



Effects of soybean genotype on the glyceollin elicitation competency of cotyledon tissues to *Phytophthora sojae* glucan elicitors

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In some soybean cultivars, wounding or hypersensitive (HR) cell death is required for the activation of the competency of immediately surrounding (proximal) cells to deploy defense responses to the cell wall glucan elicitor from the pathogen *Phytophthora sojae*. These proximal defense responses include phenolic polymer deposition and accumulation of the glyceollin phytoalexins. In Williams soybeans, elicitation competency is an induced and transient state and is triggered by two endogenous competency factors which are associated with wounded or HR dying cells. In this paper it is reported for the first time that certain cultivars, including Harosoy and Bragg, are at least partially inherently competent. That is, they do not require wound- or HR-associated factors for the glyceollin response to occur. The effects on competency of several *Rps* genes for resistance to *P. sojae* are examined in near isolines of both Williams (incompetent) and Harosoy (competent) and genes controlling nodulation by Bradyrhizobium. Preliminary results suggest that genes that are linked to the *Rps* 1, 3 and 7 loci may modulate the signaling events required for the establishment of the competent state. © 2001 Academic Press

Keywords: *Glycine max*; *Phytophthora sojae*; elicitor; elicitation; wounding; hypersensitive response; competency; nodulation; isoflavonoid; isoflavones; phytoalexin; daidzein; genistein; glyceollin; *Rps* genes; general resistance.

INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is an important oilseed and grain legume crop grown worldwide. A series of dominant host *Rps* genes confer resistance to seedling damping off and root and stem rot caused by *Phytophthora sojae* [30]. Fourteen *Rps* genes have been described so far at seven different loci, with multiple allelic forms at loci 1 and 3 [2, 3, 5, 9, 22, 26]. Nearly 50 races of the pathogen have been identified based on their specific interactions with the host *Rps* genes [1, 22, 28].

The interaction of *P. sojae* and soybean has been a model system to elucidate various biochemical aspects of host-pathogen interactions. Biochemically, the various secondary product defense responses to *P. sojae* and the elicitors responsible for their deployment have been particularly well elucidated. The secondary product pathways in soybean leading to the accumulation of the

pterocarpin phytoalexins, the glyceollins, were among the first to be thoroughly characterized [10]. This information was later complemented by the discovery of high levels of pre-formed conjugates of the isoflavones daidzein and genistein in soybean seedling tissues [21, 24] and the elucidation of their potential involvement in the mobilization of the overall complex secondary product defense responses [16, 19, 20]. In addition, the glucan elicitor from the cell wall of *P. sojae* is one of the most thoroughly characterized elicitors [4, 25, 29, 32], and putative receptors for this elicitor have been characterized [8, 12, 31]. Finally, the use of cotyledon tissues for analysis of infection and elicitor responses [4, 11, 17, 18] has helped to delineate the complex interplay of the various signaling processes and responses involved. In addition to displaying race-specific resistance to *P. sojae*, the cellular architecture of cotyledons is nearly ideal for the study of spatial and temporal aspects of response as well as cell-to-cell signaling. In particular, the recent development of a minimal-wound snapped cotyledon assay has allowed a clear separation and delineation of the various primary responses to such signals or conditions as light, glucan elicitor and wounding, which are otherwise often superimposed upon one another [18].

The culmination of these various studies has led to the following understanding of the elicitation processes

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Abbreviations used in text: GSH, glutathione; MGD, 6''-malonyl-7-O-glucosyl daidzein; MGG, 6''-malonyl-7-O-glucosyl genistein; WGE, intact *Phytophthora sojae* wall glucan elicitor; ERGE, enzyme released, soluble glucan elicitor.

involved in the deployment of the secondary defense responses in soybean. Both daidzein, the precursor of the glyceollins, and the closely related isoflavone genistein, are present at high constitutive levels in soybean tissues [15, 24] as glucosyl (daidzin and genistin, respectively) and malonylglucosyl conjugates (MGD and MGG, respectively). Genistein has been shown to be antibiotic to *P. sojae* [27]. Conjugates of both isoflavones are rapidly hydrolysed at the infection front in incompatible interactions to release the free aglycones [21]. Thus, a preformed antibiotic (genistein) and the precursor of the more elaborate antibiotic glyceollins are released. Glyceollin subsequently accumulates to high levels at the infection front in the incompatible, but not the compatible response [21].

Studies with the glucan elicitor from *P. sojae* further elucidated the deployment of these defense responses. Spatial and temporal analysis of responses to elicitor with the classical cut cotyledon assay elucidated the proximal and distal responses to elicitor [17]. The proximal responses, occurring in a few cells immediately adjacent to the treated surface, include a net hydrolysis of the conjugates of daidzein and genistein, the accumulation of glyceollin and the deposition of phenolic polymers. The distal cell response, occurring in cells as far as 30–100 cells away, involves the net and massive accumulation of conjugates of the isoflavones daidzein and genistein, hypothesized to raise the defense potential of these cells. In fact, many aspects of defense seen in infected tissues are elicited by the glucan elicitor, with two important exceptions. First of all, the glucan elicitor does not elicit the HR. Secondly, a number of complementary experiments demonstrated that the elicitation of the proximal cell responses by the glucan elicitor requires prior conditioning of cells by wound- or HR-associated “competency factors” [13]. Treatment of non-conditioned cells with the glucan elicitor results in the “default” expression of the distal cell response, the massive net accumulation of conjugates of the isoflavones daidzein and genistein. Thus, it was hypothesized that the race-specific expression of the HR actually programs the capacity of proximal cells to deploy the glyceollin and phenolic polymer responses to the general resistance glucan elicitor.

Studies using minimally-wounded snapped cotyledon tissues, allowed the further delineation of these signaling processes. It was discovered that while the glucan elicitor was a necessary and sufficient (primary) signal for daidzein conjugate accumulation, light was a necessary and sufficient signal for genistein conjugate accumulation [18]. Since daidzein and genistein share common precursors, these two processes are highly synergistic with one another. The cut and snapped cotyledon assays also allowed the clear identification of two wound- and HR-associated competency factors, CF-1 and CF-2. CF-1, the activity of which can be largely mimicked by reduced

glutathione [18], generally enhances all phenylpropanoid responses to the glucan elicitor, but diverts synthesis particularly into phenolic polymers. CF-2, which can be mimicked by orthovanadate (T. L. Graham and M. Y. Graham, unpublished), a proton pump inhibitor, specifically enables the glyceollin response to the glucan elicitor.

Thus the overall deployment of soybean defense responses involves the integration and orchestration of several signaling components and processes [16, 19, 20]. The ability to reconstitute and monitor these various responses in a discrete manner in the minimal wound snapped cotyledon assay provides a particularly rich matrix of biochemical tools for dissection of the responses. In this paper, the authors begin to use some of these tools in complement with the well characterized genetics of the soybean system. In particular, they have employed two series of near-isogenic lines to determine if specific *Rps* genes for resistance influence the signaling processes alluded to above. This would seem to be a particularly relevant analysis in that the *Rps* genes might be expected to condition certain events in the hypersensitive cell death program, which it is hypothesized in turn conditions several aspects of the proximal cell responses to the general resistance glucan elicitor.

MATERIALS AND METHODS

Chemicals

Due to the complexity of the *Phytophthora* cell wall glucan, the actual glucan elicitor species involved in the activation of defense responses in soybean tissues is still somewhat controversial. While responses can be demonstrated to a synthetic heptaglucan elicitor [8], the structure of which was based on acid hydrolyses and subsequent fractionation of the wall glucan [29], other studies suggest that optimal elicitor activity resides in larger, enzymatically (glucanase) released glucan fragments [25]. In this work, the intact glucan elicitor was used to allow the elicitor fragments to be generated *in situ*. It is felt that this most closely approximates infection with the fungus itself; indeed it has been shown that this elicitor preparation induces nearly the full range of the defense responses seen in infected tissues [17]. For comparative purposes, the enzymatically released glucan elicitor fragments were also employed [32].

The intact wall glucan elicitor (WGE) was prepared from the cell walls of race 1 of *P. sojae* (Kauf. and Gerde.) according to Ayers *et al.* [4] and as described previously [17]. Before use, the unfractionated and insoluble wall glucan preparation was sonicated and then autoclaved for 3 h in deionized double-distilled water [4].

Since the *in situ* elicitor-releasing glucanase is at least partially constitutively expressed in soybean tissues, it is

relatively easy to prepare an enzymatically released soluble elicitor from the intact glucan [32]. To partially account for possible differences in this elicitor-releasing glucanase activity in different organs and tissues, such a soluble, enzyme-released glucan (ERGE) elicitor was prepared for parallel examination in all studies reported here. This elicitor was prepared by a modification of the conditions first reported by Yoshikawa *et al.* [32]. In brief, a large excess of the intact wall glucan preparation (2 mg of wall glucan preparation in 4 ml of sterilized distilled water) was incubated with 1 ml Williams cotyledon cell-free extract for 2 h. The cell-free extract was prepared by adding water to 0.6 g tissue to a total volume of 1 ml, grinding until homogeneous and clarifying by centrifugation at 13 000 *g* for 10 min. After 2 h, the incubation mixture was boiled (100°C) for 10 min. The boiled fraction was centrifuged (13 000 *g*, 10 min) and the supernatant was passed through a 0.2 μm membrane filtration (Gelman Sciences Inc., Ann Arbor, MI, U.S.A.). The non-hydrolysed intact wall glucan was in the centrifugal pellet or retained by the membrane, whereas the soluble, sterile ERGE passed through. This elicitor was used at a concentration equivalent to the intact glucan present in the original hydrolysis mixture. As noted below, this approach gave nearly identical results as seen with the same concentration of the intact glucan, suggesting that the solubilization process at least partially mimicked *in situ* release.

Sodium metavanadate and reduced glutathione (GSH) were obtained from Sigma Chemical Company, St. Louis, MO, U.S.A. A stock solution of metavanadate was prepared at 50 mM in sterile DDW and kept at room temperature. At this concentration, orthovanadate is formed within 24 h and is stable indefinitely. Orthovanadate was diluted to the appropriate concentrations immediately before the experiment.

Growth of soybean seedlings

Seeds of various near-isogenic isolines of Williams and Harosoy soybean (*Glycine max* L. [Merr.], Table 1) [2] were grown for these studies by A. F. Schmitthenner (Ohio Agricultural Research and Development Center, Wooster, OH, U.S.A.). Seeds of A. K. Harrow and Mandarin (Ottawa) were provided by T. Anderson (Agriculture Canada, Harrow, Ontario). Seeds of *Bradyrhizobium japonicum* response mutants of the cultivar Bragg (supernodulators *nts383* and *nts 1007*, and nonnodulators *nod49* and *nod139*) were obtained from P. M. Gresshoff (Department of Botany, University of Queensland, Australia). Seedlings were grown as described previously [21]. Instead of vermiculite, healthy, sorted seeds were grown in Metromix 360 (Sierra Grace, Milpitas, GA, U.S.A.) at 26°C with 500 $\mu\text{E m}^{-2} \text{s}^{-1}$ of light and a 14 h photoperiod. The flats were immediately

watered very thoroughly for germination. After 3 days, the plants were watered every other day from the top. Plants were not fertilized.

Cotyledon assays

Cotyledons (7 or 8 day old), unless otherwise noted, were harvested in small batches and used immediately. The cut cotyledon assay [17] and the minimal wound snapped cotyledon assay [18] were performed as described previously. The concentrations of elicitors and other effectors reported below are the final concentrations in the surface droplets in the two assays. In all cases, effectors were applied first and the glucan elicitor (or an equivalent amount of water) was applied as the last treatment because the glucan elicitor induces a refractory state for the establishment of competency (T. L. Graham, unpublished). Generally the intact glucan elicitor (WGE) was applied at 20 $\mu\text{g ml}^{-1}$ and the enzymatically released elicitor (ERGE) was applied at 30 $\mu\text{g ml}^{-1}$, concentrations normally giving a half-maximal (ED_{50}) response in the cut cotyledon assay.

HPLC analyses were carried out as described previously [14]. Treated cotyledons were harvested after 48 h of incubation in the light. Only the uppermost 0.5 mm section (proximal cell layer) in the cut or snapped cotyledon assays was analysed unless otherwise noted. The 10 or 20 sliced sections per treatment were pooled together and either extracted and analysed immediately or stored intact

TABLE 1. Williams and Harosoy isolines: a brief description

Isoline	Maturity group	<i>Rps</i> gene(s)	Source of gene
(a) Williams isolines			
Williams	III	<i>rps</i>	
L75-6141	III	<i>Rps1</i>	Union
L77-1863	III	<i>Rps1-b</i>	Harrell
L75-3735	III	<i>Rps1-c</i>	Lee 68
W79	III	<i>Rps1-c</i>	Lee 68
W82	III	<i>Rps1-k</i>	Kingwa
L83-570	III	<i>Rps3</i>	PI 86972-1
(b) Harosoy isolines			
Harosoy 1XX	II	<i>Rps7</i>	Mandarin
HARO 13-3	II	<i>rps7 + Rps1-b</i>	PI 84637
HARO 13XX	II	<i>Rps7 + Rps1-b</i>	PI 84637
HARO 15XX	II	<i>Rps7 + Rps1-k</i>	Kingwa
L70-6494	II	<i>Rps7 + Rps2</i>	D54-2437
HARO 32XX	II	<i>Rps7 + Rps3</i>	PI 171442
PRX 146-36	II	<i>rps7 + Rps3-b</i>	PI 172901
HARO 43 XX	II	<i>Rps7 + Rps4</i>	PI 86050
HARO 52 XX	II	<i>Rps7 + Rps5</i>	PI 91160
HARO 62 XX	II	<i>Rps7 + Rps6</i>	Altona

XX = designated as 72 [2].

HARO 13-3 = gives a susceptible reaction to races 16, 19.

PRX 146-36 = gives a susceptible reaction to races 16, 18, 19.

at -20°C for later analysis. Each HPLC analysis takes 30 min and large numbers of samples are analysed for any given experiment. For this reason, rather than analysing multiple replicates, the approach of pooling 10–20 cotyledon subsamples was taken. Performed in this way, the HPLC analysis itself has very low variability {see [14] for statistics}. Statistical analyses are thus usually only performed as the standard error between experiments, where somewhat greater sources of variation are seen.

Wound washings from soybean cotyledon tissues

Cotyledons from 7 to 8 day seedlings were obtained as described above. Individual cotyledons were surface sterilized and cut as described for the cut cotyledon assay [17]. The cut surface of each cotyledon was supplied with $15\ \mu\text{l}$ of sterile double-distilled water and wound exudate was collected after transferring the fluid onto and off the cut surface several times with a micropipette. The droplet from the first cotyledon was then transferred to a second cotyledon surface and the process repeated, resulting in a final volume of approximately $10\ \mu\text{l}$ of wound exudate. The wound exudate obtained in this manner was applied immediately to the exposed surface of each individual cotyledon in the snapped cotyledon assay. Each treated cotyledon was then immediately supplied with $10\ \mu\text{l}$ of water or elicitor to complete the treatment.

RESULTS

Evaluation of isoflavone responses in Williams and Harosoy near-isogenic lines

The synthesis and subsequent turnover of the isoflavone conjugate pools is of central importance to both the proximal deployment of the glyceollin response and to distal defense potentiation [17, 19, 20]. Primary signals triggering the formation of daidzein and genistein

conjugate pools are the glucan elicitor and light, respectively [18]. The isoflavone responses of all isolines to light and glucan elicitor were examined first. In all cases, all lines in both the Williams and Harosoy series showed strong induction of daidzein and genistein conjugates to glucan and light, respectively. Overall, there was little genotypic variation in the isoflavone responses of these lines. Fig. 1(a) shows representative data with the Williams lines for MGD accumulation after treatment with the two different soluble glucan elicitor preparations. Although the intact glucan elicitor is generally slightly more active than the enzymatically released fragments, the uniformity of these responses (and those to light, not shown) is striking and suggests that, at least with the light and glucan isoflavone signal-response pathways, the net accumulation of the isoflavones themselves are not likely conditioned by genes linked to these *Rps* loci. An additional test proved interesting, however. In the absence of wounding and other signal molecules, it has been demonstrated that reduced glutathione (GSH) causes a massive increase in genistein conjugates, suggesting a possible link to the light-induced response [18]. Although treatment with glutathione revealed no variations in genistein accumulation with most of the lines, L77-1863, carrying the *Rps1b* gene, showed virtually no daidzein and very little genistein accumulation in response to GSH [Fig. 1(b)]. On the other hand, light induction of genistein conjugates in L77-1863 was not substantially reduced (data not shown), suggesting that the apparent GSH interaction with this line involves a GSH effect on isoflavone accumulation not shared by light. Although the Harosoy lines showed slightly higher variations in genistein and daidzein conjugate responses, the overall results were the same as with Williams, with the exception that the two lines carrying *Rps1b* (HARO 13-3 and HARO 13XX) did not show altered responses to GSH.

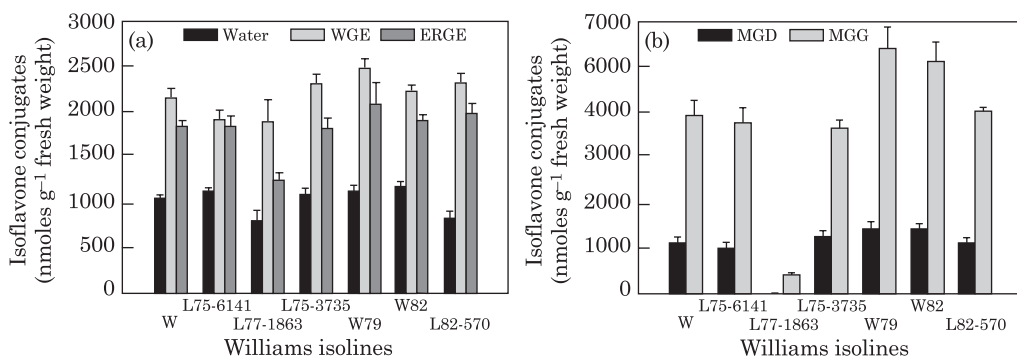


FIG. 1. Effects of genotype on isoflavone induction. (a) Effects of genotype on the induction of malonyl glucosyl daidzein (MGD) by the intact wall glucan elicitor (WGE) and enzymatic released elicitor (ERGE). Values are the average of two experiments. (b) Effect of genotype on elicitation of MGD and malonyl glucosyl genistein (MGG) by reduced glutathione. Isoflavone levels in water treated controls have been subtracted. Values are the average of two experiments.

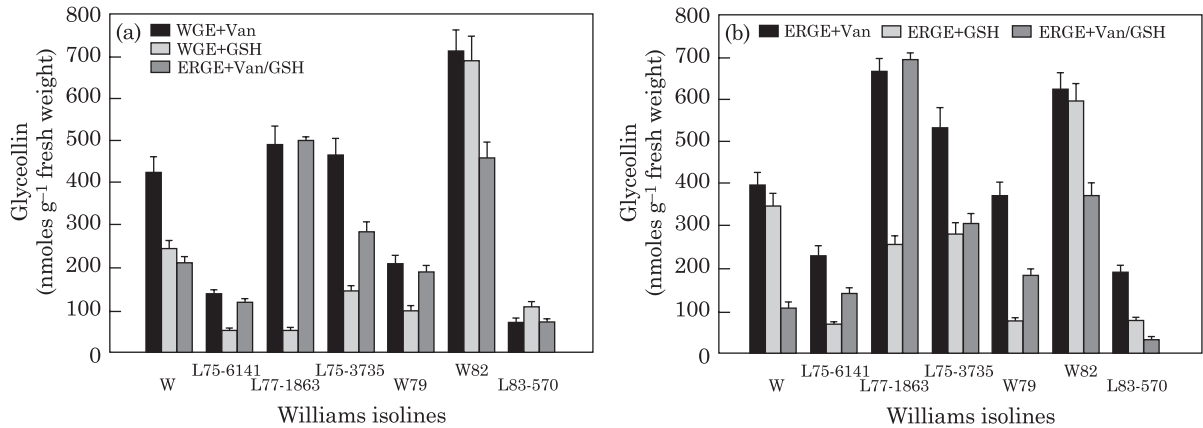


FIG. 2. Effects of glucan elicitors, glutathione and orthovanadate on the glyceollin responses of Williams isolines. Glutathione (GSH, 200 µM) and orthovanadate (Van, 100 µM) pretreatments were followed by application of 20 µg ml⁻¹ WGE [Fig. 1(a)] or 30 µg ml⁻¹ ERGE [Fig. 1(b)]. In both figures, the glyceollin levels in glucan treated controls have been subtracted from the plotted values. The values represent the averages of two experiments.

Competency for glyceollin elicitation in Williams isolines

To avoid the complications of wound-induced competency, the Williams and Harosoy isolines with different *Rps* backgrounds were analysed for glyceollin response to elicitor in the minimal-wound, snapped cotyledon assay [18]. As is true with Williams *per se*, none of the Williams isolines carrying the various *Rps* genes showed a glyceollin response to either of the *P. sojae* glucan elicitors. That is, there was no difference in glyceollin accumulation in elicitor- or water-treated cotyledons. Levels of glyceollin in these tissues averaged 30 nmol g⁻¹ fresh weight tissue, which is a typical level in control tissues. Williams isolines were non-responsive for glyceollin accumulation even at 200 µg ml⁻¹ of either elicitor in the snapped cotyledon assay (data not shown). This is over six times the ED₅₀ of the elicitor in the classical cut cotyledon assay.

However, when the exposed surface of the snapped cotyledon was supplied with glutathione or orthovanadate, mimics of CF-1 or CF-2, respectively, elicitation competency was achieved for the glyceollin response in all the Williams isolines tested, although to a varying degree (Fig. 2). Again there was very good consistency in response with the two different elicitors, as compared in Fig. 2(a) and (b). Although GSH is known to have a slight stimulatory effect on glyceollin elicitation competency [18], this effect is thought to be an indirect result of its effect on overall loading of the phenylpropanoid pathway (e.g. genistein and phenolic polymer responses), whereas orthovanadate selectively enhances glyceollin elicitation competency with no apparent direct effects on phenylpropanoid metabolism *per se*. Of the results shown in Fig. 2(a) and (b), several effects are of potential interest. First is the particularly strong elicitation of glyceollin observed in Williams 82 cotyledons (*Rps1-k*) with co-application of GSH with elicitor. Secondly, in

Williams isolines L75-6141 and L83-570, carrying the *Rps1* and *Rps3* genes, respectively, orthovanadate was comparatively much less effective in stimulating the glyceollin response when co-applied with *P. sojae* elicitors [Fig. 2(a) and (b)]. These results were confirmed and extended in a series of studies below.

Competency for glyceollin elicitation in Harosoy isolines

The Harosoy isolines used in this study have the *Rps* genes integrated into the Harosoy background, which already contains the *Rps7* gene (earlier called the “Harosoy gene”). In contrast to the Williams isolines, all the Harosoy lines tested responded to *P. sojae* wall glucan elicitors alone (in the absence of wounding or elicitation competency inducers) by accumulating relatively high levels of glyceollin in the immediate vicinity of the site of elicitor application [Fig. 3(a)]. The isolate PRX146-36 (*Rps3-b*, abbreviated to “PRX” in Fig. 3), though responding to both elicitor preparations, did not respond as strongly as the other lines tested. Although Harosoy isolines are thus partially inherently competent for glyceollin elicitation, orthovanadate when coapplied with *P. sojae* wall glucan elicitors gave an additional enhancement of induction of glyceollin biosynthesis [Fig. 3(b)].

The inherent competency of Harosoy, illustrated in Fig. 3, has been confirmed in many independent experiments with seed lots from over 6 different years. Of the various Harosoy lines, HARO 13-3 and PRX 146-36 do not carry the *Rps7* allele (Table 1). While the response of PRX 146-36 is much lower than the other lines, the response of HARO 13-3 is only slightly depressed. Interestingly, the lack of response of PRX 146-36 to elicitor was partially reversed by vanadate and HARO

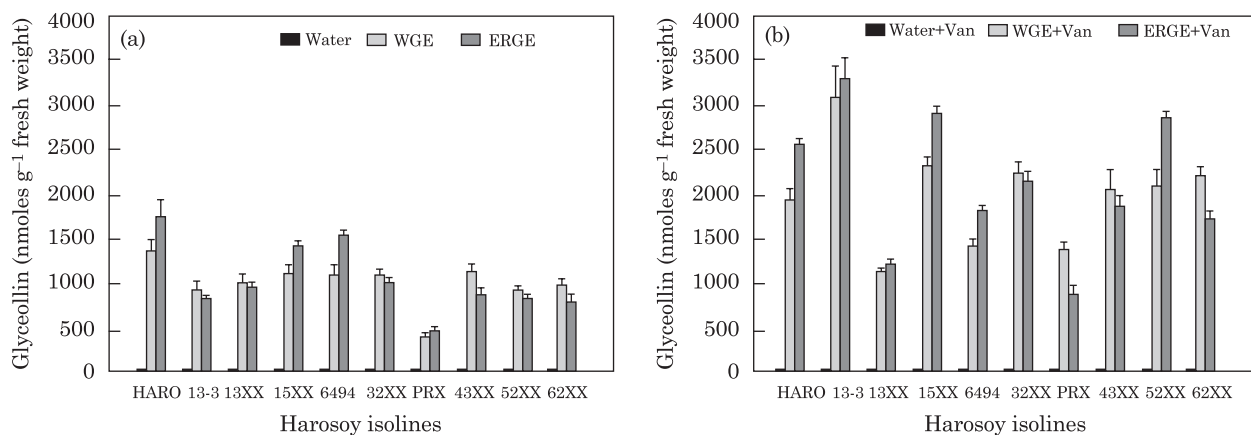


FIG. 3. Effects of glucan elicitors and orthovanadate on glyceollin responses of Harosoy isolines. Water (a) or orthovanadate [Van, 100 μM , (b)] pretreatment was followed by application of 20 $\mu\text{g ml}^{-1}$ WGE or 30 $\mu\text{g ml}^{-1}$ ERGE. (a) and (b) represent data from the same experiments. Values in (b) are net accumulations, without subtraction of the glucan controls in (a). The values represent the averages of two experiments.

13-3 (abbreviated to “13-3” in Fig. 3) was particularly responsive to vanadate [Fig. 3(b)], suggesting some deficiency in competency of these lines. The possible correlation of competency with genes linked to the *Rps7* locus was further tested by examining the responses of the parental lines for Harosoy, Mandarin (Ottawa) and A. K. Harrow. Mandarin is the source of the *Rps7* gene in Harosoy. While A. K. Harrow showed no inherent competency. Mandarin was equally as inherently competent as Harosoy (accumulation of glyceollin with elicitor alone averaged 1000–1500 nmol g⁻¹ fresh weight tissue). While the inherent competency in Harosoy may thus be derived from Mandarin and may be linked to the *Rps7* gene, further confirmation of this is necessary.

Response of the cultivar Bragg and its nodulation mutants to *P. sojae* wall glucan elicitor in the cut cotyledon and snapped cotyledon assays

Due to the potential involvement of isoflavones in recognition of rhizobia and the possible effects of soybean defense mechanisms in altering nodulation processes, the cultivar Bragg and its *B. japonicum* response mutants including the nonnodulating *nod49* and *nod139* [7] and the supernodulating mutants *nts382* and *nts1007* [6] were also analysed.

Bragg and its nodulation mutants were again analysed for elicitor competency in a minimal wound cotyledon assay. None of the mutations affecting nodulation appeared to affect any of the isoflavone or glyceollin responses (data not shown). Like Harosoy isolines, however, the cultivar Bragg and its variants seemed to be partially competent to elicitor response. When the exposed surface of the snapped cotyledon was supplied with *P. sojae* wall glucan elicitor, the non-wounded cells

responded to elicitor by accumulating glyceollin. When co-applied with elicitor preparations, GSH and orthovanadate further enhanced the wall glucan triggered levels of glyceollin as seen with the Harosoy isolines.

Further analysis of selected lines

Selected lines showing a potential genotype interaction with glyceollin accumulation were further tested to confirm and extend the above studies. These included Williams and its isolines, Williams 82 and L83-570 and Harosoy and its isolate PRX 146-36. Three different analyses were carried out. First were dose-response curves for the effects of GSH and orthovanadate. Secondly, the capacity of wound exudate from the poorly competent lines was examined for its ability to establish elicitation competency in Williams. Finally, the lines were examined in the cut cotyledon assay to determine the effectiveness of the *in situ* generation of competency factors and the possibility that the genetic variation in glyceollin response might be due to a shift in the timing of the establishment of wound-induced competency in these lines.

Dose responses with glutathione and orthovanadate in selected soybean isolines

The apparent interactions of GSH and orthovanadate with selected lines was examined over wide concentration ranges. These were 4–900 μM for orthovanadate and 12–3000 μM for reduced glutathione. Each dilution was tested in the presence and absence of WGE. Neither glutathione nor orthovanadate induced glyceollin at any concentration when applied alone on the broken surface of the cotyledons (data not shown). When coapplied with *P. sojae* elicitor, orthovanadate activated glyceollin

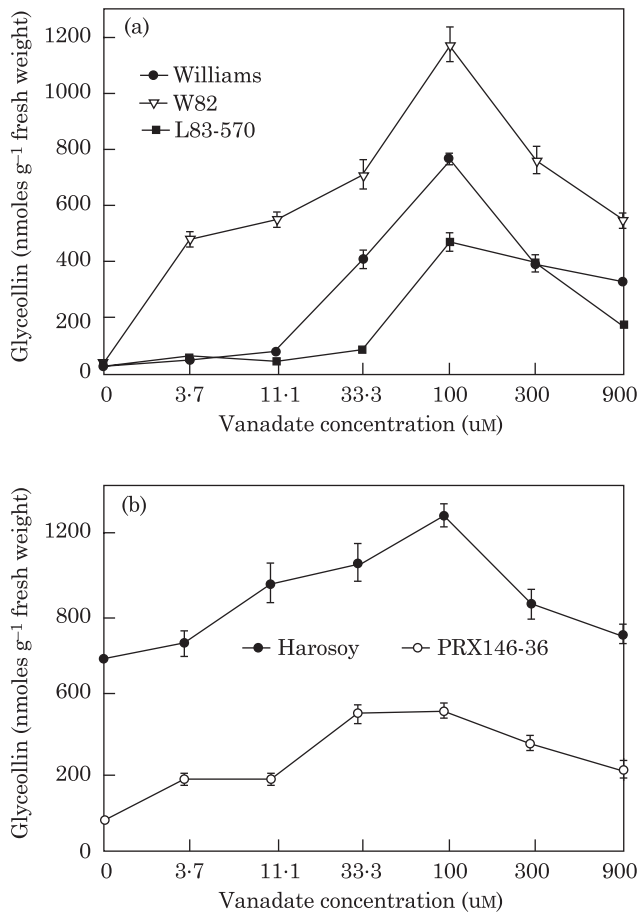


FIG. 4. Effects of increasing concentrations of orthovanadate on the elicitation competency of selected Williams (a) and Harosoy (b) isolines. The elicitor WGE was applied at $20 \mu\text{g ml}^{-1}$. Note the non-linear scale. The values represent the averages of two experiments.

elicitation competency in Williams isolines and enhanced glyceollin elicitation in Harosoy isolines (Fig. 4). Consistent with the results in Fig. 2, the dose response data support the conclusion that orthovanadate is more effective in inducing glyceollin elicitation competency in Williams 82 and less effective in inducing competency in L83-570 as compared with Williams [Fig. 4(a)]. In fact, Williams 82 appears to be particularly sensitive to orthovanadate (responding to approximately 10 times less orthovanadate than Williams), whereas L 83-570 is relatively insensitive (requiring approximately three times more orthovanadate than Williams). Results with the Harosoy isolate, PRX146-36 were also confirmed. Depending on the concentration of orthovanadate, two- to five-fold higher levels of glyceollin were induced in Harosoy as compared with PRX146-36 [Fig. 4(b)].

The unusually strong activation of glyceollin elicitation competency in Williams 82 by GSH was also confirmed

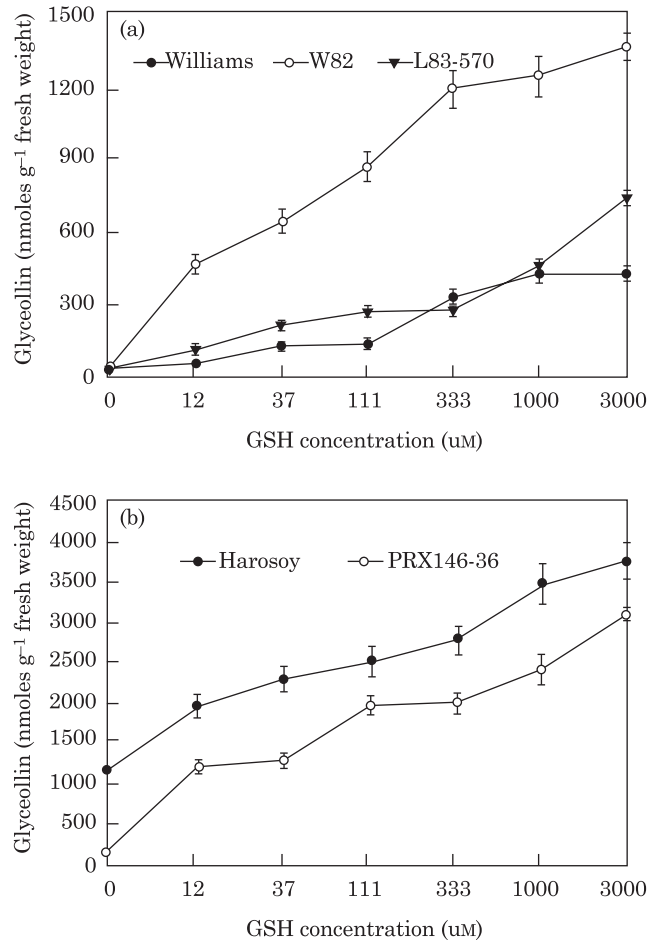


FIG. 5. Effects of increasing concentrations of reduced glutathione on the elicitation competency of selected Williams (a) and Harosoy (b) isolines. The elicitor WGE was applied at $20 \mu\text{g ml}^{-1}$. Note the non-linear scale. The values represent the averages of two experiments.

by a comparison of dose response curves for Williams isolines [Fig. 5(a)]. While Williams and L83-570 are very close in their response, Williams 82 again shows an extreme sensitivity to GSH, responding to GSH concentrations at least 81-fold lower than Williams and with a maximum glyceollin elicitation nearly five times as high. Consistent with earlier results, GSH is nearly equally effective in enhancing the elicitor-induced levels of glyceollin in both Harosoy isolines [Fig. 5(b)].

Restoration of elicitor competency with wound exudates from soybean cotyledons

To determine whether the low responses to competency inducers such as orthovanadate might represent an inherently lowered capacity to produce the endogenous competency factors, wound exudates from various Williams and Harosoy lines were applied to Williams

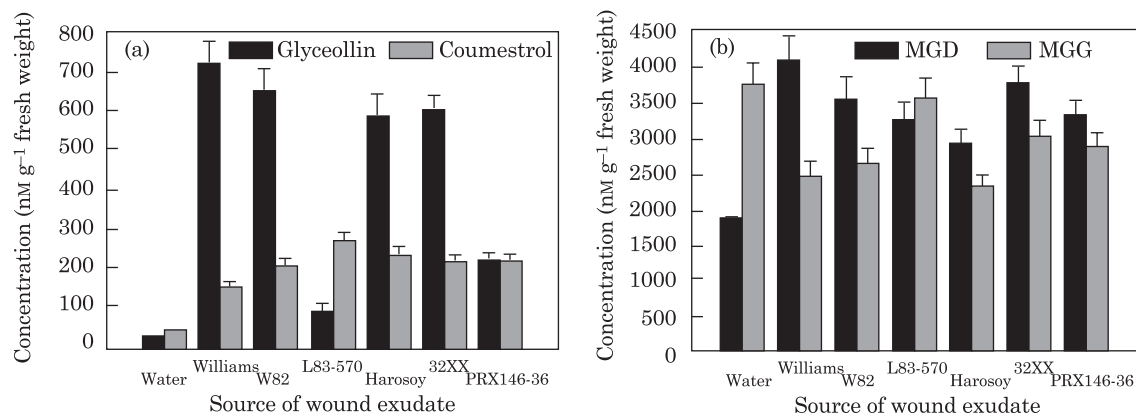


FIG. 6. Reconstitution of elicitor competency on Williams soybean by wound exudates from selected Williams and Harosoy isolines. (a) Effects on glyceollin and coumestrol. (b) Effects on isoflavone conjugates, MGD and MGG. The elicitor WGE was applied after the wound exudate at a concentration of $20 \mu\text{g ml}^{-1}$. The values represent the averages of two experiments.

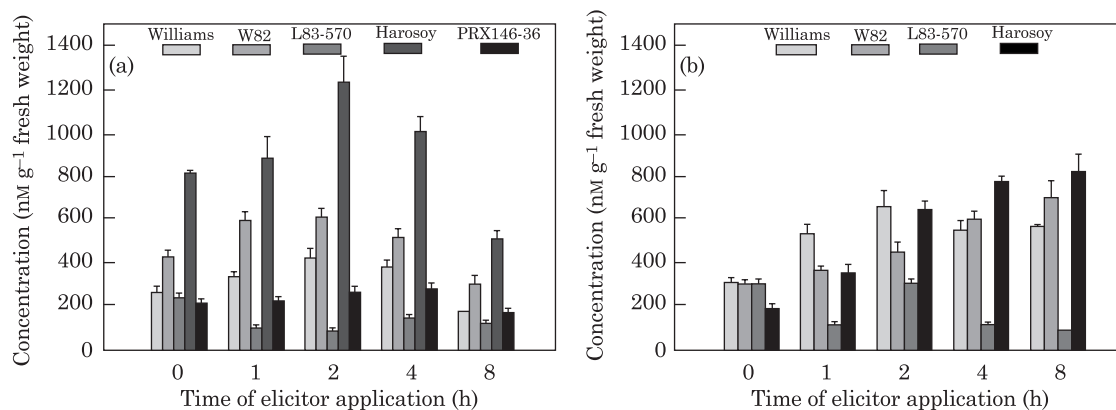


FIG. 7. Time course of establishment and duration of elicitor competency in selected Williams and Harosoy isolines in the cut cotyledon assay. At various times (0, 1, 2, 4, 8 h) after wounding and incubating in (a) light or (b) dark, the cut surface of the cotyledons was treated with $30 \mu\text{l}$ of WGE ($20 \mu\text{g ml}^{-1}$). After 48 h of further incubation in (a) light or (b) dark, the treated surface was analysed for glyceollin. The values are means of two experiments.

snapped cotyledons to determine their capacity for induction of competency [18]. No wound exudates induced glyceollin when applied alone on the exposed surface of snapped cotyledons (data not shown). Wound exudates from the Williams isolate L83-570, and the Harosoy isolate PRX146-36, were not as effective in restoring the glyceollin response as the wound exudates from other selected isolines [Fig. 6(a)]. Importantly, this seems to be a specific effect for glyceollin elicitation competency in that the levels of coumestrol and isoflavone conjugate induction [Fig. 6(b)] in response to glucan elicitor are not affected. Thus, the ineffectiveness of wound exudates from these two cultivars seems to mirror the lowered ability to establish competency in these lines using orthovanadate, suggesting that some endogenous pathway in the establishment of glyceollin elicitation competency is altered.

To complement these studies, the responses of L83-570 and PRX146-36 were examined using the cut cotyledon assay, in which the endogenous competency factors are generated *in situ*. It was established previously that wounding induces a competent state that is transient in nature [13]. Thus, for a thorough analysis, elicitor was applied at various times 1–8 h after wounding. The study was conducted both in the light and dark [18].

As shown in Fig. 7(a), in the light, response to elicitor generally gradually increased as the elicitor application was delayed 1–2 h after wounding with the maximum response seen 2 h after wounding. Later application of elicitor, for instance, 4–8 h after wounding, led to lower responses, consistent with earlier reports [13]. However, glyceollin induction was again much lower at all times in L83-570 and PRX146-36 and nearly level with time, suggesting that the induction of the transient

competent state did not occur effectively. This nicely complements the inability of the wound exudates from these lines to induce competency in Williams, suggesting a deficiency in the generation of endogenous competency factors.

When the cotyledons were incubated in the dark after wounding and elicitor application, competency was attained only gradually over an 8 h period [Fig. 7(b)]. Thus, light not only seems to increase the speed of attainment and magnitude of this inducible competent state, but it also appears to regulate the transiency of the induced state. Under these conditions, the response of L83-570 was particularly low (PRX146-36 was not examined).

DISCUSSION

It has been established previously that prior activation of cells by wound- or HR-associated elicitation competency factors is required for the proximal cell responses (glyceollin accumulation and phenolic polymer deposition) of Williams cotyledon tissues to the *P. sojae* wall glucan elicitor [13]. Stated somewhat differently, some aspects of wounding or HR cell death [19, 20] are required for the activation of the capacity of immediately adjacent (proximal) cells to respond to the glucan elicitor, which in turn elicits the general resistance responses normally expressed downstream from the HR. In contrast, isoflavone conjugate accumulation (the distal cell response) does not require the presence of such wound factors [13]. These phenomena are conserved among the majority of soybean cultivars that have been examined. It was hypothesized that if HR cell death conditions competency for response to the glucan elicitor, then the presence of the *Rps* resistance genes, which condition aspects of establishment of the HR to *P. sojae*, may influence establishment of elicitation competency.

In this paper, it is demonstrated for the first time that some soybean lines display partial inherent competency. That is, although they do not require wounding or competency factors for glyceollin elicitation, they respond to the competency inducers, GSH and orthovanadate, with increased glyceollin responses. An exhaustive comparison has been performed of two such inherently competent lines (Harosoy and Bragg) and the incompetent line, Williams. In addition, the effects of the presence of several *Rps* resistance genes and/or nodulation genes on the responses of these lines have been examined. The data presented suggest that heterogeneity in response can be found within near-isogenic variants of soybean lines carrying specific *Rps* genes.

The cultivar Harosoy originated from a cross between Mandarin (Ottawa) and A. K. Harrow. Interestingly, the cultivar Mandarin (Ottawa) which is the source of the

Rps7 gene, also has inherent competency to the glucan elicitor, while A. K. Harrow does not. Thus, it is possible that the inherent competency of Harosoy is derived from Mandarin and linked to the *Rps7* gene. Consistent with this, PRX146-36 a Harosoy line in which the *Rps7* gene is missing, shows only a slight inherent competency to glucan elicitor. Further experiments which measured the generation of endogenous competency factors, suggests that PRX146-36 is very deficient in its capacity to produce the endogenous competency factors. While HARO 13-3 (which also lacks the *Rps7* gene) shows only slightly reduced response compared with other lines, it is particularly responsive to competency induction by orthovanadate, also suggesting a lowered capacity for endogenous competency. Although results presented here are suggestive, the ultimate linkage of competency to the *Rps7* locus will require further confirmation.

The inherent competency associated with the Harosoy background is interesting in relation to recent work published by Mohr and Cahill [23] in which they demonstrate that pre-treatment of Harosoy hypocotyls with norflurazon, an inhibitor of ABA synthesis, leads to restricted lesions in normally compatible interactions and elevated glyceollin accumulation in the absence of an apparent HR. Although they suggest that this represents an uncoupling of the HR from phytoalexin accumulation, results presented here suggest that the cultivar Harosoy may not require the wound- or HR-associated establishment of glyceollin elicitation competency.

Overall the results presented here also suggest potential, though complex, interactions of genes linked to the *Rps1* locus with the establishment of competency. Unlike other cultivars, the Williams line L77-1863 (*Rps1b*) does not show the characteristic induction of genistein (and daidzein) conjugates in response to glutathione. Moreover, the CF-2 mimic, orthovanadate, is a poor inducer of glyceollin elicitation competency in L75-6141 (*Rps1*). On the other hand, Williams 82, carrying the *Rps1k* gene, is particularly sensitive to the induction of glyceollin elicitation competency by orthovanadate and GSH, the latter of which does not normally induce significant glyceollin elicitation competency. The possible effects of genes linked to the *Rps1* locus are thus complex and may reflect the different activities of the gene products associated with the different *Rps1* alleles or other linked genes. Interestingly, genes linked to the *Rps1* locus do not seem to affect isoflavone induction or elicitation competency in the Harosoy background. Perhaps their effects are overwhelmed by the inherent competency associated with the Harosoy background.

Williams isolate L83-570 (*Rps3*) also shows greatly diminished response to orthovanadate. The lack of response of L83-570 was further supported with dose response data with orthovanadate. Further confirmation came from studies with L83-570 (Williams) in which the

elicitor competent state was either not induced or very poorly induced by wounding, and wound exudate from these lines was inactive in inducing competency in the Williams background. The lack of activation by orthovanadate and the lack of the development of effective endogenous competency in L83-570 suggests that it may be compromised in some way in the establishment of the competent state. Once again, genes associated with the *Rps3* locus do not have an obvious effect in the Harosoy background. HARO 32XX (*Rps3*) shows little alteration in response, and while PRX 146-36 (*Rps3b*) shows greatly diminished response, this may be due again to the absence of *Rps7* or a closely linked gene. Although a negative effect of an *Rps* gene on elicitation competency may seem counter to the classically proposed role of glyceollin in race specific resistance, it is important to point out that the correlation of glyceollin accumulation *per se* to the expression of *Rps* genes has only been studied exhaustively in a very few race-cultivar combinations. The activation of elicitation competency for glyceollin accumulation may very well detract from the phenylpropanoid resources available for other responses, such as phenolic polymer deposition.

The super (*nts382*, *nts1007*) and non-nodulating (*nod49*, *nod139*) mutants of Bragg are altered in their symbiotic properties. The supernodulators form nodules in both the presence and absence of nitrate whereas non-nodulators are unable to nodulate and even lack curled root hairs when inoculated with *B. japonicum* [7]. Finally, the “*nts*” mutants are defective in autoregulation of nodulation, a host feedback process which optimizes the nodule number and development in normal soybeans [6]. None of these mutations appeared to affect elicitation competency to the glucan elicitor.

In conclusion, the results suggest that while Williams isolines require the exogenous presence of wound-associated competency factors for the glyceollin response of minimally wounded soybean cells to *P. sojae* wall glucan elicitors, the partial inherent competency of Harosoy and Bragg cultivars suggests that the presence of these factors might not be a universal prerequisite for response to elicitor. These lines may provide useful research tools to uncover some of the elements controlling elicitation competency. The results also suggest that genes linked to several *Rps* loci may condition some elements of competency. Genes linked to *Rps7* may be associated with the inherent competency of Harosoy. Though more complex, the data also suggest that genes linked to the *Rps1* and *Rps3* loci may condition elicitation competency in the Williams, but not the Harosoy background.

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