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Signaling in Soybean Phenylpropanoid Responses[1]

Dissection of Primary, Secondary, and Conditioning Effects of Light, Wounding, and Elicitor Treatments

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The spatial and temporal deployment of plant defense responses involves a complex interplay of signal events, often resulting in superimposition of signaling processes. We have employed a minimal-wound protocol to clearly separate and characterize the specific contributions of light, wounding, and a wall glucan elicitor preparation (PWG) from *Phytophthora sojae* (Kauf. and Gerde.) to the regulation of phenylpropanoid defense responses in soybean (*Glycine max* L. [Merr.]) cotyledon tissues. The assay also allowed us to clearly reconstitute responses to combinations of these primary signals and to examine the effects of other pathogenesis-related molecules on the responses in a defined manner. Light specifically triggers accumulation of malonylglucosyl conjugates of the 5-hydroxy-isoflavone, genistein, which is normally found in epidermal cells. PWG selectively induces accumulation of conjugates of the 5-deoxy-isoflavone daidzein, the first committed precursor of the phytoalexin glyceollin. Wounding initiates phenolic polymer deposition, a process greatly potentiated by PWG and light. Whereas glutathione selectively enhances light induction of genistein conjugates, methyl jasmonate enhances both light and PWG-induced isoflavone conjugate accumulations. Wound exudate fully activates the cell's capacity (competency) for the phenolic polymer and glyceollin responses to PWG, whereas glutathione partially restores competency, favoring coumestrol and phenolic polymer responses to PWG. Abscisic acid inhibits all induced phenylpropanoid responses.

Infection of soybean (*Glycine max* L. [Merr.]) tissues with an incompatible race of *Phytophthora sojae* induces localized hypersensitive cell death and an array of temporally and spatially coordinated phenylpropanoid defense responses in cells immediately proximal and distal to the hypersensitively dying cells. In tissues within which some cells are artificially killed by simple wounding, PWG induces cellularly coordinated phenylpropanoid defense responses that closely parallel all aspects of those of infected tissues (Graham, 1995). In cells immediately proximal to PWG treatment, the accumulation of the phytoalexin glyceollin and the deposition of phenolic esters and polymers in the cell wall are both stimulated. In distal cells, PWG induces a massive accumulation of isoflavone conjugates.

On the other hand, in predominantly unwounded (injected) or in washed wounded tissues, PWG induces only the accumulation of isoflavone conjugates, the distal cell response. This observation led to the demonstration of the elicitation CFs, which are released into the apoplast from wounded or dying soybean cells and condition immediately adjacent cells to enable the proximal cell responses to elicitor (Graham and Graham, 1994). The state of elicitor competency promoted by these factors is both induced and transient, leading to a short "window" during which cells may express the proximal defense reactions. We hypothesized that a similar state of elicitor competency is programmed in infected tissues through the release of related factors from hypersensitively dying cells. Consistent with this hypothesis, washings from hypersensitive lesions also induce elicitor competency (Graham and Graham, 1994).

In addition to the processes initiated by wounding and elicitor treatment, light has profound effects on the various phenylpropanoid responses outlined above (see Ward and Buzzel, 1983; Graham and Graham, 1991a, 1991b). Thus, the regulation of plant defense responses involves a very complex interplay of signaling processes. To effectively study the signal transduction events associated with any one of these signaling processes and to determine the relative contributions of each in infected tissues requires that one be able to clearly separate the signal-response cascades associated with each and, if possible, reconstitute combinations of the signals against a clean background.

Much of our research on the multiplicity and coordination of cellular defense responses to elicitor has been done using modifications (Graham and Graham, 1991b) of the classical cut-cotyledon assay (Frank and Paxton, 1971). In this assay, the nonpenetrable surface of soybean cotyledons is sliced off to facilitate the application of wall glucan elicitors. This wound assay also led to the initial character-

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Abbreviations: CF, competency factor; ED_{50}, median effective dose; MGD, 6″-malonyl-7-*O*-glucosyl daidzein; MGG, 6″-malonyl-7-*O*-glucosyl genistein; PSE, polymer-specific elicitor; PWG, *P. sojae* cell-wall glucan elicitor.

ization of the competency phenomenon. The competent cell state can be largely abolished by washing off the wound exudate and it can then be restored in a dose-responsive manner by adding fresh wound exudate from wounded tissues back to the washed cells (Graham and Graham, 1994).

However, the washed cotyledon assay has several limitations with regard to further characterization of the complex interplay of signal processes alluded to above. Most importantly, washing the wound exudate from the cut surface is very laborious and cannot always be done thoroughly, sometimes leaving a low-level "residual" wound background to complicate the interpretation of results. Second, very rapid or short-term responses initiated by wounding may already have been triggered before washing is completed. To overcome some of these limitations, we looked into the development of alternative assays. In the process we discovered that simple exposure of subepidermal cells by snapping soybean cotyledons causes remarkably little cell damage. Although the freshly exposed cells are responsive to external signals such as PWG and light, they do not display wound-related responses, thus providing a very clean minimal-wound background. Importantly, wound exudate fully reconstitutes all aspects of wound-associated elicitor competency to these cells. Moreover, the assay is much more reproducible than the washed-cotyledon assay.

As reported below, the snapped-cotyledon assay has allowed us for the first time to very clearly differentiate the separate primary effects of light, wounding, and PWG in planta and to reconstitute responses to combinations of these primary signals. Using the assay, we have also been able to clearly define the effects of various known wound- and pathogenesis-associated signal molecules and growth regulators on these various primary responses in a minimally wounded background. These experiments have provided us with valuable new clues concerning the regulation of phenylpropanoid responses and elicitor competency.

MATERIALS AND METHODS

Chemicals and Elicitor Preparations

Unless noted otherwise, all chemicals were reagent grade and obtained from Sigma or Aldrich. PWG was prepared from a race-1 isolate as described previously (Graham and Graham, 1991b). This cell-wall glucan preparation has carbohydrate composition and linkage nearly identical to that reported originally by Ayers et al. (1976b). Methyl jasmonate was obtained from Bedoukian Research (Danbury, CT).

Cotyledon Assays

The cut- and washed-cotyledon assays were performed as described previously (Graham and Graham, 1991b, 1994). The cut-cotyledon assay is a modification of the classical wound assay for examination of elicitors (Frank and Paxton, 1971). The washed-cotyledon assay is identical except that the wound-associated CFs are removed prior to elicitor application.

The snapped-cotyledon assay is a minimal-wound assay, allowing the treatment of intact, freshly exposed subepidermal cells to elicitor preparations. This assay was performed as follows. Soybean (*Glycine max* L. [Merr.]) seedlings (cv Williams) were grown as described previously from freshly harvested seed (Graham and Graham, 1994) except that they were grown in Metromix 360 (Grace-Sierra, Milpitas, CA) instead of vermiculite. Cotyledons from 7- to 8-d-old seedlings were removed from the seedlings by gently twisting them off the hypocotyl. Only unblemished cotyledons from seedlings of the same developmental state were collected. Once excised, the cotyledons were used as soon as possible to avoid desiccation. Each individual cotyledon was snapped into two pieces at a point one-third of its length away from the point of attachment to the hypocotyl. This smaller section was then placed immediately, petiole side down and broken-surface side up, into a Petri plate containing 5% water agar at a depth of 5 mm. At this concentration and depth the cotyledons are firmly supported with little resistance to their insertion. The cotyledons were arranged 20 to a plate (Fig. 1).

The exposed surface of each cotyledon (10 cotyledons per treatment) was treated as soon as possible with 12 μL of test solution. To facilitate application, the droplet was drawn across the surface as it was released from the micropipet, taking care not to touch the cell surface with the pipet tip. At the appropriate time (48 h if not noted otherwise), the cotyledons were removed and the uppermost 0.5 mm was sliced off to provide the proximal cell layer. Serial sections of 0.5 mm each were harvested next, if desired, to provide distal-cell populations at increasing distances from the point of elicitor treatment. The cell layers from the 10 cotyledons were then pooled for each treatment and weighed directly into microfuge tubes. These sections were then either extracted and analyzed immediately or stored intact at −80°C for later extraction. In those cases where snapped cotyledons were treated with wound exudate, wound exudate was freshly collected from cut cotyledons as described previously (Graham and Graham, 1994). The high-speed pellet from this exudate, used in some experiments, was collected at 13,000*g*.

All assays were carried out at 25°C in continuous light (150 μE m^{-2} s^{-1}) unless otherwise noted. Extraction and analysis of soluble phenolics by HPLC and phenolic polymers by the thioglycolic-acid assay were accomplished as described previously (Graham, 1991a; Graham and Graham, 1991a). In all assays, each test molecule or condition was examined individually or in combination with PWG. The concentration of PWG used in combination with any given effector is that which stimulated a half-maximal glyceollin response in the cut cotyledon assay under the specific conditions used. Choosing this concentration allowed us to readily detect both inhibitory and stimulatory effects of the effector on elicitation or competency.

Microscopy

Microscopic examinations of the snapped-cotyledon surface were carried out by direct examination in the Petri plate at 20 to 120 power using an Olympus SZH zoom

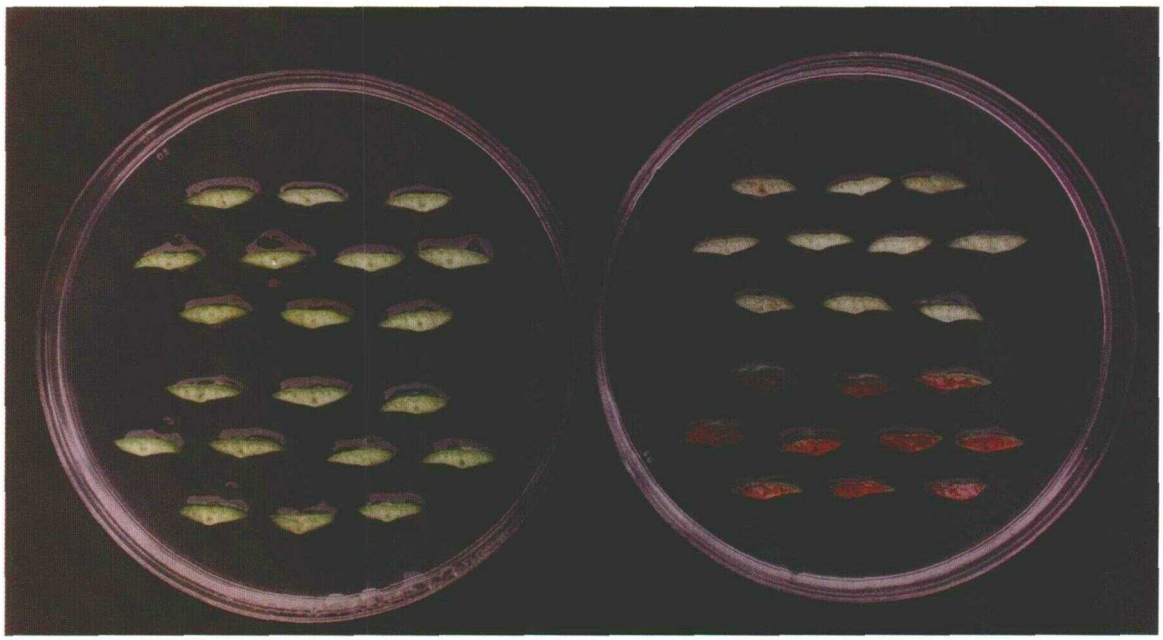

Figure 1. Formation of intensely red-colored derivatives of 3,6a,9-trihydroxypterocarpan in the snapped-cotyledon assay at 20 h. Cotyledons were treated with water (left plate, upper three rows), three cotyledon equivalents of wound exudate (left plate, lower three rows), glucan elicitor at 30 μg/mL (right plate, upper three rows), or wound exudate followed by elicitor (right plate, lower three rows).

stereomicroscope equipped with a Flexilux 90 HLU ringlight. Alternatively, cells were examined with a Nikon compound microscope at 100 and 200 power after slicing off the proximal layer as described above. Histological examinations were facilitated by staining with toluidine blue and the vital stain Evans blue (Jensen, 1962). To quantitate cell damage, random subfields of Evans blue-stained material were examined with the stereomicroscope at 100 power using an ocular in which each field was divided into a grid of 16 subfields, each containing approximately 10 cells.

RESULTS

Demonstration of the Separate Primary Phenylpropanoid Responses to Light, Wounding, and Elicitor Using the Snapped-Cotyledon Assay

Histological Evidence of Minimal Wounding in Snapped Cotyledons

To confirm the lack of substantial cell damage in the snapped cotyledons, the exposed surface was examined microscopically immediately after snapping and daily for 4 d. Cells were examined before and after staining with toluidine blue and the vital stain Evans blue.

The toluidine blue stain confirmed that nearly all of the cells on the surface were turgid and possessed intact cell walls. The process of snapping the crisp cotyledon tissues thus appeared to cleanly separate the cells along the intercellular spaces. The only immediately apparent damage that was detected were the openings associated with the few vascular elements at the point of severing and an occasional dead cell on some cotyledons. The surface cells did not dry out and remained fully turgid and viable for up to 4 d, suggesting that sufficient water is taken from the water agar through the petiole to bathe these cells with at least a thin film of extracellular fluid.

To further monitor cell damage, cell death was quantitated after staining with Evans blue as described in "Materials and Methods." In an examination of 100 random subfields from 50 individual cotyledons (16,000 cells), cell damage was determined to be 0.89% (+ 0.04% SE, $n = 100$). Qualitatively, the greatest cell damage was associated with the vascular elements and the edges of the cotyledon.

A typical example of the snapped-cotyledon assay is shown in Figure 1. The lack of browning of control tissues is clearly apparent, as are the dramatic changes associated with reconstitution of elicitor competency with wound exudate from cut donor cotyledons. The specific responses illustrated in this figure are discussed in more detail below.

Wounding Specifically Initiates the Deposition of Phenolic Polymers into the Cell Wall

A response initiated by wounding and strongly potentiated in the presence of PWG is tissue browning and the accompanying oxidative polymerization of phenolics into the cell wall. This response is conveniently followed in the cut-cotyledon assay by the thioglycolic-acid assay. It includes, but is probably not limited to, peroxidase-mediated deposition of both lignin- and suberin-like polymers (Graham and Graham, 1991a). Based on these results, PWG could either alter a primary response to wounding or act as an alternative and synergistic inducer. Applying PWG in the minimal-wound snapped-cotyledon assay should allow us to differentiate these two possibilities. We also wished

to determine if wounding released an elicitor for the phenolic polymer response and if this could stimulate phenolic polymer deposition in snapped cotyledons.

In the cut-cotyledon assay, PWG greatly enhances the rate of wound-initiated phenolic polymer deposition but not the final magnitude of the response. Thus, in the presence of PWG, phenolic polymer deposition is 10 times that in wounded controls within just 4 h, but at 24 h the maximal response (approximately 12 mg thioglycolic acid derivatives/g tissue) is similar in both cases (Graham and Graham, 1991a). The phenolic polymer responses of untreated and PWG-treated tissues in snapped cotyledons is shown in Figure 2A. Compared with cut cotyledons, maximal deposition of phenolic polymers is greatly diminished (approximately 0.4 mg thioglycolic acid derivatives/g tissue by 48 h). Staining with phloroglucinol (data not shown) suggests that the slight increase in phenolic polymers seen in both cases correlates specifically to the few severed vascular elements and damaged cells. Importantly, PWG causes little if any enhancement of phenolic polymer deposition in snapped cotyledons (Fig. 2A).

These various results from the cut- and snapped-cotyledon assays suggest that host-derived and wound-released phenolic polymer elicitor(s) might exist. Fractionation of wound exudates has demonstrated such an elicitor activity, which triggers phenolic polymer deposition even in the snapped-cotyledon assay. Preliminary characterization of this elicitor activity suggest that it is proteinaceous, heat labile, and associated with a high-speed centrifugal pellet of freshly prepared wound exudate. Attempts to solubilize and further characterize the elicitor(s) are underway.

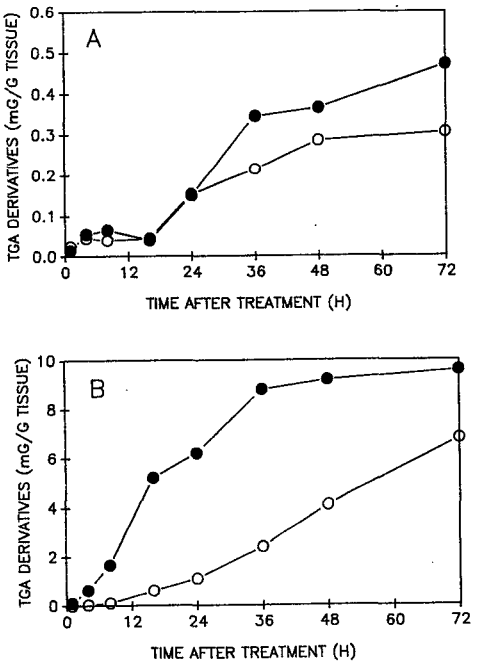

Figure 2. Responses of the snapped-cotyledon assay upon treatment of cells with glucan elicitor. A, Phenolic polymer deposition was measured in proximal cell layers at the times indicated in the snapped-cotyledon assay in the presence (●) or absence (○) of wall glucan elicitor at 30 μg/mL. Data points are the average of two experiments. SE, $n = 2$, was <12% of the mean for all data points. B, Alternatively, phenolic polymer deposition was measured using the same treatments in the presence of three cotyledon equivalents of a high-speed centrifugal pellet from wounded tissues containing the polymer-specific elicitor. SE, $n = 2$, was <9% of the mean for all data points. TGA, Thioglycolic acid.

Reconstitution of the phenolic polymer responses in snapped cotyledons in the presence of this elicitor activity (with and without PWG) is shown in Figure 2B. Both the magnitude and the rate of the response are greatly stimulated. These data compare well with those in the cut-cotyledon assay (Graham and Graham, 1991a), suggesting that all aspects of phenolic polymer deposition can be fully reconstituted in the snapped-cotyledon assay by supplying exogenous wound-released phenolic polymer elicitor(s).

Thus, phenolic polymer deposition is specifically initiated by a host-derived, wound-associated elicitor(s). The rate of polymer deposition is greatly enhanced in the presence of PWG.

Light Selectively Induces the Malonylglucosyl Conjugate of the 5-Hydroxy-Isoflavone Genistein

Light has particularly strong effects on the elicitation of the various phenylpropanoid responses in soybean cotyledons. It markedly enhances the phenolic polymer responses to wounding or wound/PWG treatment and enhances both the glyceollin (proximal) and the isoflavone (distal) responses to PWG (Graham, 1995). However, using the classical cotyledon assay, the effects of light are superimposed on those of wounding, and its independent effects, if any, have not been evaluated. This is critical information if we are to unravel the independent signaling processes involved in these various responses.

In the snapped-cotyledon assay, large accumulations of the malonylglucosyl conjugate of genistein are induced by simple exposure of the subepidermal cells to the light (Fig. 3A). This net accumulation is not seen in the dark (data not shown). It is interesting that light stimulates little accumulation of the conjugates of daidzein (Fig. 3A), although it does strongly enhance PWG-induced accumulation of these conjugates (data presented below). Moreover, there are no other detectable changes in the HPLC profiles of these tissues. Thus, simple exposure of subepidermal cells to light triggers a net and highly selective accumulation of the malonylglucosyl conjugate of genistein.

Under Minimal-Wound Conditions, PWG Selectively Induces the Accumulation of Conjugates of the 5-Deoxy-Isoflavone Daidzein

As noted in the introduction, the effects of PWG in wounded tissues are very complex, including accumulations of conjugates of both daidzein and genistein, glyceollin and phenolic polymers. To effectively study PWG-mediated signaling processes it is critical to determine which if any of these responses are specifically triggered by the glucan. In dramatic contrast to its effects in wounded tissue, when PWG alone is applied to snapped cotyledons, accumulations of the malonylglucosyl (Fig. 3B) and glu-

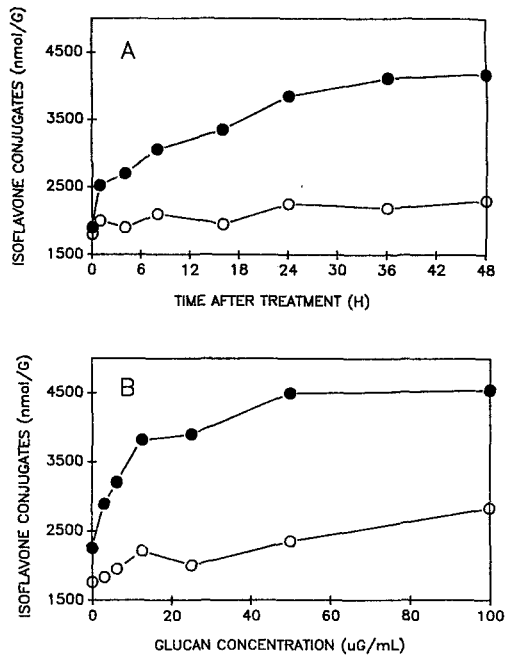

Figure 3. Effects of the simple exposure to light or the wall glucan elicitor in the snapped-cotyledon assay. A, The accumulations of the malonylglucosyl conjugates of genistein (●) and daidzein (○) were determined by HPLC in the proximal cell layers at various times after the exposure of cells in the snapped-cotyledon assay to continuous light. Data points are the averages of two experiments. SE, $n = 2$, was <6% of the mean for all data points. B, Alternatively, the malonylglucosyl conjugate of the isoflavone daidzein was determined at 48 h in proximal cell layers in the snapped-cotyledon assay at various concentrations of the glucan elicitor in continuous light (●) or dark (○). Data points are the averages of two experiments. SE, $n = 2$, was <7% of the mean for all data points.

cosyl (data not shown) conjugates of daidzein were selectively induced. To our knowledge, this is the first demonstration of such highly selective effects of the *P. sojae* wall glucan. The PWG-induced accumulation of daidzein conjugates is much greater in the light than in the dark (Fig. 3B). However, a stoichiometrically equivalent amount of free daidzein accumulates in the dark (data not shown), suggesting that, although the glucan elicitor alone is sufficient for daidzein accumulation, light may be required for effective conjugation. In contrast, PWG causes no accumulation of genistein or its conjugates in the dark, and PWG enhances light-induced genistein conjugate levels by less than 15% (data not shown).

Thus, under minimal-wound conditions, the glucan elicitor is sufficient for and selective in stimulating the accumulation of conjugates of the 5-deoxy-isoflavone daidzein, the first committed precursor of glyceollin. Moreover, although light and PWG are both primary signals in stimulating isoflavone accumulation, they are highly selective signals and the end products are distinctly different.

In the Presence of Wound Exudate, PWG Induces the Sequential Accumulation of Pterocarpans and Glyceollin

The requirement of wound-associated factors for the proximal cell responses (phenolic polymer deposition and glyceollin accumulation) to elicitor was based on washing off and restoring these wound factors to the cut cotyledon assay (Graham and Graham, 1994). Due to the potential lack of complete removal of wound factors in this assay, it is critical to confirm and complement these results by examining the effects of the elicitor and wound exudates under conditions of minimal wounding. The lack of the phenolic polymer response of the snapped-cotyledon assay to PWG and its reconstitution after treatment with a proteinaceous factor from wound exudate, were discussed above. As shown in Figure 4, application of PWG alone to snapped cotyledons also results in little to no elicitation of glyceollin. On the other hand, application of PWG in the presence of increasing amounts of wound exudate from donor cut cotyledons dramatically enhances the glyceollin response in snapped cotyledons (Fig. 4). Reconstitution of competency requires slightly more wound exudate than has been reported for the washed-cotyledon assay (Graham and Graham, 1994). This may be due to the difficulty in completely washing wound factors from the cut-cotyledon assay or it could relate to the number of cells exposed to elicitor. Based on the exposed surfaces and cell arrangement, we estimate that there are potentially 12 times as many cells responding in the cut assay.

The elicitation of glyceollin in the light in the classical cut-cotyledon assay is nearly always accompanied by the transient accumulation of intensely red-colored pigments that are spontaneously formed chemical derivatives of the 3,6a,9-trihydroxypterocarpans (Zahringer et al., 1981). The pterocarpans that have been shown to form these derivatives include 3,6a,9-trihydroxypterocarpan (also known as glycinol) and the isoprenylated compounds 4-dimethylallyl-3,6a,9-trihydroxypterocarpan (also known as glyceollidin I) and 2-dimethylallyl-3,6a,9-trihydroxypterocarpan (also known as glyceollidin II). Glycinol and glyceollidin I are precursors of glyceollin. Glyceollidin II can also be elicited in soybean leaves treated with sodium iodoacetate or *Pseudomonas syringae* bv *pisi* (Ingham et al., 1981). These same red pigments are responsible for the bright-red coloration of race-specific incompatible interactions of soybean with *P. sojae*.

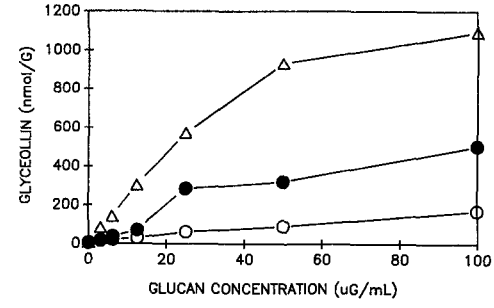

Figure 4. Effect of wound exudate on the glyceollin response to wall glucan elicitor. The glyceollin response to the elicitor was determined in the absence of wound exudate (○) or in the presence of either one (●) or three (△) cut-cotyledon equivalents of wound exudate. Data points are the averages of two experiments. SE, $n = 2$, was <9% of the mean for all data points.

Figure 1 (lower right) illustrates the clear reconstitution of this response in the snapped-cotyledon assay when wound exudate is applied immediately before the glucan elicitor. The identity of the red pigments as derivatives of glycinol and glyceollidin I and their transient formation were confirmed by HPLC profiling (Graham, 1991a) with time and by UV spectroscopy and MS according to Zahringer et al. (1981). Thus, all aspects of the cut-cotyledon response to elicitor and of the competency phenomenon are reconstituted in the snapped-cotyledon assay in the presence of wound exudate, including the early formation of the trihydroxypterocarpans and the later accumulation of glyceollin as quantified in Figure 4.

Effects of Wound- and Pathogenesis-Associated Molecules on Cellular Response

In the previous section, we described the primary signals controlling phenylpropanoid responses in soybean cotyledon tissues and the reconstitution of responses to combinations of these signals. As a complementary approach toward better delineating this interesting interaction of signals, we have investigated the effects of various known wound- and pathogenesis-associated signal molecules and growth regulators on the various responses outlined above. Our goal was to seek some insight into the possible relationship of the competency phenomenon to other signaling processes and to identify possible candidates for the CFs or messengers involved in their action.

In initial studies, a number of molecules were examined in the cut- and washed-cotyledon assays at 100 and 500 μM. The molecules tested included ABA, glutathione, methyl jasmonate, oxalic acid, salicylic acid, ethephon, ACC, *N*-methyl nicotinic acid (trigonellin), and traumatic acid. Of these molecules only ABA, methyl jasmonate, and glutathione had significant and consistent effects on the various soluble phenylpropanoid or phenolic polymer responses. These were thus chosen for more detailed examination in the snapped-cotyledon assay as described here. Each effector was examined in dose responses in the light and dark and in the presence and absence of PWG elicitor. Moreover, both soluble phenolics and phenolic polymers were measured in all experiments. The data presented here were chosen to best illustrate the key activities of each effector (some data are not shown but are described in the text).

ABA

As demonstrated above, the effects of light and elicitor can be particularly clearly differentiated in the snapped-cotyledon assay. As shown using a nonlinear scale in Figure 5A, when the classical wound hormone ABA is included in the assay, it acts as a particularly potent inhibitor of light-induced malonylglucosyl genistein formation. It is suppressive at concentrations as low as 0.3 μM (4.5 pmol/cotyledon), with an ED_{50} of approximately 1 μM (Fig. 5A). In wall glucan-treated tissues, ABA strongly suppresses the glucan-induced accumulation of MGD in the light (Fig. 5A). The dashed lines in Figure 5A represent the preformed pools of genistein and daidzein conjugates present in freshly harvested and untreated control tissues. These values are included to illustrate the fact that, although it completely suppresses the light- and glucan-induced responses, ABA apparently does not affect the preformed pools of genistein or daidzein conjugates over the time course of these experiments. Thus, ABA's effect appears to be a marked and general suppression of de novo isoflavone synthesis.

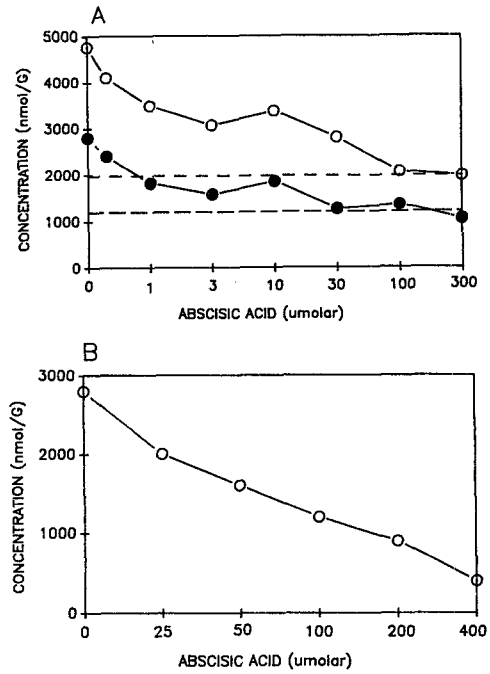

Figure 5. Effects of ABA on light- and wall glucan elicitor-induced responses at 48 h in the snapped-cotyledon assay. A, Accumulations of the malonylglucosyl conjugate of genistein (MGG, ○) or the malonylglucosyl conjugate of daidzein (MGD, ●) were followed in the uppermost cell layers of the snapped-cotyledon assay upon simple exposure of cells to the light (○) or upon exposure to 30 μg/mL of the glucan elicitor (●). The dashed lines represent the constitutive (noninduced) levels of MGG (– – –) and MGD (— — —). Data represent the mean of three experiments. SE, $n = 3$, was <7% of the mean for all data points. B, Alternatively, accumulation of glyceollin (○) was determined in the snapped-cotyledon assay in the presence of 30 μg/mL wall glucan elicitor and three cut-cotyledon equivalents of wound exudate. SE, $n = 2$, was <12% for all data points.

In the snapped-cotyledon assay in the presence of elicitor and wound exudate, ABA suppresses glyceollin accumulation as well, although its effects are much less potent (Fig. 5B). In fact, complete suppression of glyceollin accumulation is not seen, even at 400 μM. Taken together, these data are consistent with a primary effect of ABA on early events in isoflavone synthesis. The fact that glyceollin synthesis is not as effectively inhibited may be due to the presence of preformed pools of daidzein conjugates in these tissues and their possible deployment for glyceollin synthesis (Graham, 1995).

Methyl Jasmonate

Jasmonic acid has also been associated with wound responses (Reinbothe et al., 1994). When applied at concentrations as low as 3 μM (45 pmol/cotyledon), methyl jasmonate strongly enhances the light-induced accumulation of malonylglucosyl genistein (Fig. 6). In the presence of the glucan elicitor, these same levels of methyl jasmonate selectively enhance PWG-induced accumulation of malonylglucosyl daidzein. At substantially higher concentrations (above 50 μM), it inhibits glucan-induced glyceollin accumulation in wounded tissues (data not shown).

Methyl jasmonate alone (in the absence of elicitor or light) had little effect on isoflavone levels. Thus, the primary effects of methyl jasmonate appear to be a strong potentiation of the isoflavone responses initiated by light or glucan in unwounded tissues.

Glutathione

As summarized in "Discussion," glutathione has been implicated as a potential defense signal. Glutathione is present at millimolar concentrations in the cytoplasm (Meister and Anderson, 1983). When cells are wounded or cut through, as they are in the cut-cotyledon assay, a rapid release of intracellular glutathione stores might result and affect signaling processes in the apoplast. The effects of glutathione in the snapped-cotyledon assay are shown in Figure 7. The effect of glutathione alone in this assay is to strongly and selectively enhance the light-induced accumulation of malonylglucosyl genistein (Fig. 7A). Glutathione does not induce genistein conjugate accumulation in the dark, which demonstrates that its effects are stimulatory in nature. When PWG is also present, however, glutathione strongly enhances the accumulation of coumestrol but has very little net effect on daidzein, genistein, or glyceollin accumulations (Fig. 7B).

When applied under conditions in which the wound-associated phenolic polymer elicitor(s) is present (exemplified in the cut cotyledon assay, Fig. 8A), glutathione alone greatly enhances phenolic polymer deposition. In fact, this enhancement by glutathione is even greater than that observed with the glucan elicitor (Graham and Graham, 1991a). Glutathione has little effect on phenolic polymer accumulation in the snapped-cotyledon assay (in the absence of wound exudate; data not shown). Thus, there appears to be a specific interaction between glutathione and the wound-stimulated accumulation of phenolic polymers.

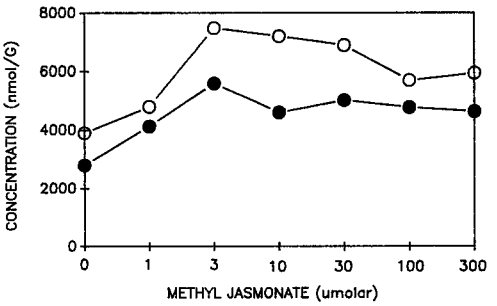

Figure 6. Effects of methyl jasmonate on MGD and MGG accumulation in the snapped-cotyledon assay. Concentrations of the malonylglucosyl conjugates of genistein (○) or daidzein (●) were determined, respectively, in the uppermost cell layers at 48 h after treatment of tissues with methyl jasmonate alone in the light (○) or immediately prior to 30 μg/mL glucan elicitor (●) in the light. Data represent the means of three experiments. SE, $n = 3$, was <6% of the mean for all data points.

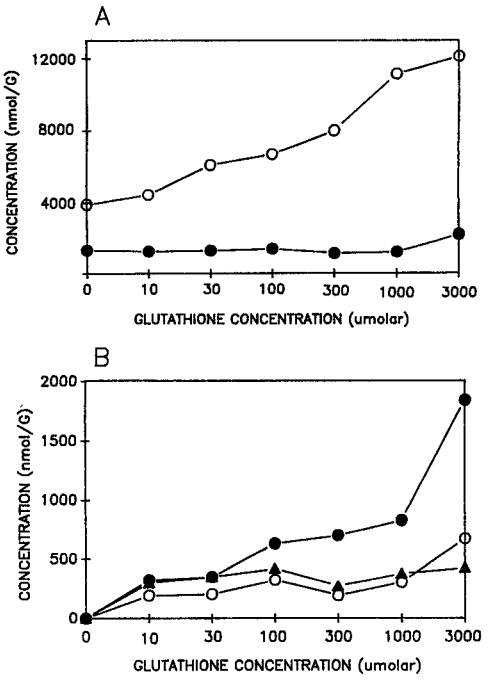

Figure 7. Effects of GSH on phenylpropanoid responses of the snapped-cotyledon assay. A, GSH was applied alone in the snapped-cotyledon assay in the light (○) or the dark (●) and the accumulation of MGG was measured. B, GSH was also applied immediately prior to 30 μg/mL of the glucan elicitor in the snapped-cotyledon assay in the light and the accumulations of coumestrol (●), glyceollin (▲), and MGG (○) were measured. Data represent the means of three experiments. SE, $n = 3$, was <7% of the mean for all data points.

In a separate set of experiments we evaluated the effects of GSSG on elicitation. In all cases it clearly lacked the specific effects noted above for GSH on genistein or coumestrol accumulations. To determine if a specific redox state is optimal for glutathione's effects, we investigated the effects of mixtures of GSH and GSSG, while the molarity of monomeric glutathione was maintained at 1 mM. As shown in Figure 8B, the activating effects of glutathione on genistein and coumestrol accumulation in the snapped-cotyledon assay required that it be at least 80% in reduced form at the time of application.

DISCUSSION

Separation of Individual Responses to Primary Signals

In the process of further characterization of the wound-associated elicitor competency phenomenon, we developed a minimal-wound soybean cotyledon assay. Due to the very low wound background of this assay, it cleanly and

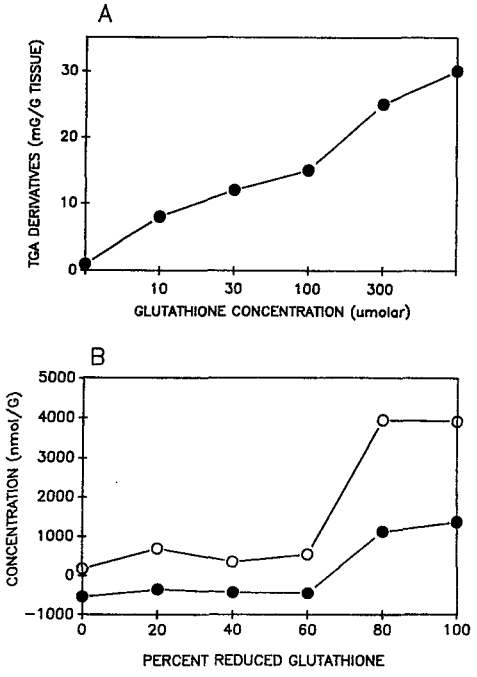

Figure 8. Effects of GSH and GSSG on elicitor competency. A, GSH was applied alone in the cut-cotyledon assay to determine its effects on wound-induced phenolic polymer deposition (●). The phenolic polymer response plotted is the difference between GSH-treated and control wounded tissues. Data represent the mean of three experiments. SE, $n = 3$, was <13% of the mean for all data points. B, Alternatively, mixtures of GSH and GSSG were applied at a total concentration of monomeric glutathione of 1 mM to the snapped-cotyledon assay. Glutathione was applied either alone or immediately prior to 30 μg/mL of the glucan elicitor to determine its effects on MGG (○) and coumestrol (●) accumulations in the uppermost cell layers, respectively. Data represent the mean of three experiments. SE, $n = 3$, was <9% of the mean for all data points.

reproducibly uncouples the wound response from other signal processes and thus has provided us with an excellent opportunity to clearly separate the independent effects of wounding, light, and PWG on cellular response. Importantly, it also allowed a clear reconstitution of the more complex cellular responses to combinations of these agents. This protocol may provide a very powerful tool for further work on signaling processes and cellular communication in this system.

The various primary responses we have described in this paper and their possible interactions are summarized diagrammatically in Figure 9. For simplicity, only the end products of the responses and what we hypothesize are the key points of regulation are shown. It should be noted that the factors (CF-1, CF-2, and PSE) shown in Figure 9B do not represent purified entities, but are hypothesized based on their specific activities in partially fractionated wound exudates (Graham and Graham, 1994). However, the induced states triggered by each factor are clearly differentiable (see below).

The relative simplicity of responses in tissues without wounding is illustrated in Figure 9A. Simple exposure of subepidermal cells to light is both sufficient for and selective in initiating the accumulation of the malonylglucosyl conjugate of the 5-hydroxy-isoflavone genistein, a metabolite normally predominant in mature tissues and in epidermal cells (Graham, 1991b). Perhaps light triggers this specific aspect of epidermal cell metabolism during seedling development. Free genistein, which is toxic to *P. sojae*, is released from its conjugates by enzymes associated with the hyphal tips of this fungus (Rivera-Vargas et al., 1993). It may thus play an important defensive role, particularly in the earliest phases of fungal penetration of epidermal tissues.

The specific effects of PWG have also been greatly clarified using the snapped-cotyledon assay (Fig. 9A). Under minimal-wound conditions, PWG is sufficient for and selectively stimulates the accumulation of the 5-deoxyisofla-

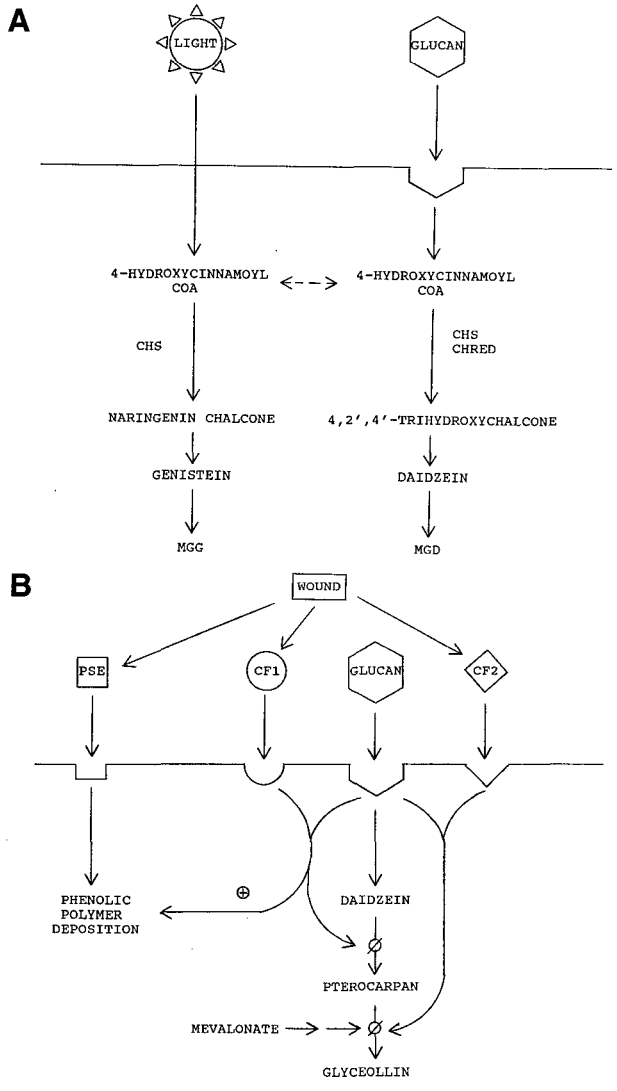

Figure 9. Phenylpropanoid responses of nonwounded (A) and wounded (B) soybean cotyledon cells to treatment with various signals alone and in combination. A plus sign (⊕) signifies a synergistic interaction. The valve sign (∅) signifies a required interaction for completion of that segment of a pathway. CHS, Chalcone synthase; CHRED, chalcone reductase.

vone daidzein. Daidzein is the first committed precursor for the formation of the pterocarpan phytoalexin glyceollin. Thus, the fungal cell-wall glucan's primary effects are consistent with its proposed role in triggering defense. In wounded, PWG-treated tissues and in infected tissues, daidzein conjugates accumulate predominantly in cells distal to the point of infection or elicitor treatment. Results reported here suggest that this response is also the "default" response of proximal cells to PWG in the absence of tissue damage.

Thus, in the absence of wounding, light and elicitor selectively trigger 5-hydroxy- and 5-deoxy-isoflavone accumulations, respectively. The enzyme that differentiates these alternative pathways is a chalcone reductase (Fig. 9A), which is thus a likely point of specific regulation in these two responses. The fact that the light and glucan responses are somewhat synergistic could be explained by the fact that 4-hydroxycinnamoyl-CoA is a common intermediate in both pathways.

Responses in wounded tissues are more complex (shown schematically in Fig. 9B). Comparisons of the cut- (Graham and Graham, 1991a) and snapped-cotyledon assay have confirmed that wounding alone is sufficient to initiate the phenolic polymer response. The use of snapped cotyledons has also allowed us to demonstrate that PWG and light are apparently not primary signals in this response, but potentiate the effects of wounding. A proteinaceous host fraction, operationally designated in Figure 9B as PSE, appears to trigger this wound response and can reconstitute the phenolic polymer responses even in snapped cotyledons.

In addition to the wound-released phenolic polymer elicitor activity, two CFs are released from wounded tissues and condition cellular response to the wall glucan (Graham and Graham, 1994). One of these factors, CF-1, sharply shifts the response of cells to the wall glucan away from the simple accumulation of conjugates of the isoflavone daidzein toward a greatly accelerated deposition of phenolic polymers and enables the accumulation of the simpler isoflavone-derived pterocarpan glycinol and the coumestan coumestrol (P.A. Abbasi and T.L. Graham, unpublished data). In the presence of a second factor, CF-2, this enhanced phenylpropanoid response to elicitor is further and specifically channeled into formation of the isoprenylated pterocarpan phytoalexin glyceollin.

The snapped cotyledon assay has allowed an important confirmation of the requirement of these wound-associated CFs for the proximal cell responses (phenolic polymer and glyceollin accumulations) under minimal-wound conditions. The glyceollin and phenolic polymer responses are not induced in the snapped-cotyledon assay in response to PWG. However, both responses can be reconstituted in this assay by co-application of PWG and wound exudates. The very low wound background of the snapped-cotyledon assay provides us with a much more reliable assay for purification and further characterization of the CFs.

It is important to point out that the wall glucan used in these studies is the native cell-wall glucan from *P. sojae*. Although it has been partially purified and characterized (Ayers et al., 1976a, 1976b), it has not been subjected to acidic (Ayers et al., 1976b; Sharp et al., 1984) or enzymatic (Cline and Albersheim, 1981; Keen and Yoshikawa, 1983; Ham, et al., 1991) hydrolysis or fractionation. Acid-released fragments have been purified and synthesized accordingly and are being used to characterize glucan-binding activities in soybean plasma membranes (Cheong and Hahn, 1991; Frey et al., 1993). A binding activity for enzymatically released glucan fragments has also been characterized (Yoshikawa and Sugimoto, 1993). The size and major structural motifs of the glucan fragments with optimal binding activities appear to differ in the acid- and enzyme-released products (M. Katitani, N. Umemoto, A. Iwamatsu, M. Yoshikawa, I. Ishida, unpublished data), and the relationships of the corresponding receptors remains to be established. Since our primary immediate objectives have been to define the multiplicity and coordination of defense responses, we chose to use the unfractionated native wall glucan because its elicitor activity is released endogenously (Keen and Yoshikawa, 1983) and it triggers the full range of defense responses occurring in infected tissues (Graham, 1995). As with any heterogeneous polymer, however, it is quite possible that multiple elicitor activities are associated with this preparation.

ABA and Methyl Jasmonate Are Potent Effectors of Induced Phenylpropanoid Responses

A few wound-induced proteins have been shown to be also inducible by ABA. Examples are protease inhibitor II in potato (Pena-Cortes et al., 1989) and peroxidases in potato and tomato (Cottle and Kolattukudy, 1982; Roberts and Kolattukudy, 1989). Thus, ABA has been considered as a possible messenger in these responses. On the other hand, Ward et al. (1989) reported that ABA inhibited defense-induced transcriptional activation of Phe ammonia lyase and glyceollin accumulation in soybean. However, only partial inhibition of these two processes was seen at very high levels of ABA (2 mM). Because these are nonphysiological levels of ABA, we reexamined the effects of ABA on a wider range of responses. Our results show that ABA was a potent inhibitor of both the light- and glucan-induced isoflavone responses, with an ED_{50} of approximately 1 μM, and a somewhat less potent inhibitor of glyceollin accumulation (ED_{50} approximately 70 μM). Since the inhibitions we report are at 30- to 2000-fold lower concentrations of ABA than reported by Ward et al. (1989), it seems unlikely that the inhibition of Phe ammonia lyase transcription could fully account for the effect of ABA on these phenylpropanoid responses. However, the results do support the notion that ABA may play a negative role in the regulation of phenylpropanoid responses in soybean.

Creelman et al. (1992) demonstrated that jasmonic acid and methyl jasmonate accumulate in wounded, dark-grown soybean hypocotyl tissues. They also demonstrated the transcriptional induction of chalcone synthase in wounded, dark-grown tissues or in light-grown tissue-cultured cells of soybean treated with methyl jasmonate. However, the independent effects of light, wounding, and methyl jasmonate were not delineated, nor was the metabolic consequence of gene activation. We have shown that

methyl jasmonate itself seems to have little effect on the accumulation of phenylpropanoids in the dark. However, at concentrations as low as 3 μM, methyl jasmonate strongly enhances the accumulations of conjugates of the isoflavones genistein and daidzein induced by light and wall glucan elicitor, respectively. Although these results are not directly inconsistent with an induction of chalcone synthase by methyl jasmonate, they do suggest that light or elicitor treatments are required for actual product accumulation and that the effects of methyl jasmonate on chalcone synthase expression should perhaps be reinvestigated in the dark. In any case, we hypothesize that jasmonic acid may be generated as a result of the wounding associated with hypersensitive cell death and that it may play an important role in conditioning or enhancing the distal cell response to PWG.

Research in other systems is also consistent with a connection between fungal elicitation and jasmonic acid. For instance, addition of various elicitors to different plant cell cultures at 10 to 500 μM induces the accumulation of jasmonic acid and a variety of secondary products (Gundlach et al., 1992; Mueller et al., 1993). Although these workers suggested a second messenger role for jasmonic acid in elicitation, direct evidence for such a role has not emerged. In work more parallel to our own, Kauss et al. (1992) demonstrated that methyl jasmonate increased the sensitivity of the coumarin response of parsley cell cultures to an elicitor preparation from *P. sojae*. Thus, in both the soybean and parsley systems, methyl jasmonate has strongly synergistic effects with elicitor. Moreover, in neither system is methyl jasmonate alone an effective elicitor, arguing once again for its role as a synergist, but not as a messenger.

GSH Partially Restores Elicitor Competency

Sulfhydryl modification has long been suspected to be involved in elicitation of phenylpropanoid defense responses. A wide range of organic and inorganic sulfhydryl-modifying reagents possess elicitor activity in various plant species (see, for example, Yoshikawa, 1978; Ingham et al., 1981; Osman and Fett, 1983; Stössel, 1984), although the effects of these molecules on elicitation in soybean appear to be indirect (Graham and Graham, 1991b). Due to these activities, the biological sulfhydryl molecule glutathione has also been investigated as a potential defense-related signal. Its activity was first examined by Gustine (1981, 1986) in investigations of the role of sulfhydryl reagents on elicitation of phytoalexins in clover. Wingate et al. (1988) reported that glutathione caused transcriptional activation of defense genes, including chalcone synthase, and phenylpropanoid phytoalexin accumulation in bean. Transcriptional activation of chalcone synthase by glutathione was also reported in alfalfa by Choudhary et al. (1990), but only in electroporated protoplasts. Furthermore, although fungal elicitor caused the intracellular accumulation of glutathione and homoglutathione in both bean and alfalfa tissue cultures (Edwards et al., 1991), glutathione was not an elicitor of phenylpropanoid phytoalexins in alfalfa. Thus, although a number of sulfhydryl-modifying agents have effects on elicitation of phenylpropanoid defense responses, their specific modes and targets of action have remained elusive.

As reported in this paper, in the soybean system GSH does not appear to function as a primary elicitor, but seems to mimic certain effects of the wound-associated CF-1, in that it specifically and markedly enhances the phenolic polymer response of tissues to PSE and the coumestrol but not glyceollin response to PWG in minimally wounded tissues. On the other hand, it enhances the genistein conjugate response to light as well. Thus, its effects may encompass but not be limited to those of CF-1. This is not surprising given the complex effects of glutathione on cells. On the one hand, it is a potent sulfhydryl modification agent and could dramatically affect the activities of proteinaceous components of these responses, such as CF-1 and the wound-released phenolic polymer elicitor fraction. On the other hand, it is a strong reducing agent and could have dramatic effects on the generation of oxidized signal molecules or end products. It is quite possible that it influences both of these processes. Currently, we are employing both cellular assays and purified plasma membrane preparations in conjunction with a wider range of redox and sulfhydryl reagents to further delineate glutathione's mode of action in the soybean system. In any case, we hypothesize that glutathione conditions cell response, much like CF-1, rather than serving as a second messenger.

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