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GENETIC AND DEVELOPMENTAL CONTROL
OF SOYBEAN COMPETENCY FACTORS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

by

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To my daughters, Sanna and Alina
ACKNOWLEDGEMENTS

I would like to express my gratitude to my adviser, Dr. T. L. Graham for his thoughtful suggestions and guidance throughout the research. I am also very thankful to the other members of my committee, Dr. A. F. Schmitthenner, Dr. S. A. Miller and Dr. W. Dietz Bauer for their suggestions and help.

Also, I would like to thank Dr. Madge Graham and other colleagues in the Laboratory for their help and encouragement. I would like to thank the Governments of Azad Jammu & Kashmir and Pakistan, and the USAID mission in Pakistan for their cooperation and financial support throughout the study.

I also acknowledge The Ohio State University and Department of Plant Pathology for their financial support during the Autumn quarter, 1994.

Finally, I express my special thanks to all my family members, particularly my parents for their patience and prayers, and my wife, Tubbasam, for her cooperation, help, and encouragement throughout the study.
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FIELD OF STUDY

Major Field: Plant Pathology

Biochemical and genetic aspects of host-pathogen interactions under the direction of Dr. Terrence L. Graham.
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Plants are capable of defending themselves against a variety of stresses including microbial attack. In some cases, they gain this capacity by acclimatizing to these conditions by continuous exposure while in other cases, it involves a response to a specific event. Although all plants are exposed to a wide range of infectious agents, they are attacked by only a few of them and show complete resistance to others. Hence it is believed that disease or susceptibility is the exception whereas resistance is the rule, under natural conditions. Plants utilize different defense strategies which are both structural and biochemical in nature to defend themselves. Both these categories of defense mechanisms can be preformed as well as inducible.

The preformed resistance mechanisms, which exist in the host before infection, involve barriers such as the cuticle and cell wall which physically resist the invasion of the pathogen, or antimicrobial compounds which are present constitutively and usually in conjugated forms (Anderson, 1982; Keen, 1992). Although the preformed resistance mechanisms play a very crucial role in avoiding the pathogen invasion, these will not be discussed in this dissertation.
The active, induced or post-infectional defense mechanisms play a major role in overall resistance. These include phytoalexins, wall-bound phenolics (like lignin and suberin), callose, hydrolytic enzymes, and the hydroxyproline-rich glycoproteins (HRGPs) (Bell, 1981; Sequira, 1983; Barz et al., 1990; Harborne, 1990; Anderson, 1991; Keen, 1992).

Phytoalexins are low molecular weight antimicrobial compounds which are both synthesized by and accumulated in plants locally near the site of challenge (Paxton, 1981). These are not present in healthy plants but are synthesized upon induction. Accumulation of phytoalexins is considered a late event because infection first leads to the activation of genes for early phenylpropanoid and flavanoid metabolism and somewhat later to the activation of genes for phytoalexin accumulation.

Deposition of covalently-linked, cell wall phenolics, which is an early host response to infection or stress (Graham and Graham, 1991c), is also locally induced and plays an important role in further strengthening the structural barrier, the cell wall (Grisebach, 1981). The hydrolytic enzymes, β-1,3 glucanases and chitinases, are accumulated both locally and systemically and are thought to be involved in degrading the pathogen wall and releasing elicitor fragments (Boller, 1987). They are responsible for the death of the pathogen as well as for the induction of host defense responses. HRGPs, which are host structural cell wall
proteins, are also accumulated locally as well as systemically upon infection and are involved in the reinforcement of the plant cell wall (Esquerré-Tugayé et al., 1979; Roby et al., 1985).

The triggering of the above mentioned and other defense responses is the outcome of the recognition process between host and pathogen (Keen, 1982; Keen, 1990; Keen, 1992). Generally, when a pathogen is recognized by the host, the outcome of the interaction is the induction of host resistance responses, an incompatible interaction. In the compatible or susceptible reaction between host and pathogen, the host defense responses are either not triggered at all or their induction is delayed until the pathogen has already been established. As described below, some specific molecules, for example cell wall glucans from the pathogen and cell wall galacturonides from the host are involved in this recognition process.

As mentioned above, the majority of plant species show resistance to most pathogens. In other words, most plant species are non-host to a majority of the pathogens or most of the plant pathogens are non-pathogenic on a majority of plant species. Either these pathogens are specifically recognized by the non-host plant species or they are unable to suppress a general recognition event, resulting in the expression of an array of host resistance mechanisms. This kind of resistance is known as general or non-host resistance. Callow (1987)
refers to it as basic incompatibility at the species level.

Some plant species, however, show a susceptible reaction with a given set of pathogens. These pathogens somehow manage to avoid recognition and establish themselves in their respective host without turning on the host defense responses. This kind of interaction between potential pathogens and their susceptible host plant species is termed basic compatibility at the species level (Callow, 1987). Even within these susceptible host plant species, some cultivars may show a resistance reaction against some races of the pathogen while a susceptible reaction against others. According to Callow’s model (1987), these are referred to as incompatibility and compatibility at the cultivar level, respectively.

Breeding for resistance is a continuous and economic effort to develop risk-free disease control strategies. It is this effort that leads to new lines resistant against the existing races of the pathogen, but at the same time providing grounds for the development of new races of the pathogen which can defeat the pre-existing resistant lines.

In incompatibility at the cultivar level, through the breeding efforts a previously non-recognized race of the pathogen is recognized and host defense is triggered, resulting in the inability of the potential pathogen to cause a susceptible reaction. New races of the pathogen develop quickly, resulting in the conversion of an incompatible interaction into a compatible one. A very recent study on the
fungal tomato pathogen, *Cladosporium fulvum*, shows that even a change in a single base-pair in an avirulence gene of the pathogen can lead to conversion of a resistance reaction into a susceptible one (Joosten et al., 1994). The pathogen again becomes able to avoid the recognition process.

*Phytophthora sojae* [Kauf. and Gerde.] is a facultative biotroph and soil-borne fungal pathogen which causes stem and root rot in susceptible cultivars of the host soybean (*Glycine max* (L) Merrill). It infects all the vegetative parts of the host, and under favorable conditions for disease development, it causes serious economic losses (Sinclair, 1982). Dominant resistance genes (*Rps* genes) in soybean condition race-specific resistance against this disease (Athow et al., 1980; Ploper et al., 1985; Buzzell et al., 1987).

The *Rps* genes in soybean, which occur at seven different loci with multiple allelic forms at two of these loci, and several races of the pathogen (there now may be more than 35, A.F. Schmitthener, personal communication) behave in a gene-for-gene manner (Keen, 1982b). The specific interaction between *Rps* genes and different races of *P. sojae* is defined as either compatible or incompatible depending upon the genotypes of the interacting agents (Schmitthener, 1985). When a race of the pathogen causes a hypersensitive-like reaction (HR) in a given cultivar of the host, the interaction is defined as incompatible. When a race of the pathogen is successful in defeating a given *Rps* gene the interaction is
said to be compatible.

The *P. sojae*-soybean association has long been proven to be a model system to elucidate the various aspects of host-pathogen interaction. For example, this model system has been extensively used to study disease resistance mechanisms. The phenylpropanoid-derived induced resistance mechanisms are involved in providing resistance to soybean against *P. sojae* infection (Ebel, 1986; Ebel and Grisebach, 1988, Graham and Graham, 1991c). In incompatible interactions, the defense response involves the accumulation of phytoalexins, the glyceollins, isoflavone conjugates, and phenolic polymers.

The *Rps*-mediated race-specific resistance against *P. sojae* correlates well with the accumulation of the glyceollins both in timing and magnitude (Stösssel et al., 1980; Keen and Yoshikawa, 1982; Hahn et al., 1985; Ebel and Grisebach, 1988; Graham et al., 1990). This pterocarpan phytoalexin, which occurs in four different isomers (glyceollins I-IV) (Burden and Bailey, 1975; Lyne and Mulheirn, 1978; Sims et al., 1972), appears to play a major role in restricting the pathogen development in the hypersensitive cell lesion. Its accumulation is more rapid and of higher magnitude in incompatible interactions. The importance of phytoalexins in this host defense response is also evident from the study of Moesta and Grisebach (1982), in which they used a specific inhibitor of an early enzyme of the glyceollin pathway. By blocking the biosynthesis of glyceollin, they were able to
demonstrate a conversion of an incompatible interaction into a compatible one.

The above mentioned inducible defense responses are not only triggered by infection, but also by specific pathogen molecules known as elicitors (Darvill and Albersheim, 1984; Yoshikawa, 1983; Yoshikawa et al., 1993). These external signal molecules from pathogens are released during infection in a host-dependent manner (Yoshikawa et al., 1981; Keen and Yoshikawa, 1983) or even released naturally during early stages of cyst germination without the presence of host glucanases (Ebel et al., 1993). Perception of these pathogen-derived signals by the host, and intracellular transduction of the message leading to the expression of defense responses, are common steps in inducible defense mechanisms.

The P. sojae-soybean association has been widely used to explore these different levels of signal perception and transduction. One of the best characterized elicitors of fungal origin is a β-linked glucan from P. sojae (Sharp et al., 1984). It is a multicomponent elicitor with the most potent activity residing in a branched β-1,3/β-1,6 heptaglucoside which is effective in the nanomolar range in glyceollin induction in the soybean cotyledon. In contrast to above strategy (i.e. partial acid hydrolysis) to determine the active elicitor fragment, Yoshikawa and coworkers (1981, 1988) used the host β-1,3 endoglucanases. These authors linked the highest glyceollin activity with the largest wall glucan
fragments. The generation of this active elicitor fragment in planta during infection can be attributed to a host β-1,3 endoglucanase which is now cloned (Takeuchi et al., 1990b). This also led to the development of transgenic tobacco plants that harbor a cloned glucanase cDNA and that are resistant to a different group of pathogens (Yoshikawa et al., 1993).

The pectic oligomers, α-1,4-D-galacturonides from plant cell walls, are also heterogeneous in nature. They have also been proposed to elicit glyceollin in infected tissues (Nothnagel et al., 1983). A synergistic role of both biotic elicitors of pathogen and host origin has been reported (Davis et al., 1986).

These and other race non-specific elicitors elicit different host defense responses in a host and cultivar non-specific manner. The cell walls of fungi are mainly composed of glucans and chitinous compounds. It appears that these act as signal molecules which are recognized by the host and so are responsible for general or non-host resistance (Yoshikawa et al., 1993). Race-specific elicitors have also been characterized in several host-pathogen systems (Keen, 1975; Anderson, 1980; Dewit et al., 1988).

Preliminary investigations on mode of action of elicitor and signal transduction suggest the presence of elicitor binding sites on the plant plasma membrane. This elicitor-receptor binding ultimately leads to the expression of the host defense responses after a cascade of biochemical events
(Cheong and Hahn, 1991; Cheong et al., 1991; Cosio et al., 1992; Yoshikawa and Sugimoto, 1993b).

Internalization of the signal or involvement of a secondary messenger cannot be overlooked if one accepts the presence and interaction of the above mentioned signals of host and pathogen origin. Even the induction of defense-related genes and products away from the site of infection or wounding implies the involvement of intercellular signal mechanisms. Several studies suggest the involvement of Ca\(^{2+}\) ions (Stäb and Ebel, 1987), generation of active oxygen species through GTP-binding protein-mediated signal transduction, (Apostol et al., 1989a; Legendre et al., 1992; Chen et al., 1993; Degousée et al., 1994; Vera-Estrella et al., 1994), salicylic acid (Raskin, 1992), jasmonic acid or its methyl ester (Staswick, 1992), protein kinases, and inositol phosphatides in the plant signal transduction network.

The multiplicity of phenylpropanoid defense responses in various cell populations of soybean tissues challenged with *P. sojae* or its elicitors and the heterogeneity of polysaccharide elicitor preparations, as mentioned above, have led our laboratory to suggest the presence of multiple signals (Graham, 1994a). Two types of signals are defined, i.e primary and secondary. The primary signals or elicitors, as discussed above, are responsible for triggering the various defense responses in soybean tissues. These include $\beta-1,3/\beta-$
1,6 heptaglucosides from the pathogen and α-1,4-D-galacturonides from the pectic fraction of host cell walls. Other primary elicitors are the glyceollin-specific elicitor (GSE) and isoflavone-specific elicitor (ISE) from the pathogen cell wall, and the phenolic polymer-specific elicitor (PSE) of host origin (T.L. Graham, unpublished results). The secondary signals, which are not well defined, may condition or modify the specific phenylpropanoid responses (Graham, 1994a). These secondary signals or cellular processes, as described below, are of host origin and are released during the wounding of soybean tissues.

The classical cut cotyledon assay is routinely performed in our and other laboratories to facilitate the application of elicitor preparations on the wounded surface (Frank and Paxton, 1971; Graham et al., 1990; Graham, 1991b). It also led to definition of the various phenylpropanoid responses in various cell layers (i.e proximal and distal) which are coordinated both temporally and spatially. The proximal cell responses include the accumulation of glyceollin and deposition of phenolic polymers, whereas distal cell responses involve accumulation of isoflavone conjugates of daidzein and genistein (Graham and Graham, 1991b). Other cotyledon assays, like the washed cotyledon assay, cotyledon restoration assay, and minimal-wounding cotyledon infiltration assay are also performed in our laboratory (Graham, 1994a; Graham and Graham, 1994a). Using these various assays has allowed a comparison
of the effects *P. sojae* wall glucan elicitor under maximal and minimal wound conditions and has led to the discovery of wound-associated competency factors which are required for the proximal cell responses of soybean to *P. sojae* wall glucan elicitor. For instance, in the minimal wound cotyledon infiltration assay there is a marked decrease in response and a significant increase in the amount of elicitor required for half maximal glyceollin response as compared to maximal wound (i.e. cut cotyledon) assays. This suggests the importance of wounding in glyceollin elicitation and elicitor activity. Another parallel approach to test the significance of wounding was the use of washed cotyledon and cotyledon restoration assays. Washing the cut surface of cotyledons immediately after wounding greatly diminishes their glyceollin response to glucan elicitor. Restoration of wound exudates to the washed surfaces of cotyledons prior to elicitor application restores the glyceollin response and elicitor competency (Graham, 1994a; Graham and Graham, 1994a).

During the process of investigating the competency phenomena, another cellular assay of particular benefit named the snapped cotyledon assay was developed (Graham, 1994b). A detailed description on this assay will appear elsewhere. It is very advantageous over the washed cotyledon and cotyledon restoration assay. Washing of cotyledon is not always completely effective and it leaves residual effects that complicate the interpretation of results. In other words, it
does not always give a blank background. The snapped cotyledon assay is a minimal wound assay and it clearly identifies wounding as a prerequisite for elicitor activity or competency.

Two parallel approaches have been used to characterize the competency factors (CFs). One approach is to investigate the wound exudate, which is a direct source of these CFs, and the other is to test various purified signal molecules as mimics of these CFs. Initial characterization of the CFs suggests the presence of at least two host factors (CF-1 and CF-2) besides the host 6-1,3 endoglucanases, which release the active elicitor fragment (GSE) from the *P. sojae* wall glucan (Yoshikawa et al., 1981). CF-1 is a "response factor" which increases the overall sensitivity of the responding soybean cells to the elicitor and is required particularly for the phenolic polymer response. CF-2 is considered a "gating factor" which specifically shifts the induced responses into glyceollin accumulation (Graham, 1994a; Graham and Graham, 1994a).

It has been hypothesized that during wounding or during the process of HR cell death, these CFs are released and make the neighboring cells competent to respond to elicitors (Graham and Graham, 1994a). It seems possible that during incompatible interactions, HR is a natural source of wounding that causes the release of CFs. Since HR is a universal, race cultivar-specific resistance response, and since a race-
specific elicitor from *P. sojae* has not been characterized yet, it seems possible that further studies on these CFs might give us an insight into host-pathogen interactions at a race-specific level and help resolve the important issues of signal transduction as well. The purification and further characterization of these CFs is still at a preliminary stage.

To better understand this competency phenomena, we have a long list of questions in front of us; for instance, the nature, stability, and specificity of these CFs, their link to host *Rps* genes, their regulation, the effect of age and developmental state of the host on these CFs, the presence or absence of receptors, their mode of action, etc. Some of these questions have already been answered during preliminary characterization of these CFs. Some may not be answered until after the purification of these factors. Questions on genetic link, age and developmental regulation, and specificity of these CFs are addressed here. These are presented below as three separate primary objectives of the study:

a) to examine the possible effects of soybean genotype on the elicitation competent state,
b) to characterize the developmental and age-related regulation of soybean CFs,
c) to determine the specificity of CFs from soybeans, other legumes and non-legumes to the soybean system.

Each objective of the study is discussed as a separate chapter.
Chapter I

Possible Effects of Soybean Genotype on the Competency Factors

INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) is an important oilseed and grain legume crop grown worldwide. Its importance, particularly for plant breeders and plant pathologists, also resides in the fact that it is a potential source of dominant host genes (*Rps*) which confer resistance to *Phytophthora sojae*, a fungal pathogen which incites seedling damping off and root and stem rot (Sinclair, 1982). Fourteen *Rps* genes have been described so far at seven different loci, with multiple allelic forms at locus 1 and 3 (Athow et al., 1980; Ploper et al., 1985; Buzzell et al., 1987; Andeson and Buzzell, 1992).

More than 27 races of the pathogen (there now may be 35, A.F. Schmitthener, personal communication) have been identified based on their specific interactions with the host *Rps* genes (Schmitthenner, 1985). A resistant or susceptible outcome of the interaction between a race of the pathogen and a specific *Rps* gene of the host is termed as incompatible or
compatible, respectively. An incompatible interaction is characterized by a universal resistance response of the host to the pathogen known as hypersensitive reaction (HR). HR is an early host response to the pathogen invasion characterized by the death of the cells immediately in contact with the pathogen. Subsequently, phenolic polymer deposition and later the rapid and large accumulation of phytoalexins, the glyceollins, in the surrounding cells further restrict the pathogen spread. In compatible interactions the pathogen invades the host without a host necrogenic reaction. The interaction is rather typified by a chlorotic host response with a delayed accumulation of host defense compounds.

It is now a well agreed fact that there is a strong correlation between Rps-mediated race-specific resistance and accumulation of the pterocarpan phytoalexins, an induced and localized host response, as revealed by a number of studies (Stössel et al., 1980; Keen and Yoshikawa, 1982; Hahn et al., 1985; Ebel and Grisebach, 1988; Graham et al., 1990).

The compatible and incompatible interactions have also been studied at the enzyme level, particularly those enzymes involved in early phenylpropanoid metabolism and glyceollin biosynthesis. The induction, both in timing and magnitude, of these enzymes also follows the same race-cultivar specific pattern as reported for glyceollin accumulation (Bonhoff et al., 1986). The level of these enzymes increase concomitantly with glyceollin accumulation in incompatible interactions.
The defense responses induced during *P. sojae* infection of soybean can also be studied with pathogen-derived molecules (β-glucans from the cell wall) known as elicitors. The β-1,3/β-1,6 branched heptaglucoside from *P. sojae*, one of the best characterized elicitors, elicits glyceollin accumulation in the soybean cotyledon (Sharp et al., 1984).

This pathogen wall glucan elicitor has proven a powerful tool in exploring the molecular aspects of *P. sojae*-soybean interactions, for instance, signal perception and transduction. Though this *P. sojae* elicitor is very useful for biochemical studies of plant resistance responses, it no longer shows the race-cultivar specificity as found in infection studies. It triggers the isoflavanoids and other defense responses in a cultivar and race non-specific manner. The universally susceptible soybean cv. Williams, which has no known *Rps* genes, responds to *P. sojae* wall glucan elicitors with a full range of defense responses.

Infection and elicitor studies using soybean cotyledon tissues has led our laboratory to more precisely define the coordination of various phenylpropanoid-derived defense responses in various cell populations of cotyledons (Graham et al., 1990; Graham and Graham, 1991a; 1991b). At least three distinct cell zones appear to be involved in the overall resistance response. The first, possibly only 1 cell in thickness, is the HR cell lesion. Cells undergoing this response are in immediate contact with the advancing hyphae.
In elicitor studies, this first zone of cellular response is not seen but can be artificially mimicked by wounding. A second zone of healthy cells, perhaps only 2-4 cells in thickness, immediately outside the HR cell lesion or the point of elicitor treatment is known as the proximal cell zone. The defense responses in this proximal cell population include: a) phenolic polymer deposition (Graham and Graham, 1991a); b) hydrolysis of isoflavone conjugates into genistein, an antibiotic directly toxic to *P. sojae* (Rivera-Vargas et al., 1993) and daidzein, a precursor for glyceollin biosynthesis (Ebel, 1986); and c) finally, the accumulation of the pterocarpan antibiotic, glyceollin (Ebel, 1986). It seems reasonable that these proximal cell responses work together to restrict the further spread of the fungus and also add to the toxic environment of the HR cell lesion, as has been hypothesized (Graham and Graham, 1994).

Finally, another zone of healthy cells, beyond the proximal cell layer and distal to the zone of initial treatment or infection, responds to elicitor or infection with a massive build up of conjugates of daidzein and genistein (Graham and Graham, 1991b). This large accumulation of isoflavone conjugates increases the defense potential of these distal cells. Conjugation may also reduce the cellular toxicity of the isoflavones as well. The very first cell zone (i.e the HR cell lesion or the wounded surface of the cotyledon for elicitor application) releases additional wound-
associated signals which are actually required for some of the proximal cell responses (Graham, 1994a; Graham and Graham, 1994a). The distal cell responses, which are induced several cells away from the site of infection or elicitor application do not depend on these wound factors (Graham, 1994a; Graham and Graham, 1994a).

The wound-released signals, required for proximal cell responses (Graham, 1994a; Graham and Graham, 1994a), are of host origin and are released during the process of HR cell death or wounding of soybean tissues for elicitor application. These signals are known as competency factors (CFs) because they make the proximal cells competent to elicitor response. Preliminary characterization and fractionation of these wound factors or signals indicate the presence of at least two host factors (CF-1 and CF-2) which make the wall glucan elicitors competent to elicit glyceollin and phenolic polymer responses. These factors are in addition to a previously described "elicitor releasing factor" which is a host enzyme, β-1,3 endoglucanase, responsible for releasing the glyceollin-specific elicitor (GSE) from the pathogen wall glucan (Yoshikawa et al., 1981).

CF-1, also known as the response factor, is heat labile. Its activity is associated with the nonsoluble fraction or low speed centrifugal pellet of crude extracts. It is required for the phenolic polymer response of proximal cells of soybean cotyledons and its effects can be mimicked by reduced
glutathione (GSH) (Graham and Graham, 1994a; 1994b; 1994c). CF-2, which is also given the name "gating factor", is relatively heat stable and required for glyceollin response (Graham and Graham, 1994a). Its effects can be mimicked by a proton pump inhibitor, orthovanadate (Graham and Graham, 1994b).

GSH is a thiol tripeptide (\(\gamma-L\)-glutamyl-L-cysteinyl-glycine) found in many living organisms. A closely related molecule, homoglutathione (\(\gamma-L\)-glutamyl-L-cysteinyl-\(\beta\)-alanine), is found in some cases, and appears to be the major biological thiol in soybean. GSH is involved in several metabolic events (Meister and Anderson, 1983). Besides its role in protection against oxidative damage by removing the free radicals (Rennenberg, 1982; Meister and Anderson, 1983), it has recently been shown to be involved in a massive induction of gene transcription for enzymes of basic phenylpropanoid metabolism and cell wall hydroxyproline-rich glycoproteins (HRGPs) in suspension cultured-cells of bean (Phaseolus vulgaris L.) (Wingate et al., 1988). This pattern of gene activation was identical to one observed with treatment of bean cells with elicitor preparations from cell walls of Colletotrichum lindemuthianum (Sacc. et Magn) Bri. et Cav. (Wingate et al., 1988). These authors proposed a role of GSH in signal transduction during the elicitor-mediated induction of phytoalexin. Likewise, Edward et al., (1991) reported on elicitation of the phytoalexin response in cell
suspension cultures of bean (*Phaseolus vulgaris* L.), but not of alfalfa, with GSH. However, an increase in endogenous tripeptide thiol levels was observed in elicitor-treated suspension cell cultures from both bean and alfalfa (Edward et al., 1991). GSH also induces pisatin accumulation in pea (Yamada et al., 1989) and causes a massive deposition of phenolic polymers in wounded soybean cotyledon (Graham and Graham, 1994b). Finally, as noted above, Graham and Graham (1994b; 1994c) have shown that GSH also restores CF-1 related glucan elicitor competency in non-wound assays.

Vanadate is known to inhibit plasma membrane ATP phosphohydrolase (ATPase) (Perlin and Spanswick, 1981; Gallagher and Leonard, 1982; Serrano, 1989) in various plant tissues. Also at higher concentrations (>50 μM), it is a known inhibitor of plant phosphatases (Saxe and Rajagopal, 1981). Hattori and Ohta (1985) reported that vanadate stimulated the accumulation of isoflavone glucosides and a transient increase in PAL activity in suspension-cultured cells of red bean (*Vigna angularis*). Moreover, the activities of some enzymes involved in stilbene synthesis in cultured cells of *Arachis hypogaea* L. were increased by orthovanadate in a similar pattern as induced by a fungal elicitor (Steffens et al., 1989). Recently, vanadate treatment of suspension cultured-cells of a liverwort, *Calypogeia granulata*, led to the enhancement of the constitutive levels of 1,4-dimethylazulene, a sesquiterpene, and the cooperative increase
in intracellular GSH levels and in activity of some of the enzymes involved in active oxygen-scavenging systems (Nakagawara et al., 1993). More recently, in a preliminary report Graham and Graham (1994b) have shown that orthovanadate is a potential mimic of wound-associated elicitor competency factor CF-2 and restores the glyceollin response of proximal soybean cotyledon cells to *P. sojae* wall glucan elicitors in non-wound assays.

Purification and further characterization of soybean CFs is still in the initial stages. Until these factors are purified and fully characterized, we have at least two options, as mentioned above, to gather more information on these wound-released signals. One option is to use mimics of CF-1 and CF-2 (i.e glutathione and vanadate, respectively) and other option is to use wound exudates (washings) which are a direct source of the CFs. Employing these two approaches, this chapter reports the evaluation of the competency phenomena in Williams and Harosoy isolines with different *Rps* backgrounds and in the cultivar Bragg and its *Bradyrhizobium japonicum* response mutants.
MATERIALS AND METHODS

Chemicals

The elicitor preparation (cell wall glucan) was obtained from race 1 of Phytophthora sojae (Kauf. and Gerde.) according to Ayers et al. (1976a) and as described previously (Graham and Graham, 1991b). The unfractionated and insoluble wall glucan preparation was sonicated and then autoclaved for 3 hours in deionized double distilled water (DDW) to release the soluble, active elicitor fragment (Ayers et al., 1976) prior to application to the cotyledon surface. This elicitor preparation contains both the glyceollin-specific elicitor (GSE) and an isoflavone specific elicitor (ISE) (T. L. Graham, unpublished data). The abbreviation, PSWG or P, will be used throughout, for convenience, to represent autoclaved elicitor fraction.

The glyceollin-specific elicitor (GSE) was prepared by a modification of conditions first reported by Yoshikawa et al., (1981). The unfractionated wall glucan preparation (2 mg of wall glucan preparation in 4 ml of sterilized DDW), obtained as described above, which represents the substrate, was incubated with a Williams cotyledon cell-free extract (1 ml) for two hours at room temperature. Briefly, 0.6 g of seven-to eight-day-old soybean cotyledons was ground in sterilized DDW (total volume 1 ml) directly in 1.5 ml microfuge tubes using a polypropylene pestle. The extract was centrifuged at
13,000 g and the supernatant used as cell free extract. After incubation with wall glucan for two hour, the incubation mixture was boiled (100°C) for 10 min to stop enzyme activity and precipitate all proteins. The boiled fraction was again centrifuged (13,000 g, 10 min.) and the supernatant was passed through a 0.2 μ membrane filter (Gelman Sciences Inc. Ann Arbor, MI) to collect the soluble elicitor fragment. This filtered fraction, which represents enzymatically released GSE, was kept in the freezer (-20°C) until use.

Orthovanadate and reduced glutathione (GSH) were obtained from Sigma Chemical Company St. Louis Mo USA. Stock solutions (50 mM) of these chemicals were prepared in sterilized DDW, and appropriate concentrations (i.e 100 μM for vanadate and 200 μM for GSH as final concentration, unless otherwise mentioned) were prepared fresh from these stock solutions right before the experiment.

Growth of soybean seedlings

Seeds of soybean (Glycine max L. [Merr.] cv. Williams and Harosoy isolines) (Table 1) were kindly provided by Dr. A. F. Schmitthenner (OARDC, Wooster). Seeds of Bradyrhizobium japonicum response mutants of the cultivar Bragg (i.e supernodulators nts382 and nts1007, and nonnodulators nod49 and nod139) were obtained from P. M. Gresshoff (Plant Molecular Genetics, College of Agriculture, University of Tennessee, Knoxville, Tennessee). Seedlings were grown as described previously (Graham et al., 1990) with little
modification. Instead of vermiculite, healthy, sorted seeds were grown in Metromix 360 (Sierra Grace, Milpitas, GA) at 26°c with 500 \( \mu \text{E.m}^{-2}.\text{s}^{-1} \) of light and a 14-hour 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.

Table 1. Williams and Harosoy isolines: a brief description.

<table>
<thead>
<tr>
<th>Isolines</th>
<th>Maturity group</th>
<th>Rps gene</th>
<th>Source of gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Williams isolines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Williams</td>
<td>III</td>
<td>\textit{rps}</td>
<td></td>
</tr>
<tr>
<td>L75-6141</td>
<td>&quot;</td>
<td>\textit{Rps1}</td>
<td>Union</td>
</tr>
<tr>
<td>L77-1863</td>
<td>&quot;</td>
<td>\textit{Rps1-b}</td>
<td>Harrell</td>
</tr>
<tr>
<td>L75-3735</td>
<td>&quot;</td>
<td>\textit{Rps1-c}</td>
<td>Lee 68</td>
</tr>
<tr>
<td>W79</td>
<td>&quot;</td>
<td>\textit{Rps1-c}</td>
<td>Lee 68</td>
</tr>
<tr>
<td>W82</td>
<td>&quot;</td>
<td>\textit{Rps1-k}</td>
<td>Kingwa</td>
</tr>
<tr>
<td>L83-570</td>
<td>&quot;</td>
<td>\textit{Rps3}</td>
<td>PI 86972-1</td>
</tr>
<tr>
<td>B. Harosoy isolines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harosoy 1XX</td>
<td>II</td>
<td>\textit{Rps7}</td>
<td>Mandarin</td>
</tr>
<tr>
<td>Haro 13-3</td>
<td>&quot;</td>
<td>\textit{rps7 + Rps1-b}</td>
<td>PI 84637</td>
</tr>
<tr>
<td>Haro 13XX</td>
<td>&quot;</td>
<td>\textit{Rps7 + Rps1-b}</td>
<td>PI 84637</td>
</tr>
<tr>
<td>Haro 15XX</td>
<td>&quot;</td>
<td>\textit{Rps7 + Rps1-k}</td>
<td>Kingwa</td>
</tr>
<tr>
<td>L70-6494</td>
<td>&quot;</td>
<td>&quot; + \textit{Rps2}</td>
<td>D54-2437</td>
</tr>
<tr>
<td>Haro 32XX</td>
<td>&quot;</td>
<td>&quot; + \textit{Rps3}</td>
<td>PI 171442</td>
</tr>
<tr>
<td>PRX 146-36</td>
<td>&quot;</td>
<td>\textit{rps7 + Rps3-b}</td>
<td>PI 172901</td>
</tr>
<tr>
<td>Haro 43 XX</td>
<td>&quot;</td>
<td>\textit{Rps7 + Rps4}</td>
<td>PI 86050</td>
</tr>
<tr>
<td>Haro 52 XX</td>
<td>&quot;</td>
<td>&quot; + \textit{Rps5}</td>
<td>PI 91160</td>
</tr>
<tr>
<td>Haro 62 XX</td>
<td>&quot;</td>
<td>&quot; + \textit{Rps6}</td>
<td>Altona</td>
</tr>
</tbody>
</table>

XX = designated as 72 (Anderson and Buzzell, 1992)  
Haro 13-3 = gives a susceptible reaction to races 16, 19  
PRX146-36 = gives a susceptible reaction to races 16, 18, 19
**Cotyledon assays**

Eight-day-old cotyledons, unless otherwise mentioned, were harvested in batches and used immediately. The cut cotyledon assay was performed in essentially the same way as described previously (Graham and Graham, 1991b).

A minimal wound snapped cotyledon assay was developed recently in our laboratory. A detailed description of this assay will be presented elsewhere (Graham, 1994b). In this assay, 8-day-old cotyledons, grown and harvested for use as described above for the cut cotyledon assay, were gently snapped, one by one, into two halves. The snapped half which is attached to the hypocotyl was inserted into a petri dish containing 0.5% water agar prepared 4-6 days prior to the assay. The exposed surface was immediately supplied with the respective treatment (15 μL of the test solution, unless otherwise noted). Twenty cotyledons were arranged per petri dish. The plates were incubated in light or dark immediately as described previously (Graham et al., 1990).

**Wound washings from soybean cotyledon tissues**

Cotyledons of appropriate age were obtained as described above. Individual cotyledons were surface sterilized and cut as described for the cut cotyledon assay (Ayers et al., 1976; Graham and Graham, 1991b). The cut surface of each cotyledon was supplied with 15 μL of sterile double distilled water and wound exudate (2-cotyledon equivalent) was collected after transferring the fluid onto and off of the cut surface several
times with a micropipet. Approximately a total volume of 10 μl is obtained during this process of collecting wound exudates from two cotyledons. The wound exudate obtained in this manner was applied immediately to the exposed surface of the cotyledon in the snapped cotyledon assay. This same cotyledon was then immediately supplied with 10 μl of water or elicitor to complete the treatment. This process was repeated until all the 20 cotyledons in a petri dish were treated.

**HPLC analysis**

Treated cotyledons were harvested after 48 hour of incubation. Only the uppermost section in the cut cotyledon assay, unless otherwise noted, was sliced as described previously (Graham, 1991a; Graham and Graham 1991b). Also, in the snapped cotyledon assay, only the uppermost 0.5 mm treated surface or proximal cell layer was sliced off (Graham, 1994b). The 10 or 20 sliced sections per treatment were pooled together and either extracted and analysed immediately for soluble metabolites through HPLC or stored intact at -80°C for later use, as described earlier (Graham, 1991a). Individual peaks were confirmed by running standards and analysing the UV spectra.

All the experiments were repeated at least once, unless otherwise specified.
RESULTS

Evaluation of elicitor competency in Williams isolines

Williams isolines with different Rps backgrounds were analyzed for elicitor response in a minimal-wound, snapped cotyledon assay. After 48 h of treatment of the exposed surface of the snapped cotyledon, the proximal cell layer (0.5 mm treated surface) was sliced off and analyzed by HPLC for isoflavonoid metabolite accumulation. Though we snap the cotyledon in two halves, the exposed surface clearly represents non-wound conditions (Graham, 1994b). Under these circumstances, all the Williams isolines tested are non-responsive to P. sojae wall glucan elicitors in terms of proximal cell responses (i.e glyceollin accumulation and phenolic polymer deposition).

In this dissertation, the accumulation of glyceollin was measured as an indication of proximal cell response. There was no difference in glyceollin accumulation in elicitor and water treated cotyledons. Levels of glyceollin in these tissues averaged 30 nmoles/gm (arrow, Fig. 1 A,B). Although the autoclaved fraction of the wall glucan also contains GSE, we still thought that the host β-1,3 endoglucanases, which are also wound generated, may be the determinative event in glyceollin response. We thus bypassed this event by also using enzymatically released GSE. All the isolines were still lacking the glyceollin response to this enzyme-released
Fig. 1. Restoration of elicitor competency by mimics of CFs in the cotyledon tissues of Williams isolines in a minimal wound assay. The exposed cotyledon surface was supplied with 10 μl of 200 μM orthovanadate (V) or 400 μM reduced glutathione (G) or a combination of both (VG) followed by 10 μl of (A) 40 μg/ml autoclaved (P), or (B) 60 μg/ml enzymatically released (GSE) fractions of P. sojae wall glucan elicitors. The arrow shows the level of glyceollin present in water, Van, GSH, PSWG or GSE treated controls. The values represent the average of two experiments (SE, n=2, ranged from 1.4 to 9.3%).
glyceollin elicitor for the proximal responses. The Wiliams isoline cotyledons were non-responsive for glyceollin accumulation even at 200 μg/ml of elicitor concentration in the snapped cotyledon assay (data not shown). However, both elicitor fractions induced malonyl glycosyl conjugates of daidzein (MGD) and genistein (MGG) in the proximal cell layer (Fig. 2 A, 3 A).

When the exposed surface of the snapped cotyledon was supplied with glutathione or vanadate, mimics of CF-1 or CF-2, respectively, the elicitor competency was restored for the proximal cell glyceollin response in all the Williams isolines tested, though to a varying degree (Fig. 1 A,B). It seems that glutathione is not as strong a restorer of glyceollin response as vanadate. However, it does restore this pterocarpan antibiotic accumulation to levels much higher than the ED$_{50}$ (100 nmole/g tissue). A strong elicitation of glyceollin response was observed with Williams 82 cotyledons (Rps 1-k) when GSH was used as restorer factor for elicitor competency. More than 700 nmoles of glyceollin per gram of tissue (fresh wt.) was recorded in such cotyledons (Fig. 1 A,B).

GSH alone only slightly induces the phenolic polymer response in all the Williams isolines. However, when coapplied with P. sojae wall glucan elicitors, GSH resulted in enhanced levels of phenolic polymer deposition. Such cotyledons turned black. Although GSH alone did not induce
Fig. 2. Effects of *P. sojae* elicitors and mimics of CFs on MGD accumulation in the cotyledon tissues of Williams isolines in a snapped cotyledon assay. The exposed surface of the cotyledons was treated with 15 µl of the test solutions (A) water (H₂O), 20 µg/ml autoclaved (PSWG), or 30 µg/ml enzymatically released (GSE) fractions of *P. sojae* wall glucan elicitors, (B) 100 µM orthovanadate (Van), 200 µM reduced glutathione (GSH), or a combination of both (V+GSH). The values represent the average of two experiments (n=2, SE, ranged from 2.4 to 13.1%)
Fig. 3. Effects of *P. sojae* elicitors and mimics of CFs on MGG accumulation in the cotyledon tissues of Williams isolines in a snapped cotyledon assay. The exposed surface of the cotyledons was treated with 15 μl of the test solutions (A) water (H2O), 20 μg/ml autoclaved (PSWG), or 30 μg/ml enzymatically released (GSE) fractions of *P. sojae* wall glucan elicitors, (B) 100 μM orthovanadate (Van), 200 μM reduced glutathione (GSH), or a combination of both (V+GSH). The values represent the average of two determinations (SE, n=2, ranged from 1.8 to 11.4%).
the biosynthesis of glyceollin, it induced a slight increase in MGD accumulation (Fig. 2 B). Moreover, it was a strong elicitor of MGG (Fig. 3 B) and coumestrol, a pterocarpan phytoalexin without a prenyl group (data not shown). There was no difference in glyceollin response in water- and GSH-treated cotyledons. In contrast, a 1-3 fold increase of MGG was observed with GSH-treated cotyledons over water-treated controls (Fig. 3 A,B).

Orthovanadate, as mentioned above, is an effective restorer of glyceollin accumulation. However, in Williams isolines L75-6141 and L83-570, it was comparatively less effective in restoring the glyceollin response when coapplied with *P. sojae* elicitor (Fig. 1 A,B). It has slight or no effects on daidzein conjugate accumulation when applied alone on the snapped surface of the cotyledon (Fig. 2 B). However, a combination of GSH and vanadate strongly elicited MGD accumulation in the cotyledon cells proximal to treatment (Fig. 2 B). In contrast, in these same cell populations, vanadate suppresses the GSH-induced accumulation of the isoflavone conjugate of genistein (Fig. 3 B). When coapplied with *P. sojae* elicitors, both vanadate and GSH (alone or in combination) enhanced the elicitor-induced accumulation of MGD (compare Fig. 4 A,B to Fig. 2 A,B). However, in the presence of glucan elicitors, vanadate alone or in combination with GSH suppresses MGG levels whereas GSH enhances accumulation of this isoflavone conjugate (compare Fig. 5 A,B to Fig. 3 A,B).
Fig. 4. Effects of P. sojae elicitors and mimics of CFs on MGD accumulation in the cotyledon tissues of Williams isolines in a snapped cotyledon assay. The cotyledons were supplied with 10 μl of 200 μM orthovanadate (V) or 400 μM reduced glutathione (G) or a combination of both (VG) followed by 10 μl of (A) 40 μg/ml autoclaved (P), or (B) 60 μg/ml enzymatically-released (GSE) fractions of P. sojae wall glucan elicitors. The values represent the average of two separate experiments (SE, n=2, ranged from 3.2 to 10.6%).
Fig. 5. Effects of P. sojae elicitors and mimics of CFs on MGG accumulation in the cotyledon tissues of Williams isolines in a snapped cotyledon assay. The cotyledons were supplied with 10 μl of 200 μM orthovanadate (V) or 400 μM reduced glutathione (G) or a combination of both (VG) followed by 10 μl of (A) 40 μg/ml autoclaved (P), or (B) 60 μg/ml enzymatically released (GSE) fractions of P. sojae wall glucan elicitors. The values are means of two separate determinations (SE, n=2, ranged from 0.5 to 7.8%).
Evaluation of competency phenomena in Harosoy isolines

Harosoy isolines, in contrast to Williams isolines, have the \textit{Rps} genes integrated into the Harosoy background. So these lines actually contain \textit{Rps} 7 in addition to the gene which was backcrossed into Harosoy. These isolines were also analyzed for elicitor response in a cotyledon non-wound assay. The results are presented in terms of isoflavone metabolite accumulation as analyzed by HPLC.

In contrast to Williams isolines, all the Harosoy lines tested responded to \textit{P. sojae} wall glucan elicitors alone by accumulating high levels of the pterocarpan antibiotic, glyceollin, in the immediate vicinity of the site of elicitor application (Fig. 6 A). The isolate PRX146-36 (\textit{Rps} 3-b), though responding to both elicitor preparations, i.e. autoclaved and enzymatically released fraction, did not respond as strongly as the other lines tested. Although Harosoy isolines are thus inherently competent for glyceollin elicitation, the mimics of CF-1 and CF-2 (alone or in combinations) when coapplied with \textit{P. sojae} wall glucan elicitors, gave an enhanced induction of glyceollin biosynthesis (Fig. 6 B, 7 A,B). Both elicitor preparations induced MGD accumulation when applied alone on the exposed surface of the broken cotyledon. A 1-5 fold increase of this isoflavone conjugate of daidzein in elicitor-treated cotyledon over water controls is evident from Fig. 8 A. As with Williams isolines, both glutathione and vanadate also cause a
Fig. 6. Inherent competency of *P. sojae* elicitors and effects of orthovanadate on the glyceollin levels in the cotyledon tissues of Harosoy isolines in a non-wound cotyledon assay. The snapped cotyledons were supplied with (A) 15 µl of water or 20 µg/ml autoclaved (PSWG) or 30 µg/ml enzymatically released (GSE) fractions of *P. sojae* wall glucan elicitors or (B) 100 µM orthovanadate (V) with and without *P. sojae* elicitors (P or GSE). The values represent the average of two determinations (SE, n=2, ranged from 3.6 to 11.4%).
Fig. 7. Effects of mimics of CFs on the glyceollin levels induced by *P. sojae* elicitors in the cotyledon tissues of the Harosoy isolines in snapped cotyledon assay. The cotyledons were treated with (A) GSH (200 μM) with and without *P. sojae* elicitors (P or GSE), (B) a combination of orthovanadate (100 μM) and GSH (V+GSH) with and without *P. sojae* wall glucan elicitors. Each data point represents the average of two experiments (SE, n=2, ranged from 4.4 to 10.6%).
slight induction of MGD. Once again, a combination of both mimics induced larger accumulations of this isoflavone conjugate (Fig. 8 B).

Interestingly and contrary to Williams isolines, the *P. sojae* wall glucan elicitors do not induce the MGG levels in Harosoy isolines in the proximal cell population. In fact, there was a 1-2 fold decrease in MGG present in the water-controls when the cotyledons were treated with wall glucan elicitors (Fig. 9 A). Vanadate seems to have a suppressive or no role in MGG accumulation, and glutathione, as seen before, appears to be a strong elicitor of this conjugate (Fig. 9 B). GSH-induced accumulation of MGG was again suppressed (50% or more) in these isolines, as seen in Williams isolines, by vanadate when a combination of both mimics was used as treatment (Fig. 9 B). No significant change in MGD accumulation was observed when GSH or vanadate (alone or in combination) were coapplied with *P. sojae* wall glucan elicitors (Fig. 10 A,B). The level of MGG was decreased as the mimics (alone or combined) were coapplied with wall glucan elicitors on Harosoy background as well (Fig. 11 A,B).
Fig. 8. Effects of *P. sojae* elicitors and mimics of CFS on MGD accumulation in the cotyledon tissues of Harosoy isolines in a snapped cotyledon assay. The exposed surface of the cotyledons was treated with 15 μl of the test solutions (A) water (H2O), 20 μg/ml autoclaved (PSWG), or 30 μg/ml enzymatically released (GSE) fractions of *P. sojae* wall glucan elicitors, (B) 100 μM orthovanadate (Van), 200 μM reduced glutathione (GSH), or a combination of both (V+GSH). The values represent the average of two experiments (n=2, SE, ranged from 1.6 to 7.8%).
Fig. 9. Effects of *P. sojae* elicitors and mimics of CFs on MGG accumulation in the cotyledon tissues of Harosoy isolines in a snapped cotyledon assay. The exposed surface of the cotyledons was treated with 15 μl of the test solutions (A) water (H2O), 20 μg/ml autoclaved (PSWG), or 30 μg/ml enzymatically released (GSE) fractions of *P. sojae* wall glucan elicitors, (B) 100 μM orthovanadate (Van), 200 μM reduced glutathione (GSH), or a combination of both (V+GSH). The values are means of two experiments (n=2, SE, ranged from 2.4 to 7.8%).
Fig. 10. Effects of *P. sojae* elicitors and mimics of CFs on MGD accumulation in the cotyledon tissues of Harosoy isolines in a broken cotyledon assay. The cotyledons were supplied with 10 μl of 200 μM orthovanadate (V) or 400 μM reduced glutathione (G) or a combination of both (VG) followed by 10 μl of (A) 40 μg/ml autoclaved (P), or (B) 60 μg/ml enzymatically released (GSE) fractions of *P. sojae* wall glucan elicitors. The values represent the average of two experiments (n=2, SE, ranged from 0.4 to 7.8%).
Fig. 11. Effects of *P. sojae* elicitors and mimics of CFs on MGG accumulation in the cotyledon tissues of Harosoy isolines in a snapped cotyledon assay. The cotyledons were supplied with 10 μl of 200 μM orthovanadate (V) or 400 μM reduced glutathione (G) or a combination of both (VG) followed by 10 μl of (A) 40 μg/ml autoclaved (P), or (B) 60 μg/ml enzymatically released (GSE) fractions of *P. sojae* wall glucan elicitors. The values are means of two experiments (n=2, SE, ranged from 1.8 to 6.4%).
Response of the cultivar Bragg and its nodulation mutants to *P. sojae* wall glucan elicitor in the cut cotyledon and snapped cotyledon assays.

The cultivar Bragg and its *Bradyrhizobium japonicum* response mutants including the non-nodulating *nod49* and *nod139* (Carroll et al., 1986) and the supernodulating mutants *nts382* and *nts1007* (Carroll et al., 1985) were analyzed for isoflavanoid metabolite accumulation in cotyledon tissues challenged with *P. sojae* wall glucan elicitor.

The elicitation studies employing the cut cotyledon assay revealed that the cultivar Bragg and its variants respond to wall glucan elicitor with isoflavanoid metabolite accumulation (Fig. 12 B). Higher levels of the pterocarpan phytoalexins, glyceollin and coumestrol were detected in cotyledon tissues proximal to the site of elicitor application (Fig. 12 B) than with Williams. In the presence of elicitor, the high levels of MGG present in water control were reduced. Only a slight increase of MGD was observed in elicitor-treated cotyledons over water-treated controls (Fig. 12 A,B).

After removing the proximal cell layer, the same cotyledons were analyzed for distal cell responses, as described previously (Graham and Graham, 1991b). Both conjugates of MGD and MGG accumulated in elicitor-treated cotyledons at levels higher than water-treated controls (Table 2). However, in these same distal cell layers, no coumestrol or glyceollin had accumulated (data not shown).
Fig 12. Effects of *P. sojae* wall glucan elicitor preparation on the metabolite accumulation in the proximal cell layer of cotyledon tissues of the cultivar Bragg and its mutants in the cut cotyledon assay. The cut surface of the cotyledons was either supplied with 30 μl of (A) water, or (B) 20 μg/ml *P. sojae* wall glucan elicitors (PSWG). Each bar represents the actual values of a single experiment.
Table 2. Effect of P. sojae elicitors on the distal cell responses of cotyledon tissues of the cultivar Bragg and its supernodulating mutants 48 h after treatment.*

<table>
<thead>
<tr>
<th>Isoflavone conjugate accumulation (n moles g(^{-1}) of tissue)</th>
<th>MGD</th>
<th>MGG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H(_2)O</td>
<td>PSWG</td>
</tr>
<tr>
<td>Bragg</td>
<td>3491</td>
<td>4986</td>
</tr>
<tr>
<td>nts 382</td>
<td>2014</td>
<td>4376</td>
</tr>
<tr>
<td>nts 1007</td>
<td>3199</td>
<td>3950</td>
</tr>
</tbody>
</table>

* actual values of a single experiment

The distal cell responses were only analyzed in cultivar Bragg and its supernodulating mutants.

Bragg and its nodulation mutants were also analyzed for elicitor competency in a non-wound assay. Like Harosoy isolines, 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 (Fig. 13 A). The mimic of CF-2, vanadate, was ineffective in triggering the glyceollin response. However, when coapplied with elicitor preparation, it effectively enhanced the wall glucan triggered levels of glyceollin (Fig. 13 A). Vanadate slightly increased MGD levels when applied alone, and it lowered the elicitor-induced
Fig. 13. Effects of *P. sojae* elicitor and orthovanadate on the isoflavonoid metabolite accumulation in the cotyledon tissues of the cultivar Bragg and its mutants in a snapped cotyledon assay. The exposed surface of the cotyledons was treated with 15 μl of water, vanadate (van), autoclaved-fraction of *P. sojae* wall glucan (PSWG), or 10 μl of vanadate (200 μM) followed by 10 μl of 40 μg/ml PSGW (V+P). Only the proximal cell layer was analyzed by HPLC for (A) glyceollin (B) MGD, and (C) MGG levels. Each bar represents the actual value (nmoles/g tissue wt.) of a single experiment.

Levels of MGD when coapplied with wall glucan preparation (Fig. 13 B). In contrast, the high levels of MGG present in water controls were suppressed or lowered by this mimic of CF-2, the elicitor preparation from *P. sojae* wall glucan, and a combination of both (Fig. 13 C). The mimic of CF-1, glutathione, was also effective in increasing the wall glucan-induced levels of glyceollin (data not shown) in the proximal cell layer of cotyledon tissues.

**Restoration of elicitor competency with wound exudates from soybean cotyledons**

In addition to analyzing all the Williams and Harosoy isolines screened for elicitor competency with mimics of CFs, we used wound exudates from selected isolines to study elicitor competency. The selection of these isolines was based on their responses to elicitors alone or when a combination of both elicitor and mimics of CF was used. Wound exudates did not induce glyceollin response when applied alone on the exposed surface of the snapped cotyledon (data not...
shown). The glyceollin levels in such wound exudate-treated cotyledons were not more than 35 nmoles/g tissue. However, these cotyledons turned black which is an indication of phenolic polymer response. Moreover, in these same cotyledons, higher levels of MGG were detected and also a slight increase of coumestrol was observed (data not shown).

*P. sojae* wall glucan elicitors, as mentioned above, are unable to induce proximal cellular responses in a non-wound cotyledon assay with Williams isolines. However, as mentioned above, the competency of these elicitors to induce glyceollin and phenolic polymer responses in non-wounded cotyledons of Williams isolines was restored when such cotyledons were supplied with mimics of CFs prior to elicitor application. Similarly, the competency of *P. sojae* wall glucan elicitors to induce these proximal cellular responses was also restored with wound exudates from these selected isolines. When the Williams cotyledons, which give blank background when treated with elicitor preparations in a non-wound assay, were treated with wound exudates prior to elicitor application, enhanced levels of phenolic polymer deposition were observed (only observational data). This was clearly evident from the extremely black color of the treated cotyledons. Such cotyledons also showed the glyceollin response to varying degrees as analyzed by HPLC 48 h after treatment (Fig. 14 A). The wound exudates from Williams isolate, L83-570, and Harosoy isolate, PRX146-36, were not as effective in restoring the
glyceollin response as the wound exudates from other selected isolines (Fig. 14 A). Interestingly, similar results were also obtained with mimics of CFs with these two isolines. The ineffectiveness of wound exudates from these two cultivars thus seems to mirror the lowered ability to establish competency in these lines.

When wound exudates were coapplied with wall glucan elicitor, somewhat higher levels of coumestrol were accumulated in the treated tissues than in the water- or elicitor-treated controls (Fig. 14 A). A similar enhanced accumulation of MGD and a slight decrease in MGG levels were detected in these same treated cotyledon tissues over wall glucan elicitor-treated controls (Fig. 14 B).
Fig. 14. Restoration of *P. sojae* elicitor competency by wound exudates from selected Williams and Harosoy isolines on Williams background in a minimal wound cotyledon assay. The Williams cotyledons were supplied with 10 µl wound washings (2-cotyledon equivalent) from Williams and Harosoy isolines followed by 10 µl of 40 µg/ml autoclaved-fraction of *P. sojae* wall glucan elicitors (PSWG). The treated surface of the cotyledons was analyzed for accumulation of (A) pterocarpan antibiotics, and (B) isoflavone conjugates. The values are means of two determinations (n=2, SE, ranged from 0.6 to 8.4%).
Dose response with mimics of CFs in selected soybean isolines

Three-fold increments in concentration of orthovanadate and reduced glutathione were used to study the restoration of elicitor competency in the above mentioned selected Williams and Harosoy isolines. The exposed surface of the snapped cotyledons was treated with mimics of CFs at increasing concentration with and without *P. sojae* wall glucan elicitor. Only the treated 0.5 mm surface layer was harvested 48 h after treatment and analyzed by HPLC for metabolite accumulation, as mentioned previously.

Interestingly, neither glutathione nor vanadate affected glyceollin induction even at very high concentration when applied alone on the broken surface of the cotyledon. However, when coapplied with *P. sojae* elicitor, vanadate restored the glyceollin inducing ability of the elicitor in Williams isolines (Fig. 15 A) and enhanced glyceollin biosynthesis induced by the elicitor in Harosoy isolines (Fig. 15 B). A similar dose response pattern of glyceollin induction was observed in both Williams and Harosoy isolines though the levels of induction at each concentration of vanadate was different in these isolines. The maximum glyceollin induction by *P. sojae* wall glucan elicitor was observed at 100 μM concentration of vanadate. At higher concentration, it seems that vanadate has a suppressive effect. Vanadate again seems not to be an effective restorer factor for glyceollin induction in the L83-570 background as
Fig. 15. Effects of increasing concentrations of orthovanadate in restoring the elicitor competency in selected Williams and Harosoy isolines in a minimal wound cotyledon assay. The snapped cotyledons of (A) Williams isolines and (B) Harosoy isolines were treated with 10 μl of respective concentration of orthovanadate followed by 10 μl of 40 μg/ml autoclaved-fraction of P. sojae wall glucan elicitors (PSWG). The data points are means of two experiments (n=2, SE, ranged from 2.8 to 7.2%).
as compared to Williams 82 background (Fig. 15 A). The same is also true for PRX146-36 which is a Harosoy isolate. More than 2-fold higher levels of glyceollin were induced in the Harosoy background as compared to PRX146-36 background (Fig. 15 B). Thus these results strongly confirm and underscore the fundamental differences in these selected lines.

In contrast to vanadate, glutathione does not suppress glyceollin induction at higher concentrations (Fig. 16 A,B). Where it is an effective restorer or enhancer factor for glyceollin induction by wall glucan elicitors, it acts progressively over a wide range of concentrations. Glutathione appears to be a relatively strong restorer of glyceollin elicitor competency in the Williams 82 isolate particularly to induce glyceollin biosynthesis even though its effects on the other lines are more of CF-1 nature (Fig. 16 A). In general, it is not as effective as vanadate particularly in Williams isolines. Thus GSH seems to have a possibly specific interaction with the Rps1-k (Williams 82) gene. GSH is equally effective in enhancing the elicitor-induced levels of glyceollin in both Harosoy isolines with higher levels in Harosoy than in PRX146-36 (Fig. 16 B).

Vanadate alone slightly enhances MGD induction and suppresses MGG accumulation at increasing concentration in both selected Williams and Harosoy isolines (data not shown). However, when coapplied with wall glucan elicitor, vanadate
Fig. 16. Effects of increasing concentrations of GSH in restoring the elicitor competency in selected Williams and Harosoy isolines in a minimal wound cotyledon assay. The snapped cotyledons of (A) Williams isolines and (B) Harosoy isolines (B) were treated with 10 µl of respective concentration of GSH followed by 10 µl of 40 µg/ml autoclaved-fraction of P. sojae wall glucan elicitor (PSWG). The values represent the average of two experiments (n=2, SE, ranged from 2.6 to 8.4%).
suppressed both MGD (Fig. 17 A,B) and MGG (Fig. 18 A,B) levels.

Glutathione, at increasing concentrations, slightly induces MGD accumulation in these selected Williams isolines and in Harosoy, but not in PRX146-36 when applied alone on the snapped cotyledon surface (data not shown). These induction levels of MGD are less than those induced by *P. sojae* elicitor, but higher than water treated controls. However, in the presence of both wall glucan elicitor and GSH, the accumulation of MGD levels was enhanced. This was only observed with lower concentrations of GSH. At higher GSH concentrations in the presence of wall glucan elicitors, MGD levels again went down (Fig. 19 A,B).

GSH, as mentioned earlier, is a strong elicitor of MGG accumulation. It induces higher levels of MGG in Williams isolines than in Harosoy isolines (Fig. 20 A,B). In Williams isolines, at increasing concentrations, GSH induced MGG at increasing levels until a maximum around 300 μM of GSH. After this concentration of GSH the MGG levels went down (Fig. 20 A). In Harosoy isolines, unlike Williams isolines, there is no or slight induction of MGG in GSH-treated cotyledons over water-treated controls, but at higher concentration of GSH (more than 300 μM) the levels of MGG also dropped (Fig. 20 B). These GSH-induced levels of MGG were significantly decreased when *P. sojae* elicitor preparation was coapplied with GSH on the exposed surface of the snapped cotyledons (Fig. 21 A,B).
Fig. 17. Effects of increasing concentrations of orthovanadate on MGD levels when coapplied with P. sojae wall glucan elicitor in selected Williams and Harosoy isolines in a minimal wound cotyledon assay. The snapped cotyledons of Williams isolines (A) and Harosoy isolines (B) were treated with 10 μl of respective concentration of orthovanadate followed by 10 μl of 40 μg/ml autoclaved-fraction of P. sojae wall glucan elicitor (PSWG). The data points are means of two experiments (n=2, SE, ranged from 1.2 to 8.4%).
Fig. 18. Effects of increasing concentrations of orthovanadate on MGG levels when coapplied with P. sojae wall glucan elicitor in selected Williams and Harosoy isolines in a minimal wound cotyledon assay. The snapped cotyledons of Williams isolines (A) and Harosoy isolines (B) were treated with 10 μl of respective concentration of orthovanadate followed by 10 μl of 40 μg/ml autoclaved-fraction of P. sojae wall glucan elicitor (PSWG). Each data point represents the average of two determinations (n=2, SE, ranged from 3.4 to 9.6%).
Fig. 19. Effects of increasing concentrations of GSH on MGD levels when coapplied with P. sojae wall glucan elicitor in selected Williams and Harosoy isolines in a minimal wound cotyledon assay. The snapped cotyledons of (A) Williams isolines and (B) Harosoy isolines were treated with 10 μl of respective concentration of GSH followed by 10 μl of 40 μg/ml autoclaved-fraction of P. sojae wall glucan elicitor (PSWG). Each data point represents the average of two experiments (n=2, SE, ranged from 3.4 to 10.2%).
Fig. 20. Effects of increasing concentrations of GSH on MGG levels in selected Williams and Harosoy isolines in a minimal wound cotyledon assay. The snapped cotyledons of (A) Williams isolines and (B) Harosoy isolines were treated with 15 μl of respective concentration of GSH. Only the treated surface of the cotyledons was analyzed for MGG. The data points are means of two experiments (n=2, SE, ranged from 2.1 to 7.8%).
Fig. 21. Effects of increasing concentrations of GSH on MGG levels when coapplied with *P. sojae* wall glucan elicitor in selected Williams and Harosoy isolines in a minimal wound cotyledon assay. The broken cotyledons of (A) Williams isolines and (B) Harosoy isolines were treated with 10 μl of respective concentration of GSH followed by 10 μl of 40 μg/ml autoclaved-fraction of *P. sojae* wall glucan elicitor (PSWG). The data points represent the average of two determinations (n=2, SE, ranged from 1.8 to 9.4%).
Unlike vanadate, GSH at increasing concentrations also induces coumestrol in these Williams and Harosoy isolines when applied alone at high concentrations on the snapped cotyledon surface (Fig. 22 A,B). These GSH-induced levels of coumestrol were significantly enhanced by wall glucan elicitor when a combination of both was used to treat the cotyledons in Williams isolines (Fig. 23 A), but not in Harosoy isolines (Fig. 23 B). In Harosoy and PRX146-36, the GSH-induced levels of coumestrol went down or were not changed by wall glucan elicitor when a combination of both was used (Fig. 23 B).

**Establishment of the elicitor competent state in wounded soybean cotyledons**

It has been established previously that the wound factors induce a competent state that is transient in nature (Graham and Graham, 1994a). The elicitor was applied 1 to 5 h after wounding to washed and non-washed surfaces of the wounded cotyledons. The maximum elicitor response was seen 2 to 3 h after wounding (Graham and Graham, 1994a). However, only Williams cotyledons were used in this study. Here a parallel approach was used to study this inducible and transient nature of the competent state in selected Williams and Harosoy isolines. In this study, however, the wounded cotyledons were not washed and elicitor was applied 0, 1, 2, 4, and 8 h after wounding. The study was conducted both in light and dark conditions. Only the proximal tissues were harvested and
Fig. 22. Effects of increasing concentrations of GSH on coumestrol levels in selected Williams and Harosoy isolines in a minimal wound cotyledon assay. The snapped cotyledons of (A) Williams isolines and (B) Harosoy isolines were treated with 15 μl of respective concentration of GSH. Only the treated surface was analyzed for coumestrol levels. Each data point represents the average of two experiments (n=2, SE, ranged from 2.4 to 7.2%).
Fig. 23. Effects of increasing concentrations of GSH on coumestrol levels when coapplied with P. sojae wall glucan elicitor in selected Williams and Harosoy isolines in a minimal wound cotyledon assay. The snapped cotyledons of (A) Williams isolines and (B) Harosoy isolines were treated with 10 μl of respective concentration of GSH followed by 10 μl of 40 μg/ml autoclaved-fraction of P. sojae wall glucan elicitor (PSWG). The data points are means of two separate experiments (n=2, SE, ranged from 3.6 to 11.2%).
analyzed for isoflavanoid metabolite accumulation by HPLC, as described previously (Graham, 1991c; Graham, 1994b).

As shown in Fig. 24 A, the glyceollin induction pattern by *P. sojae* wall glucan elicitor was similar in various isolines except in L83-570 and PRX146-36 where this competent state of elicitor response was not induced or very poorly induced by the wound or competency factors released during the wounding. The elicitor response was gradually increased as the elicitor application was delayed 1 to 2 h after wounding with the maximum response seen 2 h after wounding. If we waited longer, for instance, 4 to 8 h after wounding, this induced state of elicitor competency again started loosing its elicitor responsiveness.

When the cotyledons were incubated in the dark after wounding and elicitor application, competency was attained gradually over an 8-hour period (Fig. 24 B). Light not only seems to speed up the attainment and magnitude of this inducible competent state but it also appears to regulate the transiency of the induced state.

Incubation of wounded and treated cotyledons in light or dark has no effect on inducing the competent state in Williams isolate L83-570 (Fig. 24 A,B). Moreover, large quantities of free daidzein (Fig. 25 A,B) and genistein (Fig. 26 A,B) accumulated in these same wounded and treated cotyledons of various isolines under dark conditions except in L83-570. In this Williams isolate, higher levels of both daidzein and
Fig. 24. 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 μl of *P. sojae* wall glucan elicitor (20 μg/ml). After 48 h of incubation in (A) light or (B) dark, the treated surface was analyzed for glyceollin. The values are means of two experiments (n=2, SE, ranged from 1.2 to 11.2%).
Fig. 25. Accumulation of higher daidzein levels in elicitor-treated and dark-incubated cotyledons of selected Williams and Harosoy isolines. 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 μl of P. sojae wall glucan (20 μg/ml). After 48 h of incubation in (A) light or (B) dark, the treated surface was analyzed for daidzein. The values represent the average of two experiments (n=2, SE, ranged from 2.8 to 9.8%).
Fig. 26. Accumulation of higher genistein levels in elicitor-treated and dark-incubated cotyledons of selected Williams and Harosoy isolines. 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 μl of P. sojae wall glucan (20 μg/ml). After 48 h of incubation in (A) light or (B) dark, the treated surface was analyzed for genistein. The values represent the average of two separate experiments (n=2, SE, ranged from 3.6 to 12.4%).
genistein also accumulated in light-incubated cotyledons suggesting an inability to incorporate the glyceollin precursor, daidzein, into glyceollin formation.

THP levels were also measured in such wounded and treated cotyledons based on the peak which immediately precedes the glyceollin peak. The extinction coefficient for glyceollin was used to calculate these THP levels. The results shown in Fig. 27 A,B suggest that this immediate precursor of glyceollin biosynthesis is regulated by light. The THP levels were significantly lowered in the dark-incubated cotyledons as compared to those incubated in light. The light-incubated cotyledons turn maximally red 16-24 h after incubation (depending upon the intensity of light) which is an indication of THP production (Zähringer et al., 1981; Graham, 1994b). A decrease in THP levels in the absence of light is strongly correlated with the disappearance of the red color in the dark-incubated cotyledons in all these isolines. The presence of these red pigments might have a role in providing protection against UV damage to exposed soybean cells as has been proposed for anthocyanins in cotton (Pierce and Essenberg, 1987). The induction of THP in the light might be influenced by genistein since in the presence of light genistein levels went down whereas THP levels went up and vice versa in the dark. More detailed studies are needed to establish a link between THP and genistein.
Fig. 27. Light regulation of Trihydroxy pterocarpan (THP) in wounded and elicitor treated cotyledons. 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 μl of P. sojae wall glucan (20 μg/ml). After 48 h of incubation in (A) light or (B) dark, the treated surface was analyzed for THP. Each bar represents the average of two experiments (n=2, SE, ranged from 1.4 to 7.8%).
The wounded and water-treated control cotyledons of these isolines were incubated in light and dark conditions to see the effect of light on MGD and MGG levels. It seems that these isoflavone conjugates are also regulated by light. A 1-3 fold increase in MGD (Fig. 28 A,B) and MGG (Fig. 29 A,B) levels was seen in light- over dark-incubated cotyledons. These results suggest that light might be a common inducer of isoflavanoid metabolite accumulation in soybean cotyledon tissues. Also, the regulation of inducible and transient nature of the competent state is light-dependent.
Fig. 28. Light induction of MGD levels in wounded cotyledon tissues of Williams and Harosoy isolines. 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 μl of water. After 48 h of incubation in (A) light or (B) dark, the treated surface was analyzed for MGD. The values are means of two separate experiments.
Fig. 29. Light induction of MGG levels in wounded cotyledon tissues of Williams and Harosoy isolines. 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 μl of water. After 48 h of incubation in (A) light or (B) dark, the treated surface was analyzed for MGG. The values are means of two separate determinations.
DISCUSSION

It has been established previously that wound-associated elicitor competency factors are required for the proximal cellular responses (glyceollin accumulation and phenolic polymer deposition) of Williams cotyledon tissues to the P. sojae wall glucan elicitor. However, isoflavone conjugate accumulation in the distal cell response does not require the presence of such wound factors (Graham and Graham, 1994a).

In this study, two parallel approaches i.e., the use of mimics of CFs and/or wound exudates (washings) were used to demonstrate this competency phenomena in various Williams and Harosoy isolines (with different Rps background) and in the cultivar Bragg and its nodulation mutants. The research employed both minimal and maximal wound assays, the snapped cotyledon and cut cotyledon assays, respectively.

In minimal wound cotyledon assays, the lack of glyceollin response of all the Williams isolines tested to P. sojae elicitors further confirms that the presence of wound-released factors is a prerequisite for inducing an elicitor competent state, which is required for the proximal cell defense responses. It was also demonstrated that this glyceollin response of minimally wounded soybean cells to P. sojae wall glucan elicitors was reconstituted by the exogenous supply of mimics of CFs or wound exudates from donor soybean tissues (Fig. 1 A,B; Fig. 14 A; Fig. 15 A; Fig. 16 A).
Also as noted before (Graham and Graham, 1994a), the accumulation of isoflavone conjugates is induced in these same minimally wounded and elicitor-treated Williams proximal soybean cells without the need for competency factors. This response, which also occurs in distal cells, is thus the default response of proximal cells to elicitor. These conjugates of daidzein and genistein have been shown to be present constitutively in different soybean tissues (Graham et al., 1990, Graham, 1991b). Thus, their accumulation is apparently under normal tissue-specific and developmental regulation. This implies that they are not solely defense related metabolites. On the other hand, daidzein is a direct precursor of glyceollin and genistein is directly toxic to \textit{P. sojae} (Rivera-Vergas et al., 1993). Thus the accumulation of these conjugates would raise the defense potential of soybean tissues as hypothesized by Graham et al. (1990). The fact that the plant responds to the glucan elicitor with a local and distal massive additional accumulation of these conjugates suggests that the plant may deploy this response as an early and non-crisis (prophylactic) response to infection.

Conclusions regarding the effects of host genetics on elicitation and competency. Interestingly and contrary to Williams isolines, all the Harosoy isolines, and the cultivar Bragg and its mutants, did respond to the \textit{P. sojae} wall glucan elicitors. However, in the presence of exogenous mimics of
CFs, the wall glucan induced levels of glyceollin were further enhanced. Two alternative general explanations for these differences among these cultivars exist. First, competency might not be an essential prerequisite for elicitor response in Harosoy and Bragg as compared to Williams. Alternatively, the cultivars Harosoy and Bragg may be inherently competent to respond to wall glucan elicitors much like tissue cultured cells. Although we can make these general statements, it is difficult to hypothesize a mechanism for these differences until we know more about the underlying molecular mechanisms. Given the fact that vanadate (a proton pump inhibitor) and GSH (a redox buffering and sulfhydryl reagent) can restore elements of competency, it is possible that some elements of ion distribution across the plasmalemma (perhaps the proton pump itself) or some elements related to redox state of the cell or to protein sulfhydryl groups may be different in Williams and Harosoy/Bragg. The precise nature of the differences in these lines or whether the differences are quantitative or qualitative will require further biochemical and genetic analysis.

The background of the cultivars may itself have profound effects on the competency phenomena since all three have different background. The cultivar Williams, which is universally susceptible to all known races of \textit{P. sojae}, originated as the cross from Wayne X L67-0034 (Clark X Adams). It has no known \textit{Rps} gene in its background. The Williams
isolines carrying different Rps genes provide resistance against different races of P. sojae. These Rps genes from different sources are integrated back into Williams background (Table 1, Chapter I). For instance, W82 is a Williams isoline that carries Rps1-k, which confers resistance to races 1 to 4 of the pathogen. The source the Rps1-k gene is Kingwa.

Similarly, the cultivar Harosoy originated as the cross from Mandarin (Ottawa) X A.K. Harrow. Although this cultivar gives a susceptible reaction with race 1 of P. sojae, the RpsH or Rps? (now designated as Rps7) in its background provides resistance against races 12, 16, 18, and 19 of the pathogen (Rennie and Buzzell, 1986; Anderson and Buzzell, 1992). So the Harosoy Rps isolines actually carry the Rps7 gene in addition to the Rps gene of interest integrated back into the Harosoy background.

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 (Graham et al., preliminary results). It is possible that the inherent competency of Harosoy is due to the presence of the Rps7 gene. Consistent with this, PRX146-36 is a Harosoy line in which the Rps7 allele is missing. As shown above, it only shows a slight inherent competency to glucan elicitor. We are currently obtaining a Williams isoline in which the Rps7 allele has been introduced for testing. The ultimate correlation of competency to the Rps7 allele will require
quantitative genetics on specific segregating populations resulting from specific crosses or molecular genetic demonstration.

The cultivar Bragg (maturity group VII) originated as an F_6 plant selection from the cross between Jackson and D49-2491 (S-100 X CNS). The one parent, Jackson, is susceptible to race 1 of *P. sojae* whereas both the ancestors (i.e S-100 and CNS) of the other parent, D49-2491, are resistant to it. In fact, the ancestor CNS does contain *Rps2*. However, the cultivar Bragg has also been shown to give a susceptible reaction to race 1 of *P. sojae* (Rose et al., 1982). The nodulation mutants used in this study were isolated from the cultivar Bragg by induced EMS or γ mutagenesis (Carroll et al., 1985; Carroll et al., 1986). We chose to examine Bragg due to the availability of the nodulation mutants and to evaluate if these mutations might affect recognition events (elicitation and/or competency) with a fungal pathogen as well.

The super (*nts382, nts1007*) and non-nodulating (*nod49, nod139*) mutants 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 *Bradyrhizobium japonicum* (Carroll et al., 1986; Mathews et al., 1987). Finally, the "nts" mutants are defective in autoregulation of nodulation, a host feedback process which
optimizes the nodule number and development in normal soybeans. The supernodulating response is shoot controlled (Delves et al., 1986; Delves et al., 1987) whereas the non-nodulating character is regulated by the root (Delves et al., 1987). None of these mutations appeared to affect elicitation or competency to the glucan elicitor.

The fact that Bragg, like Harosoy, possesses inherent competency is interesting. Unfortunately, very little is known about the specific \textit{Rps} genes present in Bragg, since this is a late maturing cultivar used in southern climates where \textit{P. sojae} is not a major problem. As with Harosoy, quantitative genetic examination of the parents of Bragg and the detailed segregation of competency in specific crosses will be necessary to determine whether this competency is dominant or recessive and due to a single gene or multiple genes. Bragg should also be tested exhaustively with various races of \textit{P. sojae} to better characterize the \textit{Rps} genes present.

Although all three soybean cultivars (Williams, Harosoy, and Bragg) are susceptible to race 1 of \textit{P. sojae}, the wall glucan elicitor-induced levels of isoflavone metabolites are quite different in these cultivars. These differences in isoflavone metabolite accumulation could again be related to the background differences in these cultivars. As mentioned above, the Harosoy background contains \textit{Rps7} whereas ancestors of one of the parents of the cultivar Bragg also gives a
resistant reaction to race 1 of the soybean pathogen.

The above discussion relates to genetic differences between the Williams, Harosoy, and Bragg isolines. There are also several interesting differences between lines within a given isolate series. For example, although the competency of \( P. \text{sojae} \) elicitor to induce the glyceollin response in Williams isolines can generally be restored by orthovanadate (mimic of CF-2), in L83-570 and L75-6141, orthovanadate was not an effective factor for restoration of the glyceollin response (Fig. 1 A,B; Fig. 15 A). Thus, the presence of the \( Rps1 \) (L75-6141) or \( Rps3 \) (L83-570) genes may also condition some component of competency. In isolines L83-570 (Williams) and PRX146-36 (Harosoy), the elicitor competent state was either not induced or very poorly induced by the wounding (Fig. 24 A,B). This suggests that the cotyledon tissues of these lines might be a poor source of wound-released competency factors. When wound exudate from these lines was tested on Williams cotyledon background, it was also not effective in inducing the glyceollin response as compared to wound exudates from other lines (Fig. 14 A). In these two lines, the reduced level of glyceollin biosynthesis may also relate to \( Rps \) background (i.e., \( Rps3 \) in L83-570; \( Rps3-b \) in PRX146-36). Further studies are needed to define specific roles for competency factors in these two Williams and Harosoy isolines. Once again, this will require more exhaustive biochemical and genetic analysis.
Although reduced glutathione does not generally restore the *P. sojae* glucan glyceollin elicitor competency (it has CF-1 and not CF-2 activity), in Williams 82 (*Rps1-k*), GSH more effectively restores and enhances glyceollin accumulation than orthovanadate (Fig. 1 A,B; Fig. 15 A; Fig. 16 A). If one assumes that these isolines are true and have no differences other than the integrated *Rps* gene then it can be hypothesized that the *Rps1-k* gene may condition cells in such a manner that generation of the CF-1 state alone is sufficient for full elicitor competency. Once again, this is a very interesting finding that will require further biochemical and genetic analysis.

The connection of these genes to conditioning of competency is further confirmed by dose-response data and transiency of competency data. Thus, our preliminary genetic analysis of competency suggests several possible and very interesting connections between specific resistance genes and the generation of the competent state. Even specific genetic effects in response to the CF-1 and CF-2 mimics, GSH and vanadate, were found. These differences may provide very powerful tools for future characterization of competency.

Conclusions from these studies on other aspects of competency. Although our studies focused primarily on the initial genetic characterization of competency, several other interesting observations emerged from the work.
Although the effects of GSH are more CF-1 in nature, in Harosoy isolines, it acts as a synergist with _P. sojae_ wall glucan elicitors in enhancing the glyceollin response (Fig. 7 A; Fig. 16 B). Possibly CF-1, but not CF-2 is limiting in Harosoy. Alternatively, the mode of action of GSH may be more complex than CF-1 alone. When applied alone on the exposed surface of the cotyledons, GSH seems to have specific effects on accumulation of malonyl glycosyl conjugate of genistein in all the isolines tested (Fig. 3 B; Fig. 9 B). The accumulation of MGG, which is suppressed by vanadate, has also been shown to be induced by light (Graham, 1994b). Most recently, Graham and Graham (1994c) have proposed that the effects of GSH may encompass more than its CF-1 activity.

Sulfhydryl modifications have long been shown to be implicated in defense response. This has been demonstrated with several known abiotic elicitors (sulfhydryl reagents) that elicit host defense responses most probably by modifying plasma membrane located sulfhydryl groups (Gustine, 1981; Moesta and Grisebach, 1981; Stössel, 1984). Despite its effects as a sulfhydryl reagent, GSH is also a strong reducing agent. In this study, we do not have either direct evidence of sulfhydryl modification or oxidation being involved in GSH-induced phenylpropanoid responses. Such modes of action are quite possible based on GSH’s broad range of effects.
In nearly all elicitation studies, we found that there was an inverse relationship between the induction of glyceollin biosynthesis and the suppression of MGG accumulation. In fact scatter plots of data from over three years of experiments suggest a very high degree of correlation ($r = 0.91$) between glyceollin accumulation and a negative net accumulation of genistein (T.L. Graham, unpublished results). This clearly suggests that either genistein might also serve as glyceollin precursor and/or it might have a regulatory role in the glyceollin response. The presence of the hydroxyl group at position 5 on the A-ring of genistein, but not of daidzein increases the chances of daidzein but not genistein as a glyceollin precursor (Ebel, 1986). However, the possibility of genistein being involved in regulatory or signalling event is very interesting. Genistein has been shown to possess both steroid hormone-like (Saxena and Bhadoria, 1990) and tyrosine protein kinase inhibitor (Akiyama et al., 1987; Linassier et al., 1990) activities. Thus, genistein (which is regulated by light or GSH when applied alone) could conceivably play a regulatory role, perhaps through alteration of specific components of the signal transduction pathways involved in establishment of the competent state.

The establishment of the elicitor-competent state in wounded and elicitor-treated soybean cotyledon tissues has been reported previously as inducible and transient (Graham and Graham, 1994a). These authors proposed that wound factors
released during wounding induce a state of competency in these soybean cells, making them respond to wall glucan elicitor. This induction of the competent state is also affected by light and temperature (Graham and Graham, 1994a).

Here in this study, a similar transient induction of the competent state of soybean cotyledon tissues by wound-released competency factors was demonstrated in various isolines of Williams and Harosoy (Fig. 24 A, B). However, this transient state is modified or abolished in isolines L83-570 and PRX146-36, as mentioned above. The establishment of this P. sojae glucan elicitor competent state was also demonstrated in the cultivar Bragg and its nodulation mutants (data not shown). The cotyledons of these various isolines gave a maximum glyceollin response when elicitor was applied at least 2 h after wounding, as previously noted by Graham and Graham (1994a). However, in the dark, the attainment of elicitor competency was delayed several hours. This suggests that both induction of the elicitor competent state and its transient nature are regulated by light.

The proton pump inhibitor, vanadate, most probably affects or modifies the cellular state of practically non-wounded donor cotyledons enabling them to respond to P. sojae elicitor. Vanadate itself may induce a state of competency in the treated cotyledons. Although here in this study the inhibition of ATPase activity of plant plasma membrane by vanadate has not been demonstrated directly, the concentration
of vanadate used here (i.e., 100 μM) has been shown to inhibit ATPase activities and ion transport in various plant species (Jacob and Taiz, 1980; Dupont et al., 1981; Perlin and spanswick, 1981; Sze and Churchill, 1981; Gallagher and Leonard, 1982; Serrano, 1989). It can be speculated that the generation of electrochemical gradients of protons across the plasma membrane due to inhibition of the H⁺ pump by vanadate might be a direct or indirect signal for induction of the glyceollin response to wall glucan elicitor.

At similar concentrations (>50 μM), vanadate is also known to inhibit plant phosphatases (Saxe and Rajagopal, 1981). Since ATP is the substrate for both ATPase and phosphatases, it is possible that it is this phosphorylation event which might be involved in the signalling process that eventually leads to the induction of the glyceollin response.

Further studies are needed to elaborate the direct effects of orthovanadate on the generation of such a competent state in soybean tissues for glyceollin elicitor response. Such kinds of studies will possibly be more effective once CF-2 factor has been identified and fully characterized.
CONCLUSIONS

In conclusion, our results suggest that 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. However, the inherent competency of Harosoy and Bragg cultivars suggests that the presence of these factors might not be a general prerequisite for elicitor competency. Harosoy and Bragg may be rich in CF-2 state.

Our results also suggest that *Rps* genes may condition some elements of competency. *Rps7* may be linked to inherent competency of Harosoy. The presence of *Rps1*-a and *Rps3*-a in Williams and *Rps3*-b in Harosoy may be responsible for lowered expression of competency. GSH specifically and effectively restores the glyceollin response in Williams isolate W82. This suggests that the *Rps1*-k gene may have a specific interaction with GSH such that the generation of the CF-1 state alone is sufficient for elicitor competency. The data on dose response and timing of establishment of the competent state further confirm the connection of these *Rps* genes to conditioning competency.

In almost all the elicitation studies, we found an inverse relationship of MGG accumulation to glyceollin biosynthesis. This suggests that genistein, a tyrosine-specific protein kinase inhibitor, may have a role in the
defense-related signal transduction pathway.

The establishment of the elicitor competent state in wounded soybean cells requires an interval of 2-h-period after wounding and before applying elicitor for maximum glyceollin response. This induced state of elicitor competency is also regulated by light. The glyceollin response is considered a late event. The time required to induce a state of competency in soybean cells by CFs may be one of the factors involve in late glyceollin response.
Chapter II

Developmental and age related regulation of soybean competency factors

INTRODUCTION

The inducible accumulation of low molecular weight antimicrobial compounds known as phytoalexins is one of the important and major defense mechanisms implicated in providing resistance to soybean \textit{[Glycine max (L.) Merrill]} tissues against attempted infection by \textit{Phytophthora sojae} [Kauf. and Gerde.] (Ebel, 1986; Ebel and Grisebach, 1988). Specifically the production and accumulation of isoflavanoid phytoalexins, the glyceollins, in soybean tissues infected with \textit{P. sojae} is correlated well with differential resistance of soybean cultivars to several races of this fungal pathogen. This correlation has been shown both in timing and magnitude (i.e., rapid and large accumulation of glyceollin in a resistant or incompatible interaction and delayed and reduced levels of glyceollin accumulation in compatible interactions) (Keen and Yoshikawa, 1982; Hahn et al., 1985; Ebel and Grisebach, 1988; Graham et al., 1990).
This race:cultivar-specific resistance is conditioned by several dominant host \( Rps \) genes (14 described at seven different loci) against several known races of the pathogen (Schmitthener, 1985). The \( Rps \) genes in the host are inherited as dominant characters, and also incompatible races of the pathogen are physiologically dominant. Therefore, \( P. \) sojae-soybean system is believed to behave as gene-for-gene (Keen, 1998b).

Glyceollin accumulation can also be induced in soybean tissues with partially purified \( P. \) sojae wall glucan elicitor preparation. However, unlike infection, elicitor preparations trigger this pterocarpan antibiotic in a race:cultivar non-specific manner, i.e., the elicitor preparation from a virulent race of the pathogen can induce host defense responses in both susceptible and resistant cultivars of the host. As yet, a well characterized race-specific elicitor from \( P. \) sojae is not known. The \( \beta \)-glucan elicitor from the cell wall of this soybean pathogen alone cannot induce race-specific resistance. So it is possible that some determinitive event(s) might be occurring in planta which may be required for an HR- or race-specific resistance.

\( P. \) sojae infects all the seedling organs of soybeans and almost all these organs have been analyzed for race-specific resistance, which is characterized by rapid and large accumulations of glyceollin, as mentioned above. Race-specific resistance is expressed differently in different
seedling organs infected with P. sojae. It has been shown that both the expression of race-specific resistance and glyceollin accumulation are under intricate control by several factors such as age and/or developmental state of the specific organ and environmental conditions, particularly light and temperature (Keen, 1971; Lazarovits et al., 1981; Ward et al., 1981; Ward and Buzzel, 1983; Bhattacharyya and Ward, 1986; Ward, 1989; Graham and Graham, 1994a).

The response of a specific organ of the soybean seedling to P. sojae races is particularly influenced by the developmental age of the organ investigated. Paxton and Chamberlain (1968), in a very early study, reported the increasing resistance of mature soybean plants to P. sojae with decreasing levels of glyceollin. These authors suggested the existence of additional mechanisms of resistance in these older tissues. Keen (1971), however, related this age-related increase in resistance to higher levels of glyceollin accumulation in older soybean plants. Ward (1989) showed that immature trifoliate leaves of both susceptible and resistant soybeans are equally susceptible to virulent and avirulent P. sojae races and even to nonhost Phytophthora species. In an earlier study, Bhattacharyya and Ward (1986) demonstrated that tissues of intermediate age show both nonhost resistance and race-specific resistance to P. sojae. However, fully mature leaves and hypocotyls express increasing levels of resistance to P. sojae races irrespective of the presence of a specific
Rps gene (Lazarovits et al., 1981; Bhattacharyya and Ward, 1986).

The developmental age of hypocotyl tissues also has dramatic effects on both expression of race-specific resistance and glyceollin accumulation. In 6-day-old etiolated soybeans, hypocotyl tissues showed increasing resistance to *P. sojae* races from the top (younger) to the bottom (older). Only the upper one-third of the hypocotyl showed race-specific resistance correlating with increasing glyceollin levels (Lazarovits et al., 1981; Ward et al., 1981). The older hypocotyl tissues expressed increasing race non-specific resistance, with low levels of glyceollin accumulation.

The developmental and tissue-specific expression of HR was even reported in a different host-pathogen system. The *Cladosporium fulvum* Avr9 gene product, which is a 28 amino acid cysteine-rich peptide, elicits an HR on tomato lines carrying *Cf-9* (Joosten et al., 1994; Van den et al., 1993). This *Cf-9*:Avr9-mediated necrosis was reported recently to be confined to specific tissues and under strict developmental control (Hammond-Kosack et al., 1994).

In a recent study, Graham and Graham (1994a) have reported that competency of soybean cotyledon tissues to respond to *P. sojae* glucan elicitors through induction of the glyceollin and phenolic polymer responses is markedly affected by the age of these tissues. As the cotyledon tissues
approached senescence, they became more and more inherently competent for proximal cellular responses to wall glucan elicitors. Two wound-released signals of host origin (CF-1 and CF-2) have been described (Graham, 1994; Graham and Graham, 1994). CF-1 is required for the phenolic polymer response whereas CF-2 is necessary for the glyceollin response of proximal soybean tissues to *P. sojae* elicitors. The effects of CF-1 and CF-2 can be reproduced by reduced glutathione and orthovanadate, respectively (Graham and Graham, 1994b; 1994c). The elicitor competency for these proximal cellular responses can also be restored by wound exudates (Graham, 1994a; Graham and Graham, 1994a).

Since it has been hypothesized that wound-associated competency factors are required for glyceollin accumulation, which is a race-specific event in *P. sojae* infected soybean tissues, it is important to redefine the developmental and age related resistance responses in this perspective. This chapter reports on developmental and age-related regulation of soybean competency factors.
MATERIALS AND METHODS

Chemicals

The glucan elicitor preparation was prepared from the cell walls of race 1 of *Phytophthora sojae* (Kauf. and Gerde.) according to Ayers et al. (1976a) and as described previously (Graham and Graham, 1991b). The unfractionated wall glucan preparation was sonicated and then autoclaved for 3 h in deionized double distilled water (Ayers et al. 1976).

The glyceollin-specific elicitor (GSE) was prepared as described previously in Chapter I (Materials and Methods). In brief, the unfractionated wall glucan preparation obtained as described above, was incubated with a Williams cotyledon cell-free extract for two hours. 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) to collect the soluble elicitor fraction. The final concentration of orthovanadate used throughout was 100 µM. This was prepared freshly from a stock solution (50 mM) in sterilized DDW.

Growth of soybean seedlings

Soybean seeds (*Glycine max* L. [Merr.] cvs. Williams and Harosoy) were kindly provided by Dr. A. F. Schmitthenner (OARDC, Wooster). Seedlings were grown as described previously (Graham et al. 1990) with slight modifications as
mentioned in Chapter I (Materials and Methods).

Cotyledon assays

Cotyledons from six- to eleven-day-old seedlings, unless otherwise mentioned, were harvested in batches and used immediately. The cut cotyledon assay was performed in essentially the same way as described previously (Graham and Graham, 1991b). The snapped cotyledon assay was performed as described in Chapter I (Materials and Methods).

Wound washing from soybean tissues

Individual cotyledons were surface sterilized and cut as described for the cut cotyledon assay. The wound exudate or washing was obtained as described previously (Graham and Graham, 1994a) and also as mentioned briefly in Chapter I. Extracellular or apolastic fluids from hypocotyl tissues were obtained as follows. Hypocotyls from the same seedling age and developmental state as routinely used for cotyledon tissues (i.e. 8-d-old) were selected and cut into four 2 cm sections. The cut ends were immediately washed and blotted with sterile filter paper. The four sections represent; the top or younger zone which includes the 2 cm portion immediately below the point of cotyledon attachment, two sections from the middle of the hypocotyl, and the bottom or older zone includes 2 cm above the root zone. Fifteen replicates of each representative zone were loosely tied and placed in sterile DDW in a beaker. Another empty beaker of slightly smaller size was placed inside the beaker containing
the samples to keep the sample immersed. These beakers were placed in a vacuum chamber attached to an aspirator for vacuum infiltration for 5 min. Water was introduced into the apoplastic fluids were collected by low speed centrifugation (3,000 g, 10 min) in microfuge tubes. The extracellular washings obtained were applied to the exposed surface of the cotyledon in a snapped cotyledon assay as described previously.

Wound extracts were also obtained from the above mentioned hypocotyl sections by grinding them in sterile DDW in microfuge tubes. Representative sections were weighed directly into 1.5 ml microfuge tubes (0.6 g in all cases) in 400 µl of sterile DDW. The final volume of the extract was adjusted to 1 ml by the addition of more water. The extract was centrifuged (13,000g, 10 min) and supernatant was used as wound washing as described above.

To obtain non-wounded exudates from different zones of root, a modification of the method described by Graham and Graham (1990) was followed. The roots were grown in essentially the same manner as described by Graham and Graham (1990). However, instead of collecting exudates with cotton wicks, 2-3 cm long sections from different zones of root tissues were cut and placed in sterilized DDW in microfuge tubes (20 sections in 500 µl water). After 1 hour the fluid was removed and used as root exudate as described above.
These exudates from different zones of root also contain a small fraction of wound exudates imparted through the cut ends.

HPLC analysis

Treated cotyledons were harvested after 48 h of incubation. Unless otherwise noted, only the uppermost section in the cut cotyledon assay was sliced as described previously (Graham and Graham 1991b). Also, in the snapped cotyledon assay, only the uppermost 0.5 mm treated surface (proximal cell layers) was excised for assay as described by Graham (1994b). 10 or 20 sections per treatment were pooled together and either extracted and analysed immediately for soluble metabolites through HPLC or stored intact at -80°C for later use, as described earlier (Graham and Graham, 1991c). Individual peaks were confirmed by running standards and analyzing the UV spectra.

All the experiments were repeated at least once, unless otherwise mentioned.
RESULTS

Effects of age on the recostitution of elicitor competency with mimics of CF in Williams and Harosoy cotyledon tissues.

Williams and Harosoy cotyledons from seedlings of different ages (6 to 11 days old) were analyzed for elicitor response in a minimal wound, snapped cotyledon assay. Orthovanadate, which is a mimic of CF-2 (Graham, 1994), restores elicitor competency for the glyceollin response in Williams isolines and enhances the wall glucan-induced levels of glyceollin in Harosoy isolines. The freshly exposed surface of 6- to 11-day-old Williams and Harosoy cotyledons was treated with orthovanadate and P. sojae wall glucan elicitors. After 48 h of treatment the uppermost 0.5 mm treated surface was harvested and analyzed by HPLC for isoflavanoid metabolite accumulation.

There was absolutely no difference in the glyceollin response in Williams cotyledons treated individually with water, vanadate or P. sojae elicitors regardless of age (Fig. 30 A). However, when vanadate was co-applied with autoclaved or enzyme-released fractions of P. sojae wall glucan elicitors, the glyceollin response was age dependent, with maximum glyceollin accumulation in 8- to 9-day-old cotyledons. After 9 days the glyceollin response gradually decreased (Fig. 30 A). In Harosoy isolines, as mentioned in Chapter I, the cotyledons are inherently competent to P. sojae elicitors in
Fig. 30. Restoration of \textit{P. sojae} elicitor competency with mimic of CF-2 in an age-dependent manner. The exposed surface of snapped cotyledons (6–11 days old) of (A) Williams, and (B) Harosoy was treated with either water or vanadate (Van), or autoclaved (PSWG) or enzyme-released (GSE) fractions of \textit{P. sojae} wall glucan elicitors alone or a combination of vanadate and PSWG or GSE (V+P; V+GSE). Only the proximal cell layers were analyzed by HPLC for glyceollin accumulation. The data points represent the average of double determinations with SE (n=2) ranged from 1.3 to 10.8%. 
the minimal wound assay. However, the inherent ability of these cotyledons for glyceollin response to *P. sojae* elicitors was also influenced by the age variable. Harosoy cotyledons (6 to 11 days old) responded to *P. sojae* elicitors alone or in the co-presence of vanadate, mimic of CF-2, by accumulating glyceollin in a similar pattern as seen for Williams (close to bell-shaped curve) (Fig. 30 A,B). Quantitatively, there are very different responses in these two soybean cultivars in glyceollin accumulation. In Harosoy, *P. sojae* wall glucan induced levels of this pterocarpan antibiotic were generally much higher and were further enhanced (2-3 times) in the co-presence of the mimic of CF-2 (Fig. 30 B).

These results suggest that, at least in cotyledon tissues, both the presence of competency factors and a cellular state of particular age are responsible for increased levels of glyceollin response. Furthermore, in cotyledon tissues of very young or very old age, either one or both of these conditions is not met effectively or are modified, resulting in the reduced levels of glyceollin biosynthesis.

It seems that the levels of isoflavone conjugates of daidzein and genistein are not as strongly affected by the age of the cotyledons in both Williams and Harosoy (Fig. 31 A,B; 32 A,B). As mentioned previously, the malonyl glycosyl conjugate of daidzein (MGD) is induced in Williams cotyledons by *P. sojae* elicitors alone or in the co-presence of vanadate (Fig. 31 A). This induced accumulation of MGD is only slightly
Fig. 31. Effects of age and elicitors on MGD accumulation in a minimal wound assay. The exposed surface of snapped cotyledons (6-11 days old) of (A) Williams, and (B) Harosoy soybeans was treated with either water or vanadate (Van), or autoclaved (PSWG) or enzyme-released (GSE) fractions of *P. sojae* glucan elicitors alone or a combination of vanadate and PSWG or GSE (V+P; V+GSE). Only the uppermost treated surface was analyzed for MGD accumulation by HPLC. The data points represent the average of two experiments with SE (n=2) ranged from 2.1 to 9.4%).
Fig. 32. Effects of age and elicitors on MGG accumulation in a minimal wound assay. The exposed surface of snapped cotyledons (6-11 days old) of (A) Williams, and (B) Harosoy soybeans was treated with either water or vanadate (Van), or autoclaved (PSWG) or enzyme-released (GSE) fractions of P. sojae glucan elicitors alone or a combination of vanadate and PSWG or GSE (V+P; V+GSE). Only the uppermost treated surface of cotyledons was analyzed for MGG accumulation by HPLC. Each data point is the average of two determinations (SE, n=2, ranged from 3.4 to 12.1%).
affected by the age of the cotyledons (Fig. 31 A). However, in Harosoy isolines, the MGD levels are not effectively induced by the glucan in the older cotyledon tissues, suggesting the possibility of less availability of glyceollin precursor ultimately resulting in the reduced levels of glyceollin biosynthesis in these older cotyledon tissues (Fig. 31 B). Also, the *P. sojae* elicitor-induced levels of MGD are slightly suppressed in the co-presence of vanadate in Harosoy cotyledons.

The malonyl glycosyl conjugate of genistein (MGG) is induced by *P. sojae* elicitors and suppressed by vanadate, but not affected by age in Williams cotyledons (Fig. 32 A). In Harosoy cotyledons MGG accumulation was also not strongly affected by age, but it was suppressed by *P. sojae* elicitors, vanadate, or a combination of both (Fig. 32 B). Thus, as in many other studies, the suppression of genistein accumulation again parallels glyceollin elicitor competency.

**Effects of age of donor cotyledons for wound exudates on the reconstitution of elicitor competency in a minimal wound cotyledon assay.**

Wound exudate (2-cotyledon-equivalents) from 6- to 11-day-old Williams cotyledons was co-applied with *P. sojae* elicitors on the exposed surface of 8-day-old Williams cotyledons in a minimal wound cotyledon assay. Treated cotyledons were analyzed for isoflavone metabolite
accumulation. The results presented in Fig. 33 A,B show that wound factors from 7- to 9-day-old cotyledons are very effective in restoring the glyceollin response of P. sojae wall glucan elicitors on 8-day-old Williams snapped cotyledons. At other cotyledon ages, response is just above background. As noted in the last section, a similar age of cotyledons has also been shown to be effective for enhanced levels of glyceollin biosynthesis with wall glucan elicitors in the co-presence of mimic of CF-2. Very similar results were obtained with both autoclaved and enzyme-released fractions of P. sojae wall glucan elicitors (Fig. 33 A,B). Coumestrol also follows a similar trend to glyceollin but at reduced levels (Fig. 33 A,B).

These results suggest that aging of intact cotyledon tissues within a specific limit is required for maximum glyceollin response. Before and after this limit, the natural conditioning of these tissues for glyceollin response is either incomplete or has been modified. Even wound exudate from younger or older cotyledon tissues when co-applied with wall glucan elicitor on the exposed surface of the snapped cotyledons of similar or different age has no specific effects other than as mentioned above (data not shown). In any case, both generation of the wound factors as well as a cellular state of a specific-age group of cotyledon tissues (i.e 7-9 day) are very important for maximum glyceollin response and elicitor competency.
Fig. 33. Restoration of *P. sojae* glucan elicitor competency with wound exudates from soybean cotyledons in an age-dependent manner. The exposed surfaces of 8-d-old snapped cotyledons of Williams were supplied with wound exudates (2 cotyledon-equivalents) from 6- to 11-day-old Williams cotyledons followed by (A) autoclaved and (B) enzyme-released fractions of *P. sojae* glucan elicitor. Only the proximal cell layers were analyzed for pterocarpan antibiotic accumulation. The data points represent the average of two experiments with SE (n=2) ranged from 2.4 to 13.2%.
In these same Williams cotyledon tissues, both wall glucan elicitor fractions induced MGD levels in a similar fashion as glyceollin (Fig 34 A,B). However, the induction of MGG accumulation followed an almost opposite pattern to glyceollin biosynthesis in such cotyledon tissues (Fig. 34 A,B). Particularly, in very older tissues, as the induction of glyceollin levels was reduced the accumulation of MGG was enhanced (Fig. 34 A,B). This suggests that in older tissues genistein might add to the increased level of resistance since its antibiotic role has been demonstrated (Rivera-Vargason et al., 1993). Although the structure of genistein is quite similar to daidzein, which is thought to be a precursor for glyceollin biosynthesis, the presence of hydroxyl group at position 5 on the A-ring of genistein reduces its chances of being a glyceollin precursor (Ebel, 1986). A similar inverse relationship of genistein to glyceollin accumulation has been seen in nearly all induction studies. Genistein formation may simply be an alternative to daidzein as glyceollin precursor or genistein may play a regulatory role in the response. This later possibility is interesting since genistein has been shown to possess both steroid hormone-like (Saxena and Bhadoria, 1990) and tyrosine protein kinase inhibitor (Akiyama et al., 1987; Linassier et al., 1990) activities.
Fig. 34. The isoflavone conjugate accumulation in wound exudate and P. sojae elicitor treated Williams cotyledons in a minimal wound assay. The exposed surfaces of 8-d-old snapped cotyledons of Williams were supplied with wound exudates (2 cotyledon-equivalents) from 6-11 days old Williams cotyledons followed by (A) autoclaved and (B) enzyme-released fractions of P. sojae glucan elicitors. Only the uppermost treated surface of cotyledons was analyzed by HPLC for MGD and MGG accumulation. Each data point represents the average of two experiments (SE, n=2, ranged from 2.4 to 13.6%).
Effects of cotyledon age on isoflavone metabolite accumulation in a maximal wound assay.

Williams and Harosoy cotyledons (6 to 11 days old) were also analyzed for *P. sojae* elicitor response in a maximal wound or cut cotyledon assay. Only the proximal cell layer was analyzed for the cellular response. The glyceollin response again increased in an age-dependent manner, with a maximum response seen in 8-day-old cotyledons of both Williams and Harosoy soybean (Fig. 35 A, B). As the cotyledon tissues approached maturity, they lost this glyceollin response in a gradual manner. In both younger and older cotyledon tissues, the levels of this pterocarpan antibiotic are still well above the toxic levels required for ED$_{50}$ (i.e. 100 nmoles/g of tissue). These results further confirm the observations above on glyceollin response in a minimal wound assay both with mimics of CF-2 and with wound exudate from cotyledon tissues.

Although wound-released signals are required for *P. sojae* elicitor responses, elicitor competency and the generation of CFs are also regulated by the age of tissue. As mentioned above, both the cellular state of cotyledons receiving the treatments and the wound factor donor cotyledons of a particular age are required for maximum elicitor competency. In the maximal wound assay, neither of the isoflavone conjugates were induced by the elicitors in the older cotyledon tissues (Fig. 35 A, B.). MGD followed a similar pattern of induction as glyceollin in Williams cotyledons.
Fig. 35. Effects of age on isoflavone metabolites accumulation in P. sojae glucan elicitor-treated cotyledons in a maximal wound assay. The wounded surface of cut cotyledons (6-11 days old) of (A) Williams, (B) Harosoy was treated with 30 μl of P. sojae wall glucan elicitor (autoclaved fraction). Only the proximal cell layers were harvested and analyzed for proximal cell responses by HPLC. Each bar represents the average of two experiments with SE (n=2) ranged from 3.4 to 9.8%.
(Fig. 35 A). In Harosoy cotyledons, its levels were gradually decreased (Fig. 35 B).

**Restoration of elicitor competency with wound exudates from various developmental zones of hypocotyl and root tissues.**

Intercellular washing fluid (IWF) from different zones of hypocotyl tissues of Williams soybean did not fully restore elicitor competency when co-applied with *P. sojae* glucan elicitor preparation on the snapped Williams cotyledons (8-day-old) (Table 3). The level of glyceollin, MGD, and MGG accumulation in such treated cotyledons were not much different from water- or elicitor-treated controls (Table 3). However, the induction of coumestrol by *P. sojae* elicitor in these same cotyledons was greatly enhanced by IWF. As noted above (Chapter 1), the elicitation of coumestrol appears to be associated with the CF-1 competent state. Thus CF-1 derived competency may be due to a factor associated with the apoplast, at least in some tissues. On the other hand, these results also suggest that at least certain aspects of competency, particularly relating to the CF-2 state, is either not present extracellularly at all or not to the extent required for glyceollin response.

When the wound extracts obtained by grinding various developing zones of hypocotyl tissues was coapplied with *P. sojae* glucan elicitor, elicitor competency was fully restored (Fig. 36 B). The wound factors from different developing zones
Table 3. Isoflavone metabolite accumulation by *P. sojae* glucan elicitor in the co-presence of intercellular washing fluids from hypocotyl sections in a minimal wound assay.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Glyceollin (nmoles/g tissue)</th>
<th>Coumestrol</th>
<th>MGD</th>
<th>MGG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>40</td>
<td>36</td>
<td>2022</td>
<td>3750</td>
</tr>
<tr>
<td>H1</td>
<td>64</td>
<td>272</td>
<td>1757</td>
<td>3628</td>
</tr>
<tr>
<td>H2</td>
<td>60</td>
<td>252</td>
<td>2057</td>
<td>3836</td>
</tr>
<tr>
<td>H3</td>
<td>66</td>
<td>260</td>
<td>2280</td>
<td>3702</td>
</tr>
</tbody>
</table>

* IWF was obtained from different zones of hypocotyl tissues as described in "Materials and Methods". H1= top, H2= mid, H3= bottom sections of hypocotyl tissue. 10 μl of IWF or water was coapplied with 10 μl of glucan elicitor (40 μg/ml) on the snapped surface of cotyledons. Only the proximal cell layers were analyzed for the proximal cell responses.

of hypocotyl tissues responded similarly for glyceollin response as from cotyledon tissues. For instance, wound extracts from hypocotyl tissues of younger age (H1, H2) were less effective in restoring elicitor competency (Fig. 36 B). The wound extracts from hypocotyl tissues of intermediate age (H3) gave the maximum glyceollin response, whereas this response was again reduced when the source of wound extracts was mature hypocotyl tissues (H4). Similar results were also obtained for coumestrol accumulation. However, coumestrol was also induced by wound extracts alone (Fig. 36 A,B). The levels of MGD and MGG were also increased by *P. sojae* elicitor (slight induction in case of MGG) in the co-presence of wound extracts (Fig. 36 A,B).
Fig. 36. Effects of *P. sojae* glucan elicitor and wound extracts from hypocotyl tissues of soybean on isoflavone metabolite accumulation in a minimal wound assay. The wound extract from different developing zones of hypocotyl tissues (H1 = top 2 cm section immediately below cotyledon attachment; H2 and H3 = 2 cm each from H1 in a downward direction; H4 = 2 cm bottom section above the crown) was obtained as described in Materials and Methods. The snapped cotyledons were treated with either (A) wound extract alone, or (B) wound extract followed by *P. sojae* wall glucan elicitor. Only the uppermost treated surface of cotyledons was analyzed for metabolite accumulation by HPLC.
As seen for cotyledon tissues, the natural conditioning of competency factors from hypocotyl tissues at a specific age may be required for maximum glyceollin response.

The exudates from different zones (sections) of Williams roots were also analyzed for their restoration of elicitor competency on Williams cotyledon background in a minimal wound assay as described above. Root exudates from all these sections restored the *P. sojae* elicitor competency for proximal cellular responses, particularly, the glyceollin accumulation (Fig. 37). However, it seems that the effect of root exudate is again more of CF-1 nature. The treated cotyledons turned black, which is an indication of the phenolic polymer response. Also, the coumestrol levels were strongly induced in such treated cotyledons. With the co-addition of root exudates, the *P. sojae* glucan elicitor induced high levels of MGD, but not MGG accumulation (Fig. 37).

Again, exudate from very young (RT, first 2 cm section including the root tip) and very old (R4, a 2 cm section before the root hair zone) root tissues restored the glyceollin response, but not as effectively as the exudates from the root tissues of intermediate age (R1 and R2) (Fig. 37).

The CFs distributed differently in various soybean tissues may contribute to different expression of phenolic polymer and glyceollin responses in such tissues.
Fig. 37. Restoration of *P. sojae* elicitor competency to Williams cotyledons by exudates from different zones of root tissues in a minimal wound assay. The root exudates were coapplied with *P. sojae* wall glucan elicitor on the exposed surface of 8-d-old Williams snapped cotyledons. The proximal cell layers were harvested and analyzed for isoflavone metabolite accumulation by HPLC.
DISCUSSION

The induction of host defense responses both in infection and elicitation studies is influenced by several environmental and plant factors such as light, temperature, age, and the developmental state of the specific organ. As mentioned in the introduction, the role of glyceollin in age-related resistance is still somewhat controversial. For instance, increasing resistance in older soybean plants and tissues to \textit{P. sojae} has been reported with decreasing levels of glyceollin (Paxton and Chamberlain, 1968; Lazarovits et al., 1981; Ward et al., 1981). On the other hand, some studies have shown that age-related resistance correlates well with the increased levels of glyceollin biosynthesis (Keen, 1971; Graham and Graham, 1994a).

Graham and Graham (1994a) have demonstrated that as soybean cotyledon tissues approach maturity they become more and more elicitor responsive for the glyceollin response. These authors suggested that older cotyledon tissues become inherently competent to \textit{P. sojae} glucan elicitors.

In this study, two different approaches were employed to investigate the influence of age and developmental state on the competency phenomenon. These approaches are based on the two important variables of the competency phenomenon which apparently interact directly and may lead to glyceollin response to glucan elicitors. One variable is the restorer
factor (mimics of CFs or wound exudates) and the other one is the cellular state of the cotyledon tissue receiving the treatment. This investigation was undertaken by keeping one variable constant and changing the other. For instance, the restorer factor (vanadate) was kept constant and the cellular state of the cotyledon tissue used was varied from 6 to 11 days old. Similarly, the cellular state of the cotyledon tissue was kept constant (8-d-old) and the restorer factor (wound exudate) was varied from 6 to 11 days old cotyledon tissues.

Although Graham and Graham (1994a) reported that the inherent competency of Williams cotyledons increased steadily with age, we did not see such effects in these studies. However, the inherent competency of Harosoy and the inducible competency of Williams and Harosoy were strongly age dependent. The results reported here have consistently shown that there is a broad maximum in competency which occurs in tissues of intermediate age, (7-9 days in cotyledons). This same maximum relates to the inherent competency of Harosoy and to the ability of wound exudates or CF mimics to further enhance competency in Williams or Harosoy tissues. Thus, the inherent competency of tissues may relate to their sensitivity to factors which generate the competent state. On the other hand, the activity of competency factors from wounded donor cotyledons showed a similar maximum, suggesting that the presence or generation of competency factors differ with age.
Thus, both the availability of competency factors and the sensitivity of cells to these factors may vary with age.

Tissue age and developmental state are known to dramatically alter several physiological parameters. Also, wounding and age-related changes in several of these parameters seem to run in parallel. Of particular relevance to our current results is the fact that the oxidative potential of plant tissues is highly age dependent (Apostol et al., 1989b; Legendre et al., 1993). Similar changes in the oxidative potential can be induced in plant tissues by wounding (Apostol et al., 1989a; Legendre et al., 1993). Since GSH may act at least in part through a redox mechanism (and plays a key role in scavenging damaging oxidative molecular species in plant and animal tissues), it may be that overall changes in the redox state of tissues with time relate to GSH levels or response to this reducing sulfhydryl reagent. Testing of endogenous levels of GSH and related natural redox agents (such as ascorbate) as a function of age and wounding would shed further light on this possibility.

It is intriguing that there is a maximum in the competency phenomenon in intermediate aged cotyledons tissues. Ward and coworkers have noted that young leaf tissues are universally susceptible to host and non-host Phytophthora species (Bhattacharyya and Ward, 1981; Ward, 1989). Perhaps this is due to the lack of an effective mechanism to ensure glyceollin production in these young tissues. The lack of
generation of competency factors or of response of cells to these factors might be such a mechanism. Intermediately aged leaf tissues respond in a host and race-specific manner to *Phytophthora*. Since generation of the competent state has also been suggested to result in race-specific expression of the phenylpropanoid defence responses in soybean (Graham and Graham, 1994a), possibly the maximum in competency in intermediately aged tissues corresponds to this maximum in race-specific resistance. Finally, older tissues show universal resistance to *Phytophthora* species. Older tissues are much more highly lignified and, as noted above, accumulate more of the fungitoxic isoflavanoid genistein. Perhaps these types of non-host and non-race specific mechanisms underly this age-related resistance. Thus, there is a very intriguing correlation between the age-relatedness of competency and race-specific resistance. These aspects of cellular response should be further examined.

Maximum elicitor competency factor activities are similarly present in hypocotyl and root tissues of intermediate developmental age. Interestingly, the competency factors released from these tissues into the apoplast (hypocotyl) or exudates (roots) seem to be more CF-1 in nature. This may relate to the primary importance of secondary wall thickenings (such as lignin) in these tissues. Perhaps phenolic polymer responses will be found to play more important role than the glyceollin response in such tissues.
In this regard it is interesting that the glucan elicitor has been reported to be a relatively poor elicitor of glyceollin in soybean roots (J. Ebel, personal communication). Also, although glyceollin was shown to be induced in a race-specific manner in soybean roots, an unknown barrier to the pathogen's penetration of the stele was considered to be the determinative event in compatible vs incompatible interactions (Hahn et al., 1985). Finally, although the glyceollin response is induced in a race-specific manner in hypocotyl tissues, this is clearly seen only in the upper 1/3rd of etiolated (dark grown) hypocotyl. Glyceollin independent mechanisms of resistance are responsible for underlying age- and light-related resistance in these tissues (Paxton and Chamberlain, 1968; Lazarovits et al., 1981; Ward et al., 1981)
CONCLUSIONS

In this study the effects of age and developmental state of various soybean tissues on the competency phenomena were investigated. Both a mimic of CF-2 and wound factors were used to determine these effects. We found that wound factors from soybean tissues of intermediate age are very effective in conferring *P. sojae* elicitor competency. This suggests that CFs may be involve in conferring HR- and race-specific resistance. The results also suggest that both the presence of CFs and the sensitivity of soybean cells to these CFs may vary with age. The universal susceptibility of tissues of very young age to non-pathogens correlates well with the lack of efficacy of CFs from tissues of very young age to confer elicitor competency.

The reduced glyceollin response in older tissues suggests that lignification or some additional mechanisms of resistance (e.g. genistein accumulation) might be involved in increased resistance of these older tissues. The CFs released from hypocotyl and root tissues into the apoplast are more of CF-1 in nature. This is consistent with the fact that these tissues are rich in phenolic polymers, which may be an alternative source of resistance.
Chapter III

Specificity of competency factors from soybean, other legumes, and non-legumes to the soybean system.

INTRODUCTION

Elicitors are compounds which induce a series of metabolic events in plants eventually resulting in host resistance responses. This resistance response is accomplished mainly by the accumulation of compounds such as phytoalexins. Several biotic and abiotic compounds have been described to have elicitor activity (Darvill and Albersheim, 1984; Yoshikawa et al., 1993). Pathogen-derived elicitor molecules are now extensively used in different systems to study the molecular aspects of mechanisms of disease resistance. A number of race:cultivar-specific and non-specific elicitors have been isolated from plant pathogenic fungi (Keen, 1975; Anderson, 1980b; Darvill and Albersheim, 1984; Yoshikawa, 1983; Dewit et al., 1988; Yoshikawa et al., 1993;). Generally, these pathogen elicitors induce a typical host response in a particular host system similar to that seen upon infection. For instance, β-glucan from the cell walls of Phytophthora sojae (Kauf. and Gerde.) triggers the
isoflavanoid phytoalexin response in soybean (*Glycine max* L.). This antimicrobial compound is also accumulated during infection of an incompatible cultivar of soybean with an incompatible race of *P. sojae*.

Although product accumulation is a very crucial event, it is a consequence of a series of events during host-pathogen interaction. The recognition of the pathogen signal by the host is actually the very first step which ultimately leads to the accumulation of metabolites after a series of signalling events. It is thought that pathogen-derived elicitor molecules are recognized by the plasma membrane-bound host receptors leading to the activation of defense responses (Cheong and Hahn, 1991; Cheong et al., 1991; Cosio et al., 1992; Yoshikawa and Sugimoto, 1993b).

*P. sojae* phytoalexin elicitors lack plant host specificity. They induce host defense responses in a cultivar-nonspecific manner in soybean and host-nonspecific manner in other legumes and even non-legumes. An elicitor fraction from this soybean pathogen can induce phaseollin accumulation in *Phaseolus vulgaris*. It also increases the activities of a key enzyme of phenylpropanoid metabolism, PAL, and an enzyme of flavanoid branched pathway, chalcone synthase in this host (Dixon and Lamb, 1979; Hahlbrock et al., 1981). This elicitor fraction from *P. sojae* is also effective in inducing PAL activity in non-legumes such as parsley and sycamore cultured cells (Ebel et al., 1976), although the
active elicitor in this case has been characterized as a polypeptide.

Wounding of tissues is routinely performed, particularly in elicitor studies, to facilitate elicitor application. It is now established that wounding does not just facilitate elicitor application, but it is a pre-requisite for some of the elicitor-induced host responses (Graham, 1994a; Graham and Graham, 1994a). The responses such as glyceollin accumulation and phenolic polymer deposition in soybean are induced by P. sojae elicitors only in the presence of wound-released factors or signals. These wound-released signals are known as wound-associated elicitor competency factors (Graham and Graham, 1994a).

In addition to these P. sojae-soybean interactions, wounding has also been shown to be a pre-requisite for sesquiterpenoid responses of potato tubers to P. infestans elicitor, (Bostock et al., 1983). Moreover, increasing tuber age as well as aging of potato tubers after wounding have been shown to enhance the sesquiterpenoid responses in elicitor-treated tubers (Henfling and Kuc, 1979), just as the glyceollin response was enhanced in soybean cotyledon tissues treated with P. sojae glucan elicitor several hours after wounding (Graham and Graham, 1994). Finally, the last reactions of glyceollin formation (i.e., isoprenylation of trihydroxypterocarpan) in soybean require isopentenyl units from the acetate-mevalonate pathway, the same pathway leading
to the sesquiterpenoids (Ebel, 1986).

These intriguing similarities between the two host-pathogen systems have prompted us to investigate the wound exudates from the potato system for their restoration of glyceollin elicitor competency in the soybean system. We also wished to determine if the wound factors from other legumes and non-legumes can restore the elicitor competency in the soybean system. A preliminary account on this work has appeared (Abbasi and Graham, 1994).
MATERIALS AND METHODS

Plant materials

Seeds of soybean (*Glycine max* L. [Merr.] cv. Williams) were kindly provided by Dr. A. F. Schmitthener (OARDC, Wooster). Seedlings were grown as described previously (Graham et al., 1990) with some modifications described in chapter I (Materials and Methods). Seeds of chickpea (*Cicer arietinum* L.), pea (*Pisum sativum* L.), and navy beans (*Phaseolus vulgaris* L.) were purchased locally from grocery stores. These seeds were grown under conditions similar to those described for soybean seed. Potato tubers (*Solanum tuberosum*), carrots (*Daucus carota*), parsnips (*Pastinaca sativa*), and turnips (*Brassica napa*) were also purchased fresh from the local grocery stores.

Chemicals

The elicitor preparation was the cell wall glucan of race 1 of *Phytophthora sojae* (Kauf. and Gerde.) prepared according to Ayers et al. (1976a) and as described previously (Graham and Graham, 1991b). The unfractionated wall glucan preparation was sonicated and then autoclaved for 3 h in deionized DDW (Ayers et al. 1976). This preparation contains both the glyceollin-specific elicitor (GSE) and an isoflavone specific elicitor (ISE) (T.L. Graham, unpublished results). An enzyme-released glyceollin-specific elicitor (GSE) was also prepared by a modification of conditions first reported by

Yoshikawa et al. (1981) as described in Chapter I (Materials and Methods).

**Cotyledon assays**

Eight-day-old cotyledons, unless otherwise mentioned, were harvested in batches and used immediately. The cut cotyledon assay was performed in essentially the same way as described previously (Graham and Graham, 1991b). The snapped cotyledon assay was used as described in Chapter I.

**Wound washings from soybean tissues and other plant material**

Individual cotyledons were surface sterilized and cut as described for the cut cotyledon assay (Graham and Graham, 1991b). Wound washings were obtained in essentially the same manner as described previously (Graham and Graham, 1994a) and also as outlined in Chapter I (Materials and Methods). Chickpea and pea cotyledons of the same age i.e, 8-day-old, were used to collect wound exudate. However, navy bean cotyledons were used at five days because older cotyledons became senescent. Wound exudate was collected in a similar manner as outlined in Chapter I (Materials and Methods) for soybean cotyledons. Cotyledons of all three of these legumes (i.e chickpea, pea, and navy beans) were smaller in size, so wound exudates obtained from 3 cotyledons (instead of 2 cotyledons, in case of soybean) were used to treat the exposed surface of individual snapped soybean cotyledons, as described in Chapter I (Materials and Methods).
Storage organs of potato, carrots, parsnips, and turnips were also used to collect wound exudate. Potato tubers were surface sterilized by wiping the tuber surface with 70% ethanol followed by several washings with sterilized DDW. These tubers were sliced vertically under sterile conditions with a slicer adjusted to 2 mm thickness. Wound exudate was collected immediately from individual slices by washing the wounded surface with sterilized DDW in essentially the same way as mentioned in Chapter I (Materials and Methods) for soybean cotyledons. Similarly, wound exudates were obtained from carrot and parsnip storage tissues. However, instead of using a slicer, vertical discs (4 mm thickness) were first cut with a cork borer and then sliced with a sterile razor blade.

All exudates were adjusted to approximate cell surface equivalency. That is, wound exudate from the same surface area was used for each plant species. The starting concentration was adjusted to approximately 2 soybean cotyledon-equivalents. The microfuge tubes containing these wound exudates were kept at 4°C until use.

**Characterization of competency factors from potato**

Potato wound exudates were fractionated into supernatant and pellet fractions by spinning them down at 13,000 g for 10 min. The pellet was washed with sterilized DDW and suspended in water (total volume 1200 µl). Both supernatant and resuspended pellet fractions were boiled at 100°C for 10-15 min to determine the heat stability of potato competency.
factors. The precipitated materials of boiled fractions were removed by centrifuging at 13,000 rpm for 10 min. Serial dilutions were made in sterilized DDW from these boiled fractions as well as from control fractions on a volume:volume basis. 10 μl of each dilution (i.e., 1:8, 1:4, 1:2, 1:1, 1:0) was applied on the exposed surface of the snapped cotyledons followed by 10 μl of water or PSWG (40 μg/ml) to determine the restoration of elicitor competency by these various wound exudate fractions as described previously.

**HPLC analysis**

The treated cotyledons were harvested after 48 h of light incubation. Only the uppermost 0.5 mm treated surface or proximal cell layer was sliced off as described by Graham (1994b). 10 or 20 sections per treatment were pooled together and either extracted and analyzed immediately for soluble metabolites through HPLC or stored intact at -80°C for later use, as described earlier (Graham, 1991a).

Unless otherwise specified, all the experiments were conducted twice.
RESULTS

Restoration of elicitor competency with heterologous wound factors.

The glyceollin response to *P. sojae* glucan elicitors was restored by wound exudates from cotyledon, hypocotyl, and root tissues of soybean in a minimal wound cotyledon assay. This was demonstrated in Chapters I and II above. Wound factors from other legumes and non-legumes were tested to determine if they can restore glyceollin elicitor competency. The results presented here are based on isoflavone metabolite accumulation in the proximal cell layers of the treated cotyledon tissues.

Wound exudates from all legumes (chickpea, pea, navy bean) and non-legumes (potato, carrot, parsnip, turnip) tested restored the glyceollin response of soybean to *P. sojae* wall glucan elicitors to a varying degree (Fig. 38 A,B). Moreover, coumestrol levels were also induced in such wound exudate and elicitor-treated cotyledons. Wound exudates alone did not induce the biosynthesis of glyceollin in soybean cotyledons (data not shown). The glyceollin response in such wound exudate-treated cotyledons was similar to water-treated controls. Potato and carrot wound exudate seemed to be more effective in restoring the elicitor competency than that from other non-legumes and even legumes. In the presence of wound exudate from parsnip, *P. sojae* elicitors induced higher levels of coumestrol than glyceollin (Fig. 38 A,B).
Fig. 38. Restoration of *P. sojae* elicitor competency with heterologous wound factors in a minimal wound cotyledon assay. Wound exudates from these legumes and non-legumes were obtained as described in "Materials and Methods". The exposed surface of Williams snapped cotyledons was supplied with wound exudate followed by (A) 40 μg/ml autoclaved (PSWG), or (B) 60 μg/ml enzyme-released (GSE) fractions of *P. sojae* glucan elicitors. Only the proximal cell layers were harvested and analyzed by HPLC for glyceollin and coumestrol accumulation. The values represent the average of two experiments (n=2, SE, ranged from 3.8 to 11.4%).
Both chickpea and pea (but not navy bean) wound exudates restored the elicitor competency by accumulating higher glyceollin levels (Fig. 38 A,B). In the presence of wound exudates, the *P. sojae* elicitor-induced levels of MGD were significantly enhanced (Fig. 39 A). However, the elicitor-induced levels of MGG were suppressed in the presence of wound factors (Fig. 39 B). In the case of wound exudates from navy beans, the higher levels of MGD and MGG may simply reflect the lower glyceollin accumulation in such wound exudate and elicitor-treated cotyledons (compare Fig. 38 A,B to Fig. 39 A,B).

It is possible that both potatoes and carrots might contain higher levels of competency factors than other legumes and non-legumes tested. Also, the wound factors from these two genera are either more selective in restoring the glyceollin response in soybean cotyledon tissues to *P. sojae* elicitors (CF-2 like activity). Restoration of elicitor competency with these heterologous wound factors suggests that the competency phenomena may be widespread and conserved across a wide range of plant species.

It has been demonstrated that aging of potato tubers several hours after wounding enhances the sesquiterpenoid response in elicitor-treated tubers (Henfling and Kuc, 1979). This prompted us to investigate whether the wound factors from aged potato slices can more effectively confer the elicitor competency to soybean tissues. Slices of potato tubers were
Fig. 39. Effects of P. sojae glucan elicitors on the isoflavone conjugate accumulation in the co-presence of heterologous wound factors in a minimal wound cotyledon assay. Wound exudate from various legumes and non-legumes was obtained as described in "Materials and Methods". The exposed surface of Williams snapped cotyledons was supplied with wound exudate followed by 40 μg/ml autoclaved (PSWG), or 60 μg/ml enzyme-released (GSE) fractions of P. sojae glucan elicitors. Only the proximal cell layers were harvested and analyzed by HPLC for (A) MGD and (B) MGG accumulation. The values are means of two experiments (n=2, SE, ranged from 3.4 to 9.8%).
cut as described in "Materials and Methods". Wound exudate was obtained 0 h or 16 h after wounding and incubating the slices in light at 25°C. Similarly, wound exudates were obtained from carrot, parsnip, and turnip discs. Wound factors (2 cotyledon-equivalents) from these aged slices and discs also conferred elicitor competency to soybean tissues in a minimal wound, snapped cotyledon assay (Table 4). Similar results were obtained with enzyme-released fractions of P. sojae wall glucan elicitor (GSE) in the presence of these aged-wound factors (data not shown). The glyceollin response was enhanced by wound factors from aged discs of parsnip and turnip whereas it was lowered by wound factors from aged carrot discs (Table 4). However, aging of potato slices had little effect on CFs from the wound exudate.

In the presence of autoclaved fractions of P. sojae elicitors, the fresh wound exudate (0 h) from carrot and parsnip discs induced high levels of coumestrol. However, this coumestrol response was lowered by wound factors obtained from aged discs of these two genera (Table 4). The isoflavone conjugates of daidzein and genistein (MGD, MGG) were only slightly changed depending upon the glyceollin response. For instance, the levels of MGG were always suppressed or lowered when the glyceollin levels were enhanced (Table 4).
These results suggest that aging of tissues might alter the efficacy of wound factors. This effect either may be specific or might need different conditions for increased effectiveness, and also might be related to the stability of competency factors from these various genera. Also, aging may have specific effects on the levels of CFs. For instance, aging of parsnip and turnip discs may enhance CF-2 but lower CF-1 levels. Similarly, both CF-1 and CF-2 states may be decreased in aged carrot discs.

**Table 4.** Effects of aging on the activity of wound factors for the restoration of elicitor competency in a minimal wound assay*.

<table>
<thead>
<tr>
<th>Source of wound factors</th>
<th>Hours after wounding</th>
<th>Isoflavanoid metabolites accumulation (nmoles/g of tissue)</th>
<th>Glyceollin</th>
<th>Coumestrol</th>
<th>MGD</th>
<th>MGG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato</td>
<td>0 h</td>
<td>1656</td>
<td>458</td>
<td>3306</td>
<td>1928</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16 h</td>
<td>1690</td>
<td>469</td>
<td>2782</td>
<td>1722</td>
<td></td>
</tr>
<tr>
<td>Carrot</td>
<td>0 h</td>
<td>1628</td>
<td>746</td>
<td>3159</td>
<td>2212</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16 h</td>
<td>766</td>
<td>524</td>
<td>3510</td>
<td>2747</td>
<td></td>
</tr>
<tr>
<td>Parsnip</td>
<td>0 h</td>
<td>412</td>
<td>928</td>
<td>2465</td>
<td>1763</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16 h</td>
<td>2019</td>
<td>415</td>
<td>3463</td>
<td>1462</td>
<td></td>
</tr>
<tr>
<td>Turnip</td>
<td>0 h</td>
<td>499</td>
<td>524</td>
<td>2744</td>
<td>2460</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16 h</td>
<td>724</td>
<td>400</td>
<td>2651</td>
<td>1895</td>
<td></td>
</tr>
</tbody>
</table>

* Wound factors were co-applied with autoclaved fraction of *P. sojae* glucan elicitor on the exposed surface of Williams snapped cotyledons. Only the uppermost treated surface of cotyledon was analyzed by HPLC for isoflavone metabolite accumulation. The values are the averages of two separate experiments (SE, n=2, ranged from 1.2 to 7.8%).
Dose response with heterologous wound factors.

Wound exudates from potato slices and carrot discs were diluted with sterilized double distilled water (DDW) on a volume/volume basis. Each dilution was tested for elicitor competency on a Williams cotyledon background in a minimal wound assay as mentioned above. The concentration of both these wound exudates before dilution was approximately 2-cotyledon-equivalents.

Potato wound exudates were effective at very low concentrations in conferring the glyceollin elicitor competency to soybean tissues. As the concentration of wound exudate was increased, both the glyceollin and coumestrol responses of soybean cotyledon tissues to *P. sojae* wall glucan elicitor were also increased (Fig. 40 A). The concentration of potato wound exudates has no effect on MGD accumulation by *P. sojae* wall glucan elicitor except at very high concentrations where MGD levels were suppressed (Fig. 40 B). On the other hand, the isoflavone conjugate of genistein (MGG) was suppressed in a biphasic manner by the *P. sojae* elicitor in the presence of potato wound factors (Fig. 40 B).

As seen for potato wound exudate, diluted carrot wound exudate was also effective in enhancing the glyceollin and coumestrol responses of soybean cotyledon tissues to *P. sojae* glucan elicitor (Fig. 41 A). However, the levels of coumestrol at each dilution were comparatively higher than the glyceollin levels (Fig. 41 B). The isoflavone conjugate of
Fig. 40. Effects of increasing concentrations of potato wound factors on the restoration of P. sojae glucan elicitor competency in a minimal wound cotyledon assay. Potato wound exudate (2-cotyledon-equivalents) was diluted in sterilized DDW on a volume:volume basis. These various dilutions were applied on the exposed surface of the Williams snapped cotyledons followed by 40 μg/ml of P. sojae wall glucan elicitor (PSWG). Only the uppermost treated surface of the treated cotyledons was harvested and analyzed by HPLC for accumulation of (A) the pterocarpan antibiotics, and (B) the isoflavone conjugates of daidzein and genistein. Each data point represents the average of two experiments (n=2, SE, ranged from 1.4 to 13.4%).
Fig. 41. Effects of increasing concentrations of carrot wound factors on the restoration of *P. sojae* glucan elicitor competency in a minimal wound cotyledon assay. Carrot wound exudate (2-cotyledon-equivalents) was diluted in sterilized DDW on a volume:volume basis. These various dilutions were applied on the exposed surface of the Williams snapped cotyledons followed by 40 µg/ml of *P. sojae* wall glucan elicitor (PSWG). Only the uppermost treated surface of the treated cotyledons was harvested and analyzed by HPLC for accumulation of (A) the pterocarpan antibiotics, and (B) the isoflavone conjugates of daidzein and genistein. The data points represent the average of two experiments (n=2, SE, ranged from 3.2 to 13.6%).
daidzein was only slightly changed, whereas MGG accumulation was suppressed by increasing concentrations of carrot wound exudate (Fig. 41 B).

The effect of carrot wound factors is more of CF-1 in nature. The effectiveness of potato wound exudates for increasing glyceollin response to soybean tissues may be related to an effect on the isopentenyl groups required for the final reactions of the glyceollin biosynthesis (CF-2 like activity). This enhanced elicitor competency for the glyceollin response in Williams cotyledons by potato wound factors may be related to the presence of both CF-1 and CF-2 factors in the wound exudate.

Partial characterization of potato competency factors.

Wound exudates were obtained and fractionated as described in "Materials and Methods". The soluble (supernatant) and nonsoluble (pellet) fractions of potato wound exudates were evaluated for elicitor competency. The results suggest that CF-1 activity is associated with both soluble and nonsoluble fractions of the potato wound exudate. This was clearly evident from the black color of the treated cotyledons with both fractions of the wound exudates. As these wound exudate fractions were diluted, the black color of the treated cotyledons was also diluted. This CF-1 related activity was completely destroyed in soluble and nonsoluble fractions of the wound exudate after boiling for 10 min at
It has been shown in the soybean system that the CF-1 factor is heat labile, but its activity is primarily associated with the low speed pellet (Graham and Graham, 1994a). The CF-1 factor in the potato system also seems to be heat labile; however, its activity is associated with both supernatant and pellet fractions of the wound exudate.

The CF-2 wound factor is required for the glyceollin response of soybean tissues to \textit{P. sojae} glucan elicitors (Graham and Graham, 1994a). In the soybean system, the activity of CF-2 factor has been shown to be heat stable and associated with supernatant fraction of the wound exudate (Graham and Graham, 1994a). The activity of CF-2 wound factor (i.e., glyceollin response) is also associated with the supernatant fraction of the wound exudate in the potato system (Fig. 42). However, unlike that from soybean wound exudates, the CF-2 factor from potato wound exudate is heat labile. The glyceollin response was only slightly induced with boiled soluble fraction (Fig. 42). Moreover, the glyceollin levels in such treated cotyledons were well below the toxic levels required for the ED$_{50}$. 
Fig. 42. Partial fractionation and characterization of potato wound factors for the glycineollin response in a minimal wound cotyledon assay. Potato wound exudate was fractionated into soluble and nonsoluble fractions and these fractions were tested for heat stability as described in "Materials and Methods". The various fractions were diluted in sterilized DDW on a volume:volume basis and then applied on the exposed surface of Williams snapped cotyledons with and without P. sojae wall glucan elicitor (PSWG, 40 μg/ml). Only the proximal cell layers of the treated cotyledons were harvested and analyzed by HPLC for the glycineollin response. The similar results were obtained in a second set of experiment.
DISCUSSION

It was demonstrated in previous chapters that the glyceollin response of non-wounded soybean cells to \textit{P. sojae} elicitors can be restored by supplying exogenous wound exudates from different soybean tissues. For instance, the wound exudate from Harosoy cultivar restored the glyceollin elicitor response in Williams cotyledon tissues. Similarly, the wound exudate from hypocotyl and root tissues can partially restore the elicitor competency in cotyledon tissues. This suggests a wide distribution of competency factors among various cultivars and tissues within this genus.

In this Chapter, it was established that wound factors from other legumes and even non-legumes can confer elicitor competency for the glyceollin response in minimally wounded soybean cotyledon cells. This suggests that competency factors are widespread and that some elements of the competency phenomenon may be conserved even across a wide range of plant species in different families.

The host necrogenic response to pathogens has been hypothesized as a natural source of wounding and subsequent release of competency factors (Graham and Graham, 1994a). Intercellular washing fluid from \textit{P. sojae} infected soybean cotyledons (incompatible interactions) were shown to restore the elicitor competency, whereas those from healthy tissues did not (Graham and Graham, 1994a). Compatible infections
generally either do not cause a necrotic host response or this response is seen as a secondary tissue collapse well after the pathogen has passed through the host tissues. The absence or active suppression of hypersensitivity is thus a key element in the compatibility (virulence) of a potential pathogen. Yet other elements may be involved in the ability of the pathogen to actively colonize host tissues, obtain required nutrients and cofactors and/or successfully reproduce. These later elements condition pathogenicity and aggressiveness. Importantly, a pathogen must first avoid the hypersensitive response in order to successfully deploy those traits associated with pathogenicity or aggressiveness. Thus, hypersensitivity is a universal form of resistance that must be avoided for a pathogen to be successful.

It is an accepted notion that under natural conditions resistance is the rule whereas susceptibility is the exception. Since hypersensitivity is the determinative event in most attempted infections, this widespread resistance against pathogens could be related to the widespread presence of wound-related competency factors in plant species. Only a few specialized pathogens are able to invade their respective hosts successfully without excessive wounding resulting in the compatible interaction or susceptibility. Under natural conditions, excessive wounding of invaded tissues by unspecialized and non-host pathogens could also lead to the release of the competency factors which might be necessary for
a particular host defense response.

As with the competency factors, branched β-glucans and chitinous compounds are widely distributed in the cell walls of fungi. Both these molecules have been shown to possess elicitor activities. For instance, the glucan elicitor used in this study was isolated from the cell walls of *P. sojae*. These signal substances from the cell walls of fungi may be recognized by plants resulting in the expression of host defense responses. Taken together, the widespread presence of host secondary signals (i.e., wound-associated competency factors), as demonstrated in this study, and primary or external signals from pathogens suggest that these may be involved in general or non-host resistance as well as in race-specific resistance initiated by hypersensitive cell death.

As mentioned in the Introduction, both the soybean-*P. sojae* and potato-*P. infestans* systems share some similarities in their respective response to elicitors. For example, wounding is required for the sesquiterpenoid phytoalexin response of potato tubers to fungal elicitors, and this response is enhanced by wounded and wounded-aged tubers (Bostock et al., 1983). The wounding of soybean tissues has also been shown to be required for the glyceollin response to *P. sojae* elicitor (Graham and Graham, 1994a). As shown for the potato system, aging of soybean cotyledon tissues after wounding has also been reported for maximum glyceollin response to glucan elicitor. It is possible that in both
these systems wound-released factors may induce a state of competency for a maximum response. Also, the isoprenylation of THP for glyceollin formation requires isopentenyl units from the sesquiterpenoid pathway. The increased CF-2 like activity of potato wound factors suggest that it may have some effects on isopentenyl units for the final reactions of glyceollin biosynthesis.

The various effects of heterologous wound factors suggest that these wound exudates from different genera may contain different amounts of CFs or even different CFs. Potato seems to be rich in both CF-1 and CF-2. The preliminary characterization of CFs from potato wound exudates suggests that CF-1 like activity is associated with both soluble and non-soluble fractions. The increased elicitor competency for the glyceollin response with potato wound factors could be related to the presence of both CF-1 and CF-2 factors. This suggests that potato wound factors may possibly affect both phenylpropanoid and mevalonate metabolisms.

The effects of carrot and parsnip wound exudates are more of CF-1 like since these wound factors effectively induced the coumestrol levels in the presence of glucan elicitor. This suggests that wound exudates from these two genera may have specific effects on phenylpropanoid metabolism since coumestrol is a pterocarpan phytoalexin and it does not require the isopentenyl units from mevalonate pathway.
Wound exudates from aged parsnip and turnip discs are very effective in conferring the glucan elicitor competency. The results suggest that, in parsnip and turnip discs, aging may enhance the CF-2 factor. We also found that wound exudates from aged storage tissues have a lowered CF-1 activity.

In initial fractionation and characterization of potato CFs, we found that CF-1 is associated with both soluble and non-soluble fractions and it is very sensitive to heat whereas CF-2 activity, which is also sensitive to heat, is totally associated with soluble fractions of the wound exudate. This suggests that potato CFs may be different from soybean CFs.

Although our results are very preliminary, they suggest several future research efforts. First, there is a need to examine elicitation (non-host, host, and race-specific) in the other plants represented in the study and determine if competency is indeed a conserved phenomena. The need for competency in potato is actually reasonably well established, although the wound and age-related factors have not yet been characterized.

Second, it would be of great interest to purify competency factors from different plant species and see if they are related or un-related to those in soybean. Regardless, their purification will add potential tools and understanding of mode of action of competency.
CONCLUSIONS

The *P. sojae* wall glucan elicitor competency to induce the glyceollin response was reconstituted with homologous and heterologous wound factors on minimally wounded soybean cotyledon tissues. This suggests that some elements of the competency factors are widespread and may be conserved across a wide range of plant species. Moreover, the widespread presence of these secondary signals of host origin and the primary elicitor signals from cell walls of pathogens suggests that both these signals may be involved in general or non-host resistance as well as in hypersensitive-mediated race-specific resistance.

The effects of wound exudates suggest that potato may be a rich source of both CF-1 and CF-2 factors whereas carrot and parsnip may contain more of CF-1 than CF-2. Aging may lead to lower levels of CF-1 whereas it may enhance the CF-2 factor in parsnip and turnip.

Preliminary characterization of potato CFs suggests that these CFs may be different from those characterized from soybean.
GENERAL CONCLUSIONS

The elicitation of the glyceollin response in soybean tissues by *P. sojae* glucan elicitor requires pre-conditioning by wound-associated competency factors. A minimal wound cotyledon assay, which gives a blank background, allows reconstitution of glucan elicitor competency with mimics of CFs or wound exudates. Both mimics of CFs and/or wound exudates were used to undertake this study.

First, the competency phenomena was investigated in different soybean cultivars and isolines with different *Rps* backgrounds. All the Williams isolines tested require an exogenous supply of wound factors for elicitor competency, whereas Harosoy and Bragg soybeans seem to be at least partially inherently competent. The results suggest that the cultivars Harosoy and Bragg may be rich in CF-2 factor. We also found that *Rps* genes may condition some elements of competency. *Rps7* may be responsible for inherent competency in Harosoy. The presence of *Rps1-a* and *Rps3-a* in Williams and *Rps3-b* in Harosoy may lead to lowered expression of competency. Also, *Rps1-k* in the Williams background may increase the sensitivity of cells in such a manner that only CF-1 state is sufficient for the glucan elicitor competency.
This \textit{Rps}-mediated conditioning of competency is further confirmed by dose response data and transiency of competency data. Almost all the elicitation studies suggest that genistein may have a regulatory role in the glyceollin response.

Second, in our investigation of age and developmental regulation of competency factors, we found that tissues of intermediate age are very effective in conditioning the elicitor competency for maximum glyceollin response. This suggests a role of CFs in race-specific resistance. The results also suggest that both the presence of CFs and the sensitivity of soybean cells to these CFs for elicitor competency may vary with age. The fact that CFs are distributed differently in different tissues may contribute to different expression of phenolic polymer and glyceollin responses in the various tissues.

Finally, regarding specificity of CFs, we found that both homologous and heterologous wound factors can restore \textit{P. sojae} elicitor competency suggesting that CFs may be conserved across a wide range of plant species. The results suggest a possible role of CFs in non-host or general resistance as well as HR-mediated race-specific resistance.
LIST OF REFERENCES


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