhizogenes* strain K599 as described for the cut cotyledon protocol in ''Materials and Methods.'' Cotyledons are shown at 2 d (A), 6 d (B), 21 d (C), 28 d (D), and 34 d (E) after infection with K599. Also shown are roots of a cotyledon transformed with an IFS RNAi construct at 24 d under white light (F) and long wavelength UV light with a GFP barrier filter (G). The two roots showing the green fluorescence were confirmed by HPLC to be silenced for isoflavone accumulation.

phytoalexins (Ebel, 1986). Genistein may play multiple roles in defense in that it possesses antibiotic activity against several pathogens (Kramer et al., 1984; Rivera-Vargas et al., 1993) and it has been implicated in the establishment of elicitation competence for the glyceollin response (Graham and Graham, 1999, 2000; Graham et al., 2000).

Although soybean has long been one of the plants of choice for chemical and biochemical studies on the isoflavone pathways, it has historically been a difficult plant in which to routinely pursue the types of molecular genetic approaches that are so vital to obtaining genetic confirmation for the importance of key processes or events. This is due to the difficulties in obtaining mutants in this ancient tetraploid plant and the lack of high frequency transformation protocols. Nonetheless, several important improvements have recently been made in molecular genetic tools for soybean. Soybean has a large expressed sequence tag database derived from a diverse set of expression libraries. To complement this, several forms of gene chips are now available that will allow more quantitative and global gene expression analyses. Moreover, important advances in soybean transformation have recently been made. Of particular pertinence to this report, transformation with *Agrobacterium rhizogenes* is both high frequency and genotype independent (Cho et al., 2000), allowing productive avenues to gene overexpression and RNAi gene silencing in transformed root tissues.

Isoflavone synthase (IFS) is the key enzyme in the formation of the isoflavones. It is encoded in soybean by two genes, IFS-1 and IFS-2, which have been cloned and examined in some detail by several groups (Akashi et al., 1999; Steele et al., 1999; Jung et al., 2000; Yu et al., 2000). Gene silencing of IFS is particularly interesting in regard to elaborating its function in defense and symbiosis. Here, employing a high frequency hairy root protocol using cotyledon tissues, we show that the silencing of IFS in roots initiated from cotyledons is highly effective and in addition leads to effective spread of silencing throughout the nontransformed cotyledon tissues. As described in more detail below, this is potentially an important finding since soybean cotyledons have been a useful model organ for the examination of cellular and biochemical processes, including defense responses to various elicitors and the cell-to-cell communication involved in their deployment (Graham and Graham, 1991, 1996).

RESULTS AND DISCUSSION

Transformation of Cotyledons with *A. rhizogenes* and RNAi-Mediated Silencing of IFS in Transgenic Hairy Roots

Figure 1 shows pictures representing the different phases of callus and root growth following transformation of cotyledons with *A. rhizogenes* strain K599.

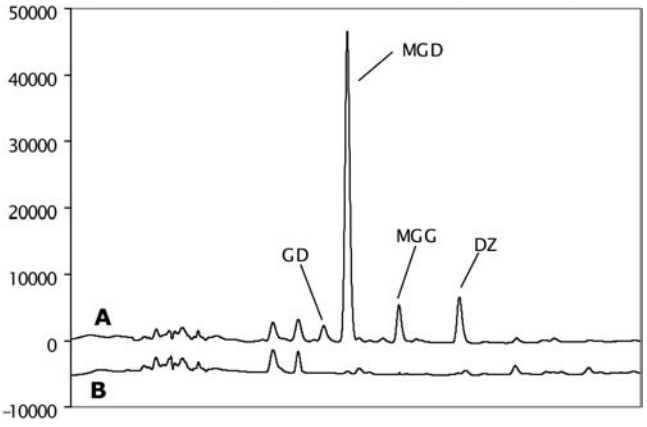

Figure 2. Reduced levels of isoflavone metabolites in roots transformed with the IFS RNAi construct. Individual roots showing GFP fluorescence were harvested from cotyledons transformed with a vector control (A) or the IFS RNAi silencing construct (B) and subjected to HPLC metabolic profiling. Both chromatograms are on the same scale, but the IFS RNAi chromatogram (B) has been offset slightly for clarity. The peaks shown include glucosyl daidzein (GD), malonyl-glucosyl daidzein (MGD), malonyl-glucosyl genistein (MGG), and free daidzein (DZ).

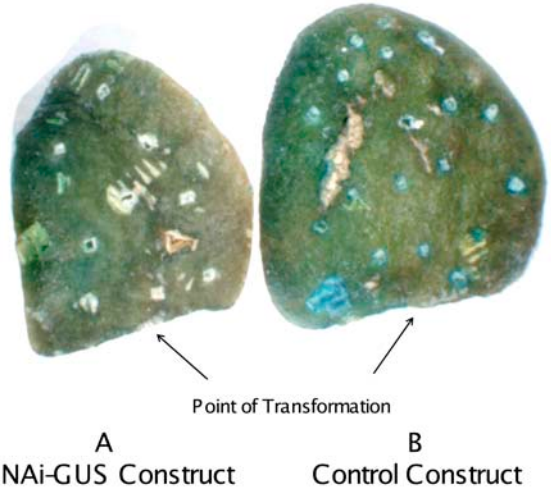

Figure 3. Expression of the IFS1:GUS reporter gene in cotyledons transformed with control and GUS RNAi constructs. Cotyledons from a transgenic soybean line carrying the GUS gene under the control of the IFS1 promoter were transformed with *A. rhizogenes* carrying a GUS RNAi construct (A) or control vector (B) to induce GUS silencing as described in "Materials and Methods." Wounding with a hemostat was used to induce IFS expression. The point of transformation with *A. rhizogenes* is indicated. Tissues were stained for GUS expression 24 h after wounding.

Within 48 h, the inoculated cut surface shows a browning response, except where the major vein has been cut through (Fig. 1A). After just 6 d, a ridge of callus has grown along the severed vein (Fig. 1B), and by 21 d, white friable callus has proliferated along the vein and roots have begun to form (Fig. 1C). By 28 d, abundant roots have formed (Fig. 1D; averaging about 10 roots/cotyledon). In some cases, more than 30 roots can form per cotyledon over time (Fig. 1E).

The presence of vector control or IFS RNAi constructs in K599 did not affect the timing or efficiency of root formation. Transformation of roots with the appropriate vector control or IFS RNAi constructs was monitored by root fluorescence due to a green fluorescent protein (GFP) marker. Of the 3 roots shown in Figure 1F, 2 of them have been transformed by K599 carrying a binary vector containing an IFS RNAi construct and a sUbi:GFP marker located on the same T-DNA (Fig. 1G). The remaining one might have developed as an adventitious root or is transformed by the wild-type Ri plasmid also present in the K599 strain. On average, we observed about 10 roots forming on each cotyledon and approximately 50% of the roots were successfully transformed with our binary vector carrying the IFS RNAi construct. Transformation of roots with the IFS RNAi can be further examined by HPLC metabolic profiling of individual roots. Typical HPLC profiles for vector control and IFS RNAi transformed roots are shown in Figure 2. Of 30 transformed (GFP+) roots screened, 24 showed over 95% reduction in isoflavones when compared to vector controls. Isoflavone levels in the remaining 6 roots were reduced by 60% to 94%. As a quantitative reference point, control levels of the malonyl-glucosyl conjugate of daidzein (MGD in Fig. 2) are as high as 6,000 nmol/g tissue in the root tip (Graham, 1991a). The timing and efficiency of transformation was similar in Williams, Williams 79, Williams 82, and Harosoy lines.

Silencing of β-Glucuronidase Occurs in Tissues Distal to the RNAi Transformation Site in a Transgenic IFS:β-Glucuronidase Reporter Line

Our first attempt at observing possible silencing in cotyledon tissues distal to the site of transformation (nontransformed tissues) was to express a β-glucuronidase (GUS) RNAi silencing construct in a stably transformed soybean line expressing the GUS gene under the control of the IFS1 promoter (Subramanian et al., 2004). Previously, we have shown that IFS mRNA is not expressed in normal cotyledon tissues but is in wounded tissues (M.Y. Graham, unpublished data). Hence, we wounded the transgenic cotyledons by clamping or piercing with the tip of a hemostat to trigger IFS:GUS expression. As expected, GUS expression was seen in the newly wounded regions of the cotyledons treated with the K599 strain carrying a vector control (Fig. 3B). The tan colored areas in Figure 3B were areas where natural scars were present prior to transformation. However, when the cotyledons were treated with the K599 carrying a GUS RNAi construct, the cotyledon did not display observable GUS expression despite wounding (Fig. 3A), suggesting that there was distal silencing after K599 transformation.

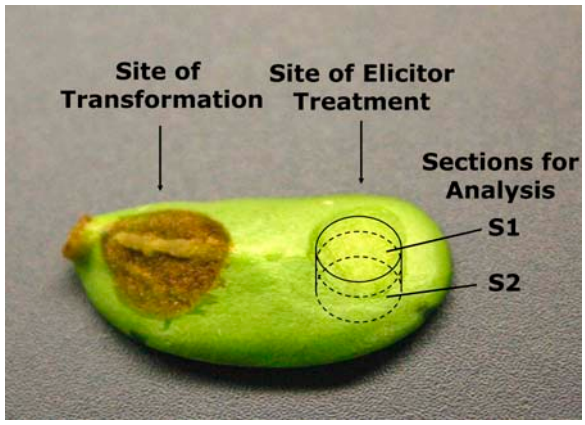

Figure 4. Cut cotyledon protocol used to assess distal silencing. Cotyledons were transformed with control or IFS RNAi constructs at the site of transformation. At various times after transformation (shown is 6 d), a second horizontal cut was made at the distal end of the cotyledon for treatment with either water or elicitor (30 μg mL^{-1} WGE from *P. sojae*). At various times posttreatment, a column of cells was subsequently harvested from the treated area and divided into 2 transverse sections, S1 and S2, for HPLC or real-time RT-PCR analysis.

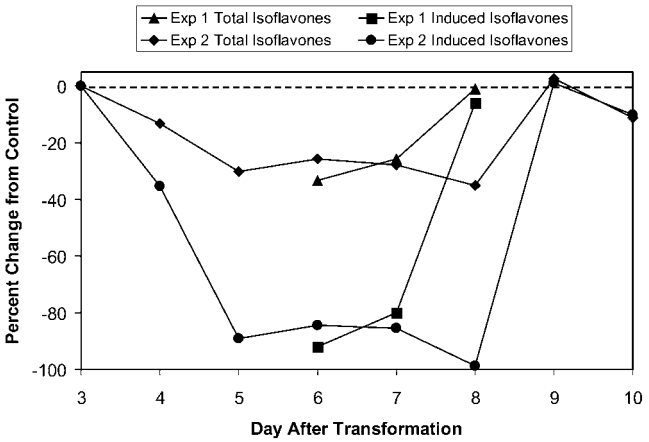

Figure 5. Time course of establishment and duration of distal silencing. Cotyledons were transformed and treated as described in Figure 4. Results shown here are from the S1 section of WGE-treated tissues from 2 experiments. The change in total isoflavones is expressed as the percent change for WGE-treated IFS RNAi transformed tissues as compared to the WGE-treated vector control. The change in induced isoflavones is expressed as the percent that this value represents of the total isoflavones induced by WGE (in the WGE-treated vector control as compared to an untreated vector control).

Distal Silencing of IFS Genes Is Most Efficient 5 to 7 d after Transformation

Our success with distal silencing of the GUS reporter gene prompted us to undertake a more detailed investigation of this form of silencing of the IFS genes in cotyledon tissues. Using the general protocol pictured in Figure 4 and described in ''Materials and Methods,'' a series of 5 time course experiments were performed to first evaluate the timing of establishment of gene silencing in transformed tissues and in nontransformed tissues at various distances from the site of transformation. These included experiments with time points ranging from 4- to 21-d-post K599 infection. In each case, the cotyledons were treated with water or the *Phytophthora sojae* wall glucan elicitor (WGE) at the appropriate time point after infection with *A. rhizogenes* as noted in Figure 4. Three of these time course experiments were performed at Ohio State University using the cut cotyledon protocol shown in Figures 1 and 4. A fourth was performed at the Donald Danforth Plant Science Center using a different cotyledon protocol kindly provided to us by Dr. Chris Taylor (Donald Danforth Plant Science Center, St. Louis). The fifth experiment was performed at Ohio State University using modifications of the vertical multicut procedure (Savka et al., 1990) as described in ''Materials and Methods.'' In all of these experiments, we used HPLC metabolic profiling to monitor the effects of IFS RNAi silencing on responses to wounding alone and to WGE in different sections of nontransformed cotyledon (S1 and S2, Fig. 4). The five experiments were quite consistent with one another in terms of overall observations. Thus, effective distal silencing was observed using three different protocols in two different laboratories. For simplicity, we describe only representative results to illustrate the key observations.

Our initial efforts examined wider time points after transformation. It was clear from these various efforts (over periods of 7–21 d) that distal silencing was most apparent at early time points. In Figure 5, we show results from 2 subsequent time course experiments, in which we attempted to determine a tighter time frame for the phenomenon. These experiments were carried out using the cut cotyledon and vertical multicut protocols over 6 to 8 d and 4 to 10 d, respectively. The results presented are for the effects of silencing on WGE responses in S1. For each experiment, we show the effects of silencing on total isoflavone levels (all daidzein, genistein, and glycitein metabolites) as well as on the isoflavone responses induced in response to WGE (as compared to untreated controls). As can be seen, the distal silencing effect, as measured by the reduction of isoflavone pools, was established within just 5 d after K599 infection. In both experiments, silencing was still apparent at 6 and 7 d, while the results of the 2 experiments varied as to the silencing seen at 8 d. Additional experiments performed at these various time points confirmed that silencing is reproducibly seen for a 3-d period (5–7). At these early time points, just the beginning of a callus ridge was apparent on the transformed cotyledons (illustrated in Figs. 1B and 4). The friable whitish callus typical of root formation was not yet apparent. Thus, distal silencing is established as an early event following transformation before transformed hairy roots emerge.

Importantly, from the HPLC results, the effects of silencing appear to be associated predominantly if not solely with induced responses. While silencing led to only about a 30% to 40% reduction of total isoflavones, it led to an 80% to 100% reduction in WGE-induced isoflavone responses. For quantitative comparisons, the total pools of preformed isoflavones in nonelicited cotyledon tissues were approximately 3,200 nmol/g tissue; WGE treatment induced an additional 900 to 1,300 nmol/g tissue. These results suggest that distal silencing is effective but that the turnover of preformed isoflavones may be relatively slow. This is consistent with results on isoflavone turnover in chickpea (Barz and Hoesel, 1979), which have shown that the isoflavone conjugate pool is quite stable (half lives for various isoflavone conjugates range from 50 to >320 h depending on the tissue).

Table I. *Summary of IFS RNAi silencing of WGE responses in distal tissues over six experiments*

Values are the percent change in total isoflavone levels from the WGE-treated vector control. SES (n = 6) are at the 95% confidence level.

Section	S1	S2
Average	−37.1	−42.0
SE	7.4	5.9

Table II. *Summary of IFS RNAi silencing of WGE-induced responses in distal tissues for various soybean cultivars*

Data are a summary of results for two independent experiments. Values are percent change in total isoflavone levels of WGE-treated tissues from WGE-treated vector controls ± SE ($n = 2$, 95% confidence level).

Cultivar	S1	S2
Williams	−29.2 ± 4.2	−34.9 ± 0.4
Williams 82	−39.3 ± 4.8	−48.6 ± 4.2
Harosoy	−31.0 ± 2.4	−32.7 ± 3.2

The wounding and exposure of the cut cotyledon tissues to light also induces isoflavone accumulation (Graham and Graham, 1996) and accumulation of IFS mRNA (M.Y. Graham, unpublished data). Distal silencing of isoflavone responses in these wounded, light exposed tissues was also effective and silencing of both wound control and WGE-induced responses were seen in both sections S1 and S2. Shown in Table I is a summary of the silencing observed for WGE-induced responses in S1 and S2. Results are presented as the average of 6 independent experiments performed at 6 or 7 d posttransformation using all 3 of the various transformation protocols. The data shown are the percent reduction in total isoflavone pools. Silencing was also independent of the soybean lines examined in these studies. The average percent silencing of total isoflavone pools for 3 soybean cultivars at 7 d posttransformation is shown in Table II. Time course experiments, however, revealed that the timing of establishment and duration of silencing varied within a day or so with the different cultivars (data not shown).

Distal Silencing Leads to Lowered Levels of the Appropriate IFS mRNA Transcripts

If the marked suppression of induced isoflavones seen in the above experiments is truly due to distal posttranscriptional gene silencing, we would expect to see lower levels of IFS mRNAs in elicitor treated tissues. Figure 6 shows the results of real-time reverse transcription (RT)-PCR for WGE-induced responses in section S1 at various times following treatment with WGE. These data are from an experiment where samples were harvested 6 d after transformation. As can be seen, accumulations of mRNA for both IFS1 (Fig. 6A) and IFS2 (Fig. 6B) in vector controls (VG) begin as early as 4 h, peak at 8 h, and are again lower by 16 h. The results closely parallel northern-blot analyses for a similar experiment on nontransformed cotyledons (M.Y. Graham, unpublished data). On the other hand, the peak in mRNA accumulation for both IFS genes is strongly suppressed in the IFS RNAi distally silenced tissues (IG). Levels of mRNA at the 8-h peak are essentially reduced to background levels (175-fold for IFS1 and 130-fold for IFS2). These results are consistent with the nearly total silencing of WGE-induced isoflavone accumulation as seen by HPLC and illustrated in Figure 5. Samples for real-time RT-PCR were also sampled at 8 d from Experiment 1 as summarized in Figure 5. In this case, no reduction in IFS mRNA accumulation was seen (data not shown), consistent with the lack of silencing at 8 d as shown in Figure 5.

The exact molecular mechanism of the observed distal silencing is not clear. Long-distance transport of protein and RNA via phloem has been shown to play major roles in regulating noncell-autonomous development and cellular responses in plants (Voinnet and Baulcombe, 1997). Recently, Yoo et al. (2004) demonstrated that small interfering RNA can enter and move through phloem of several species. Their observation provides a possible explanation of our distal silencing.

Effect of IFS Gene Silencing on Infection by *P. sojae*

We have begun to systematically examine the effects of IFS silencing on the infection of both root and

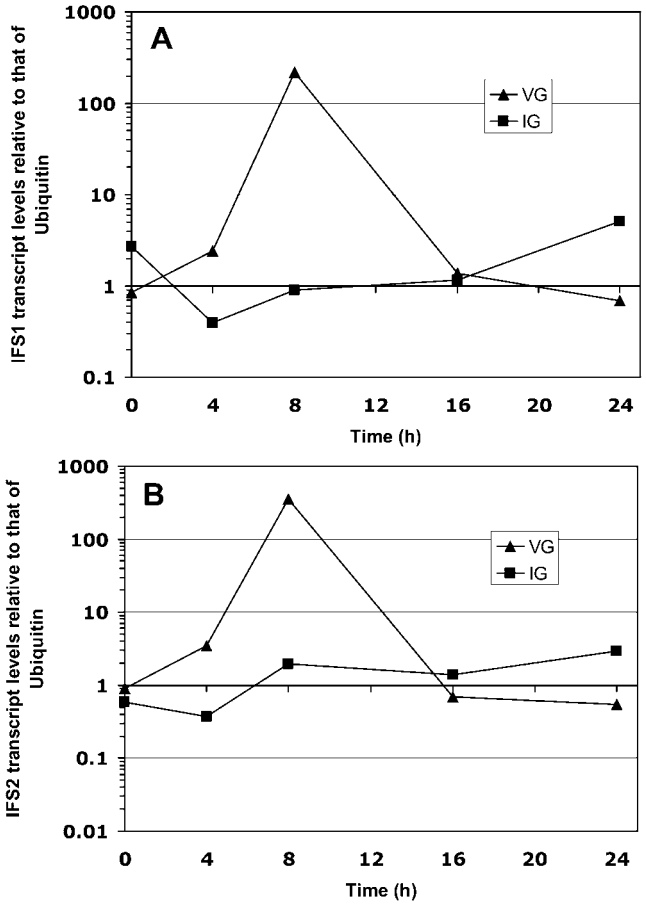

Figure 6. Real-time RT-PCR analyses of IFS expression in control and distally silenced IFS RNAi tissues. Real-time RT-PCR was performed as described in "Materials and Methods" using primer pairs specific for *IFS1* (A) or *IFS2* (B) on samples harvested at the various times shown from cotyledons transformed and treated as described in Figure 4. Data shown are for the S1 section of WGE (30 μg mL^{-1}) treated cotyledons for vector control (VG) or IFS RNAi transformed tissues (IG).

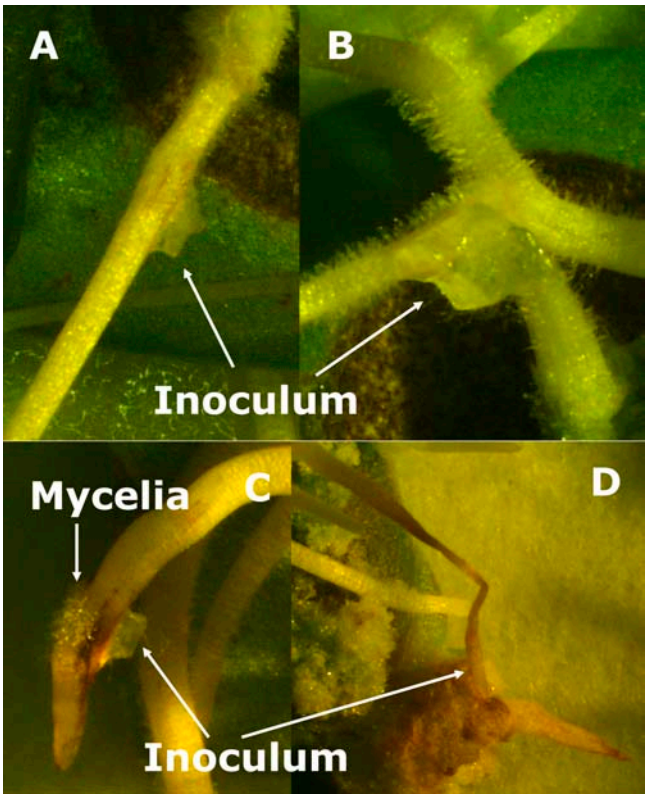

Figure 7. Infection of control and IFS silenced roots of Williams 82 with race 1 of *P. sojae*. Race 1 of *P. sojae* (incompatible on Williams 82) was used to infect vector control (A and B) and IFS RNAi transformed (C and D) roots. Transformed roots were identified by GFP fluorescence before inoculation. Inoculum was applied as a small square agar block (0.2 cm^3) of actively growing mycelium. Pictures were taken 24 h (A and C) and 48 h (B and D) after infection.

cotyledon tissues by *P. sojae*. Here we present some initial data from these studies. In interpreting these results, it is important to remember that silencing of IFS in roots leads to a nearly 100% reduction in total isoflavone pools (Fig. 2) due to their de novo formation during root emergence, while silencing in cotyledons leads to an 80% to 100% reduction only in induced isoflavone responses. As shown in Figure 7, A and B, infection of roots of the cv Williams 82 by race 1 of *P. sojae* normally leads to a resistant response due to the presence of the Rps 1k gene for resistance to race 1. Infection of IFS RNAi silenced root tissues, on the other hand (Fig. 7, C and D), led to a breakdown of resistance. Within 24 h, infection had progressed through the root tissues, with mycelia emerging from the root surface (Fig. 7C) and in 48 h, the root rot in these silenced tissues had progressed down the root (Fig. 7D), and the underlying callus had also begun to rot. Thus, a near total silencing of isoflavone accumulation severely compromised this R gene-mediated resistance in transformed tissues.

The effects of IFS RNAi silencing on *P. sojae* race 1 infection of cotyledon tissues for the cv Williams and cv Harosoy are shown in Figure 8. Williams is universally susceptible to all races of *P. sojae*. Although Harosoy carries the Rps7, it is susceptible to race 1. As can be seen, IFS silencing leads to enhanced susceptibility (rate of lesion spread) for both Williams and Harosoy, although the effect is much stronger in Harosoy. These quantitative differences in enhanced susceptibility of these two cultivars may relate to the phenomenon of partial resistance (a nonrace-specific resistance also called general or field resistance). Williams has high partial resistance, while Harosoy has very low levels of this form of resistance (Thomison et al., 1991; S. St. Martin, personal communication). Thus, induced isoflavone responses may be more important to the inhibition of lesion spread in a low partial resistance background. Infection of Williams 82 gave the expected resistant response (no lesion spread) in both vector control and IFS RNAi transformed tissues. Thus, while infection of susceptible lines can be enhanced in tissues in which induced

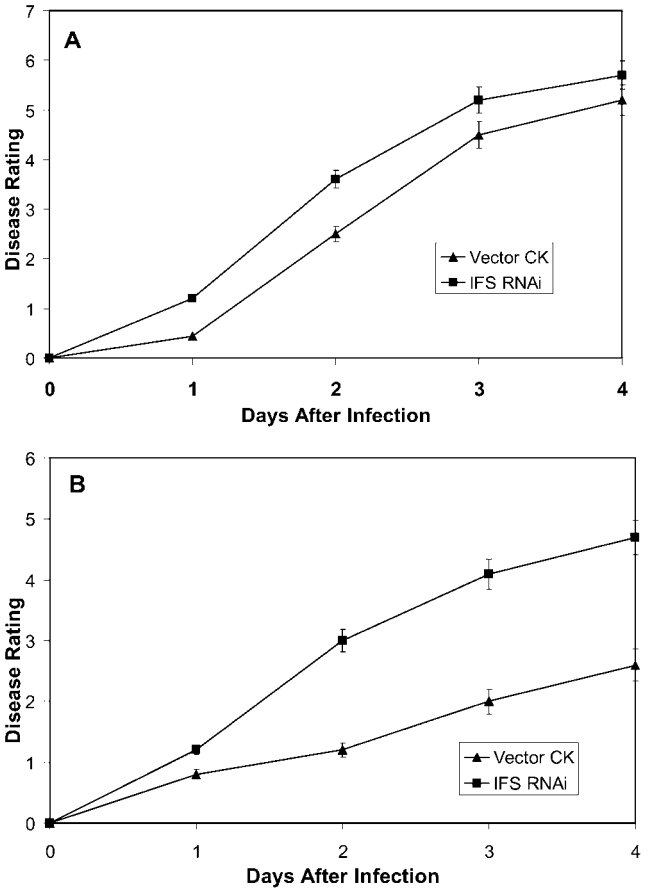

Figure 8. Disease progress curves for infection of control and IFS silenced Williams and Harosoy cotyledons with *P. sojae*. Six days after transformation with vector control or IFS RNAi constructs, cotyledons of cv Williams (A) or cv Harosoy (B) were infected at a site distal to the transformation point (same areas as the zone treated with elicitor in Fig. 4) with a small agar block of actively growing mycelium of Race 1 of *P. sojae*. Both cultivars are susceptible to Race 1, but they differ in their levels of partial resistance. Disease ratings were taken every 24 h. Values are the average disease rating for 20 cotyledons ± SD.

isoflavone responses are compromised, the presence of preformed isoflavone pools or other factors may be adequate to provide race-specific resistance in Williams 82.

These various results support earlier biochemical work with these same cultivars. In an early study (Graham et al., 1990) comparing infections of Williams and Williams 82 with race 1 of *P. sojae*, it was noted that the levels of preformed conjugates of daidzein, the immediate precursor of the phytoalexin glyceollin, in cotyledons are several times those necessary for a median effective dose accumulation of glyceollin and that these conjugates are rapidly hydrolyzed in incompatible Williams 82 (but not compatible Williams) infections to release free daidzein at the infection front. This work is consistent with that reported here in suggesting that preformed conjugates may be sufficient for race-specific resistance conferred by the Rps1k gene. In other work (Park et al., 2002), glyceollin accumulation was found to correlate well to an induced form of lesion-limiting resistance in susceptible soybean lines. This echoes the current study in that inducible isoflavones may play a role in certain quantitative forms of nonrace-specific resistance. Finally, when isoflavone pools are nearly completely suppressed (as in the root studies reported here), all forms of resistance appear to be compromised. Additional silencing studies, employing RNAi silencing of a wider range of defense genes and race-cultivar interactions, are under way.

CONCLUSIONS

In this paper, we have demonstrated that transformation of soybean cotyledon tissues with *A. rhizogenes* carrying an RNAi silencing construct for IFS leads to effective silencing of isoflavones in transformed hairy roots and also to distal silencing of induced isoflavone responses in nontransformed tissues distal to the point of transformation. Silencing of isoflavone levels is >95% in most transformed roots. Such a high degree of silencing is not unexpected in roots due to the fact that the roots are emerging from transformed callus and any isoflavone accumulation that takes place is occurring after posttranscriptional silencing has already been established. However, silencing also leads to an 80% to 100% reduction of induced isoflavone responses (including those to both wounding and treatment with the cell WGE from *P. sojae*) in tissues distal to the transformation site. Real-time RT-PCR data confirm that both IFS1 and IFS2 are nearly completely posttranscriptionally silenced in these distal tissues. Distal silencing is equally effective in at least two genotypes. It is a relatively early event following transformation but is somewhat transient in nature, lasting for a 3- to 4-d period (5–8 d) after transformation. Silencing leads to significant effects on the infection of both roots and distal, nontransformed cotyledon tissues, by *P. sojae*, leading to a breakdown of R gene-mediated race-specific resistance in roots and to increased susceptibility of cotyledon tissues in the experimental conditions under study. These observations provide molecular genetic evidence for the role of isoflavones in soybean disease resistance.

The soybean cotyledon system has long been a model system for the study of induced defense responses to pathogen elicitors and infection (Frank and Paxton, 1971; Ayers et al., 1976; Graham and Graham, 1991, 1996). Due to their size, their rapid availability (7 d postgermination), ease of manipulation, and very simple cellular architecture, soybean cotyledons have many advantages for the analysis of biochemical and cellular aspects of signal-response and cell-to-cell signaling. The demonstration of distal gene silencing in this organ adds a powerful tool for analysis of gene function in soybean. This is particularly important for soybean due to the fact that many of the tools available for other model systems (mutational analysis, routine genetic transformation) have been difficult in this ancient tetraploid plant. However, we believe that the distal silencing of genes in the soybean cotyledon system may provide a useful platform for the analysis of the function of many plant genes, particularly those whose function can be examined over a period of several days or those for which only transient silencing is desired (e.g. genes for which long-term silencing would be lethal or cause other undesired secondary effects).

MATERIALS AND METHODS

Chemicals

Unless otherwise noted, all chemicals were purchased from Sigma Chemical (St. Louis). The WGE from *Phytophthora sojae* was isolated from race 1 of the pathogen using modifications of the methods of Ayers et al. (1976) as described previously (Graham and Graham, 1991). The intact wall glucan was autoclaved at a concentration of 200 μg mL^{-1} for 3 h to release soluble elicitor fragments. Unless otherwise noted, the elicitor was applied to soybean (*Glycine max*) tissues at 30 μg mL^{-1}, a concentration that leads to approximately one-half saturation of response.

Plant Materials

Soybeans L. Merr. were grown in a Conviron Growth Chamber (26°C, 14-h photoperiod, 750 μEs) by spreading 50 mL of soybean seed (approximately 150 seeds) onto a bed of coarse vermiculite in 5- × 7-inch flats. Seeds were covered with an additional three-quarters to 1 inch of vermiculite and the flats watered with one-half strength Peter's solution to run off. Flats were then top watered with this nutrient solution every other day until they were used in various protocols. At day 3 to 4, when the seedlings were just emerging, seed coats were moistened by spraying with water and carefully removed. This is necessary to ensure the highest yield of unblemished cotyledon tissues. Cotyledons were harvested at day 7 by gently twisting them off the hypocotyl. Only unblemished cotyledons were employed for all protocols. One flat of soybean usually yields approximately 200 to 250 usable cotyledons. It is very important that the cotyledons be at the correct developmental stage rather than relying strictly on chronological age. The ideal cotyledons bend only slightly before snapping in two. If they are dark green and dense, they are too young. If they are flaccid or beginning to turn light green to yellow, they are too old.

The GUS transgenic soybean line was generated previously and described in part by Subramanian et al. (2004). GUS activity was assayed as described previously (Subramanian et al., 2004).

Agrobacterium rhizogenes Cultures

A. rhizogenes strain K599 (Savka et al., 1990) was the kind gift of Dr. Chris Taylor. Wild-type or transformed strains were maintained by culturing on yeast extract peptone agar. For transformed strains, all media contained 50 μg mL^{-1} kanamycin. Stock cultures were grown in Luria-Bertani broth and stored long-term in 40% glycerol at −80°C. Cultures for plant inoculation were grown in 10 mL of Luria-Bertani broth (with kanamycin as appropriate) at 25°C for 2 d. Before inoculation, the tubes were spun down at 2,500*g* in a tabletop centrifuge for 20 min or until a relatively tight pellet of the bacteria was obtained. The K599 pellets were drained briefly and then gently resuspended in 10 mM $MgSO_4$ to a final OD_{600} of approximately 0.3 for inoculation of cotyledon tissues.

Construction of IFS and GUS RNAi Vectors and Transformation of *A. rhizogenes*

The RNAi construct used to silence IFS transcripts (IFS RNAi) was constructed as follows. A 521-bp fragment that was more than 99% identical between the IFS1 and IFS2 coding regions was selected and was amplified by PCR using primers containing 2 sets of restriction sites at the 5′ end. The forward primer sequence was 5′-CCCAAGCTTGGTACCTCTGCACTTC-GCTCCCACACC-3′ and the reverse primer sequence was 5′-CCCTCTA-GACTCGAGTGGAGATGGTGCTGTTGGTCC-3′. These PCR products were cloned into CGT2255 (a kind gift from Dr. Chris Taylor) in opposite orientations on either side of a pKANNIBAL (Wesley et al., 2001) intron sequence to create an invert repeat construct driven by the figwort mosaic virus (FMV) promoter (Sanger et al., 1990). This IFS RNAi construct was cloned into pCAM-sUbi:GFP that was obtained by removing the 35S:GUS fragment from pCAMGFPGUS (Subramanian et al., 2004). The newly constructed pCAM-sUbi:GFP-IFS RNAi vector was transformed into *A. rhizogenes* K599 by electroporation.

The RNAi construct used to silence GUS transcript was constructed similarly, with the FMV promoter driving an inverted repeat of 432-bp coding region of GUS gene (*uidA*), separated by pKANNIBAL intron sequence in the CGT2255 vector. The forward primer was 5′-CCCAAGCTTGGTACCCGCG-TCTGTTGACTGGCAGG-3′ and the reverse primer was 5′- CCCTCTAGACT-CATATTCCCGTGCACTTGCGGACGGG-3′. The GUS RNAi fragment was introduced to AKK1426B and the resulting vector used for transformation.

The appropriate vectors without RNAi sequences were used for all control transformations.

Soybean Cotyledon Transformation with *A. rhizogenes*

In most cases, a variant of the classical cut cotyledon elicitor-response protocol (Graham and Graham, 1991) was used. Individual cotyledons were surface sterilized by wiping with an alcohol swab soaked in 70% ethanol. The alcohol swab was rung out slightly before use, so that it was wet but not dripping. The surface sterilized cotyledon was then cut by making a small, roughly circular (0.4-cm diameter) cut about 0.3 cm from the petiole end of the cotyledon. Each cotyledon was then transferred to a petri plate, which was inverted with a 9-cm sterile Whatman Number 1 filter paper placed in the lid to form a wet surface on which the cotyledons were placed. Ten cotyledons were placed in a 2-3-3-2 pattern and the cut surface was then treated with 20 μL of *A. rhizogenes* suspension. Plates were then wrapped in Parafilm and placed in a Percival Incubator at 22°C on a 12-h light cycle. Light levels varied slightly throughout the chamber but averaged about 350 μEs.

Two other protocols were used in some experiments. First was a cotyledon protocol kindly provided by Dr. Chris Taylor (unpublished data). The second protocol was a modification of the vertical multicut cotyledon protocol described by Savka et al. (1990). In this protocol, rather than making a cut parallel to the cotyledon surface, the cotyledons were cut vertically four times at the same point near the petiole end. Cuts were made with a razor blade through a 10-μL droplet of inoculum such that the bacteria were directly introduced into the wound site.

Protocols for Assessment of Distal Silencing

The protocol we used to harvest various sections of the soybean cotyledon to assess the distal silencing of IFS at the metabolic (HPLC) and mRNA levels is a simple modification of the cut cotyledon assay described previously (Graham and Graham, 1991). It is shown pictorially in Figure 4. It entails the application of either water or WGE to a cut surface at a point distal (approximately 0.3–0.4 cm) to the point of transformation with K599 carrying either a control vector or an IFS RNAi construct. At various times after treatment, which depended on the experiment, a column of cells was harvested from the treated region of the cotyledon using a number 3 cork borer. These columns of cells were then cut so that approximately the upper one-third constituted S1 and the lower two-thirds constituted S2 as shown in Figure 4. Because of the many treatments and conditions involved in our experiments and the amounts of tissue required, we did not analyze individual sections but pooled the sections for a given treatment for all 10 replicates within a plate. We have employed this strategy in many experiments and the average SE for HPLC analyses is less than 10% (Graham, 1991b).

HPLC Analyses

HPLC and quantification of the isoflavones were performed as described previously (Graham, 1991b). Peak identities were confirmed by retention times against well-established standards and by their UV spectra.

Real-Time RT-PCR

Quantitative RT-PCR assays for IFS1 and IFS2 were performed essentially as described in Subramanian et al. (2004). The primer pairs used for these assays were synthesized by Integrated DNA Technologies (Coralville, IA) and the sequences were as follows: GmIFS1 (forward, 5′-GTTCCAAGGGCA-CATAGTCTCGTT-3′; reverse, 5′-GCAACTGCGATGGCAAGACACTA-3′); GmIFS2 (forward, CACTGTTCCAAGGGCACATAGTCTTGTC; reverse, GAT-GATACAATGTGCATGGAAGGGC); and GmUbi (forward, 5′-TCTGACAC-CATTGACAATGTG-3′; reverse, 5′-CTTCTGGATGTTGTAGTCAGC-3′).

P. sojae Infection of Cotyledon Tissues

Soybean cotyledons show both partial and race-specific resistance responses to *P. sojae* infection (Graham et al., 1990; Park et al., 2002). *P. sojae* cultures, infection protocols, and soybean disease ratings were as described previously (Graham et al., 1990; Park et al., 2002). In the cotyledon infection studies, the disease rating used was as follows: 0, no observable spread from the point of inoculation; 1, <10% of the cotyledon area was infected; 2, 10% to 30% of the cotyledon infected; 3, 30% to 50% of the cotyledon infected; 4, 50% to 70% of the cotyledon infected; 5, 70% to 90% of the cotyledon infected; and 6, 90% to 100% of the cotyledon infected.

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