Plant Physiol. (1994) 104: 917–923

Cyclic β-1,6-1,3-Glucans of *Bradyrhizobium japonicum* USDA 110 Elicit Isoflavonoid Production in the Soybean (*Glycine max*) Host[1]

Karen J. Miller*, Jill A. Hadley, and David L. Gustine

Graduate Programs in Genetics (K.J.M.) and Plant Physiology (K.J.M., D.L.G.), Departments of Food Science (K.J.M., J.A.H.) and Agronomy (D.L.G.), The Pennsylvania State University, University Park, Pennsylvania 16802; and United States Department of Agriculture, Agricultural Research Service, United States Regional Pasture Research Laboratory, University Park, Pennsylvania 16802 (D.L.G.)

High levels of cyclic β-1,6-1,3-glucans (e.g. 0.1 mg mg^{-1} of total protein) are synthesized by free-living cells as well as by bacteroids of *Bradyrhizobium japonicum* USDA 110 (K.J. Miller, R.S. Gore, R. Johnson, A.J. Benesi, V.N. Reinhold [1990] J Bacteriol 172: 136–142; R.S. Gore and K.J. Miller [1993] Plant Physiol 102: 191–194). These molecules share structural features with glucan fragments isolated from the mycelial cell wall of the soybean (*Glycine max*) pathogen *Phytophthora megasperma*. These latter glucans have been shown to be potent elicitors (at nanogram levels) of the phytoalexin glyceollin in *G. max*. Using the well-characterized soybean cotyledon bioassay, we now show that the cyclic β-1,6-1,3-glucans of *B. japonicum* USDA 110 are also biologically active elicitors of glyceollin production (but at microgram levels). We further show that both classes of β-glucans elicit the production of the isoflavone daidzein within soybean cotyledon wound droplets.

Bacteria within the genera *Rhizobium* and *Bradyrhizobium* participate in agriculturally important symbiotic associations with leguminous plants. These symbiotic associations lead to the development of nitrogen-fixing nodules on the roots of the legume host. During the development of the root nodule, a complex exchange of molecular signals occurs between both organisms. Studies during the past several years have provided evidence that oligosaccharides and polysaccharides of *Rhizobium* and *Bradyrhizobium* are important signal molecules (see recent reviews by Fisher and Long, 1992; Leigh and Coplin, 1992). Indeed, unequivocal evidence from several laboratories has revealed that lipo-oligosaccharides of *Rhizobium* and *Bradyrhizobium* species act as signal molecules that elicit root hair deformation, cortical cell division, and nodule organogenesis in the respective plant host (see reviews by Fisher and Long, 1992; Franssen et al., 1992; Hirsch, 1992).

The plant flavonoids represent a second important class of signaling molecule during legume nodulation. Indeed, flavonoids appear to have multiple roles during several stages of nodule development. These roles are indicated from the following observations: flavonoids act as inducers of nodulation genes in several *Rhizobium* and *Bradyrhizobium* strains (Phillips, 1992; Göttfert, 1993); certain flavonoids have been shown to inhibit the induction of nodulation genes in *Rhizobium* and *Bradyrhizobium* strains (Firmin et al., 1986; Peters and Long, 1988; Kosslak et al., 1990; Hungria et al., 1991); flavonoids may act as chemoattractants for *Rhizobium* and *Bradyrhizobium* (Aguilar et al., 1988; Armitage et al., 1988; Caetano-Anollés et al., 1988; Barbour et al., 1991); certain flavonoid compounds have been shown to stimulate the growth rate of *Rhizobium* strains (Hartwig et al., 1991), whereas others may be inhibitory or transiently inhibitory to the growth of rhizobial and bradyrhizobial strains (Pankhurst and Biggs, 1980; Parniske et al., 1991a; Kape et al., 1992); exposure of roots to *Rhizobium* and *Bradyrhizobium* strains leads to an increase in flavonoids within root exudate (van Brussel et al., 1990; Recourt et al., 1991, 1992a, 1992b; Schmidt et al., 1992; Dakora et al., 1993); genes encoding enzymes involved in flavonoid biosynthesis are induced during nodule development (Estabrook and Sengupta-Gopalan, 1991; Yang et al., 1992; Estabrook et al., 1993); exposure of plants to inhibitors of flavonoid biosynthesis leads to a dramatic reduction in nodule formation (Schmidt et al., 1992); and flavonoids themselves may enhance the formation of spontaneous nodules on legume roots (Hirsch et al., 1991; A. Hirsch, personal communication).

In addition to signaling roles for the flavonoids during legume nodulation, this class of molecules is also induced when legumes are exposed to pathogenic microorganisms. The induced biosynthesis of certain flavonoids, which have been referred to as "phytoalexins," appears to represent an important defense response for the plant, because many of these compounds have been shown to be inhibitory to various plant pathogens (see Darvill and Albersheim, 1984; Ebel, 1986; Ebel and Grisebach, 1988, for reviews). Furthermore, the prevention of phytoalexin biosynthesis using metabolic inhibitors has been shown to lead to an increased sensitivity of plants to various fungal and/or bacterial pathogens (see Darvill and Albersheim, 1984; Ebel and Grisebach, 1988, for reviews).

The multiple effects of legume flavonoids on symbiotic and pathogenic microorganisms may be important for effective root nodulation. For example, the flavonoid composition of root exudate may influence the proportion of symbiotic and pathogenic microorganisms in the vicinity of the legume

[1] Supported by National Science Foundation grant DCB-9103924.

* Corresponding author; fax 1–814–863–6132.

root. In this regard, it is particularly intriguing that recent studies have indicated that rhizobial lipo-oligosaccharides may act as elicitors of flavonoid biosynthesis in the legume root (van Brussel et al., 1990; Recourt et al., 1991, 1992a; Spaink et al., 1991). In the present study, we show that a second class of rhizobial oligosaccharide, the cyclic β-1,6-1,3-glucans of *Bradyrhizobium japonicum*; also may act as an elicitor of flavonoid biosynthesis in the corresponding legume host.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Bradyrhizobium japonicum USDA 110 and *Agrobacterium tumefaciens* C58 have been previously described (Miller et al., 1990). *Rhizobium leguminosarum* biovar *trifolii* TA-1 was kindly provided by Dr. Michaël Breedveld (The Pennsylvania State University, University Park) and has been previously described (Breedveld et al., 1990). *B. japonicum* AN279 (*nodB*::Tn5-*lacZ*) was kindly provided by Dr. Gary Stacey (University of Tennessee, Knoxville) and has been previously described (Sanjuan et al., 1992).

B. japonicum USDA 110 and *A. tumefaciens* C58 cultures were grown to the mid-logarithmic phase in yeast extract mannitol medium (Miller et al., 1990). For some experiments *B. japonicum* USDA 110 was cultured in a defined growth medium (Gore and Miller, 1992). *B. japonicum* AN279 was grown to the mid-logarithmic phase in defined growth medium (Gore and Miller, 1992) containing kanamycin (100 μg mL^{-1}). *R. leguminosarum* biovar *trifolii* TA-1 was cultured as previously described (Breedveld et al., 1990).

Preparation of Cyclic β-Glucans

The cell-associated cyclic β-1,6-1,3-glucans of *B. japonicum* strains USDA 110 and AN279 and the cell-associated cyclic β-1,2-glucans of *A. tumefaciens* were extracted into methanol-water using the modified Bligh and Dyer procedure previously described (Miller et al., 1988). These glucan preparations were purified using three different column chromatography steps. The extracts were initially fractionated on a Sephadex G50 column. Fractions containing cyclic β-glucans were then further fractionated on a Sephadex G25 column. Next, the neutral cyclic β-glucan fractions were isolated using a DEAE-cellulose column. These chromatography procedures have been previously described (Miller et al., 1990; Gore and Miller, 1993). Finally, the purified neutral cyclic β-glucan fractions were desalted on a Sephadex G15 column using 7% (v/v) 1-propanol as previously described (Gore and Miller, 1993).

Extracellular neutral cyclic β-1,2-glucans were isolated from cultures of *R. leguminosarum* biovar *trifolii* TA-1 as described by Breedveld et al. (1990). This extracellular cyclic β-1,2-glucan fraction was desalted on a Sephadex G15 column as described above.

Fungal Glucan Elicitor Preparation

Fungal glucan elicitor was generously provided by Dr. Michael Hahn (The University of Georgia, Athens). This glucan elicitor fraction, which is referred to as "void glucan elicitor," was isolated from partial acid hydrolysates of the mycelial cell walls of *Phytophthora megasperma* f. sp. *glycinea* after column chromatography of the hydrolysates on a Bio-Gel P2 column (Hahn et al., 1992). This preparation was used as standard elicitor in the soybean (*Glycine max*) cotyledon bioassay described below.

Soybean Cotyledon Bioassay

The soybean cotyledon bioassay was performed as described by Hahn et al. (1992) with some modifications. Seeds of the soybean cv Williams 82 (obtained from Illinois Foundation Seeds, Inc., Champaign, IL) were planted between a lower 2.5-cm layer of potting soil (Fafard mix No. 3S, Conrad Fafard, Inc., Agawam, MA) and an upper 2.5-cm layer of fine vermiculite. A 5-cm layer of coarse vermiculite was placed at the bottom of the planter. The seedlings were watered daily with tap water that had been purified using a reverse osmosis system. A photoperiod regime of 14 h of illumination (PPFD approximately 300 μmol m^{-2} s^{-1} [400–700 nm]) and 10 h of darkness was used. The temperatures during the light and dark cycles were 24 and 19°C, respectively. The RH was maintained at approximately 75%. Cotyledons were detached from 9- to 11-d-old seedlings at the time when the primary leaves had just expanded.

Detached cotyledons were surface sterilized for 5 min in a 5% Clorox solution and then extensively rinsed with sterile, deionized water. Tissue sections were removed from the cotyledons as described by Hahn et al. (1992). Wounded cotyledons were transferred to sterile Petri dishes (10 cotyledons per dish), and 100 μL of sample solution were applied to the entire wound surface of each cotyledon. Two dishes, each containing 10 cotyledons, were used for each sample analyzed. Sample solutions of glucans were prepared in 4 mM sodium acetate, 3 mM sodium bicarbonate as described by Hahn and co-workers (1992) and contained between 0 and 50 μg of Glc equivalent (as determined by the phenol method [Miller et al., 1990]) per 100 μL of sample solution.

The cotyledon dishes were incubated in the dark at 25°C for 20 to 24 h. After incubation, all 10 cotyledons were transferred to a beaker containing 20 mL of deionized water. These solutions are referred to as "wound-droplet solutions." Analysis of the wound-droplet solution was performed by HPLC as described below.

HPLC Analysis

Aliquots of wound-droplet solutions (10 mL) were extracted with 2 volumes of ethyl acetate at room temperature for 5 min. After centrifugation and removal of the upper ethyl acetate phase, the wound-droplet solution was extracted one additional time with 1 volume of ethyl acetate. Ethyl acetate extracts were combined and brought to dryness under nitrogen or under vacuum. Dried samples were stored at −20°C until the time of HPLC analysis when they were resuspended in 300 μL of ethyl acetate.

HPLC analysis of ethyl acetate extracts was based on procedures previously described (Graham, 1991). Briefly, injections (5 or 10 μL) were made onto an octadecyl reverse-

phase 250-mm column with a particle size of 5 μm (IBM Instruments, Inc., Danbury, CT). Chromatography was performed using Waters chromatography pumps (models 6000A and M45; Waters Associates, Milford, MA) at room temperature using a linear gradient of 10 to 65% acetonitrile in water (adjusted to pH 3 with glacial acetic acid) for 25 min. Following this gradient, a step increase to 100% acetonitrile was performed for 3 min, followed by a return to 10% acetonitrile. The column was washed with 10% acetonitrile in water (pH 3) for 12 min prior to application of the next sample. A flow rate of 0.9 mL min^{-1} was used. Peaks were detected using a Waters 990 photodiode array detector (Waters Associates). Daidzein and glyceollin were identified based on retention times, co-elution with authentic standards, and UV spectra. Quantities of daidzein and glyceollin within ethyl acetate extracts were determined from peak height values measured at 287 nm (peak height at 287 nm was found to be linearly proportional to the amount of glyceollin and daidzein injected). Glyceollin values represent the total of glyceollin I and glyceollin II present within each extract.

Chemicals

Daidzein standard was obtained from ICN Biochemicals (Cleveland, OH). Glyceollin I standard was very kindly provided by Dr. John Giannini (St. Olaf College, Northfield, MN).

RESULTS

Application of "Void Glucan Elicitor" Preparations to Cotyledon Wounds Results in Elevated Levels of Glyceollin and Daidzein within Cotyledon Wound Droplets

When void glucan elicitor, as opposed to buffer alone, was applied to wounded soybean cotyledons, glyceollin was found to accumulate within wound droplets (Fig. 1, A and B). This result is consistent with results from previous studies that have revealed glyceollin accumulation within cotyledon tissue and/or wound droplets to be a primary response to the application of fungal glucan elicitor preparations (Graham, 1991; Graham and Graham, 1991; Hahn et al., 1992). The chromatographic profile shown in Figure 1B reveals that two isomers of glyceollin were separated using this HPLC system. The peak eluting at approximately 26.5 min was found to correspond to glyceollin I based on retention time and UV spectrum. The peak eluting immediately prior to glyceollin I (at approximately 26 min) likely represents glyceollin II. The UV spectrum of this peak was found to be essentially identical with that of purified glyceollin II (spectrum provided by Dr. Noel Keen, University of California, Riverside). Furthermore, it has previously been shown that glyceollin II is not fully resolved from glyceollin I and elutes just prior to glyceollin I using similar HPLC conditions (Graham and Graham, 1991; T.L. Graham, personal communication).

When the amount of void glucan elicitor applied to wounded cotyledons was varied, the glyceollin response was found to be saturable (Fig. 2). Indeed, this response was found to saturate at approximately 50 ng of void glucan elicitor (applied per cotyledon), consistent with findings re-

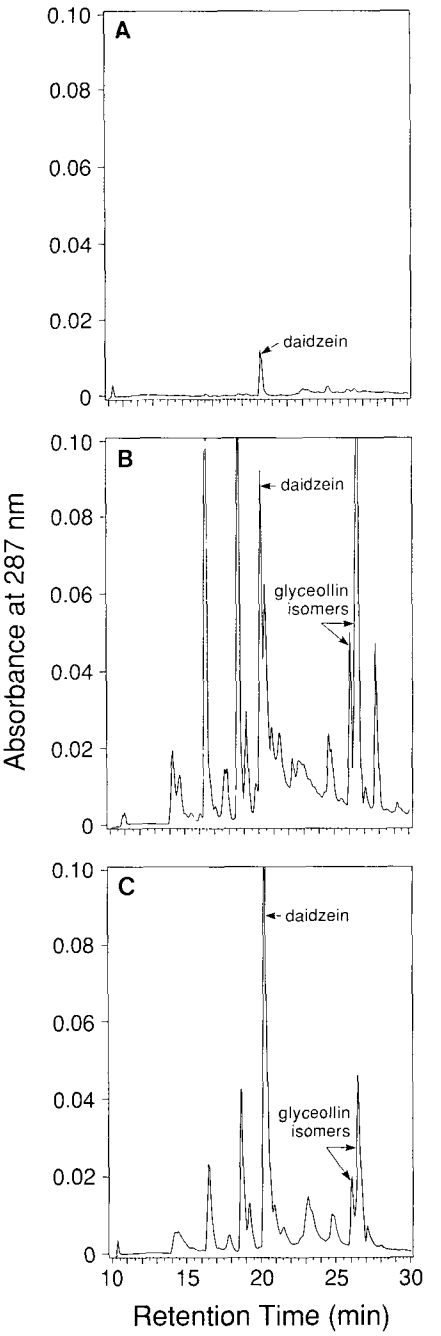

Figure 1. HPLC profiles of ethyl acetate extracts of wound-droplet solutions. A, Buffer control. A buffer solution (100 μL of 4 mM sodium acetate, 3 mM sodium bicarbonate) was applied to each of 10 wounded cotyledons. A 10-μL aliquot of the ethyl acetate extract (derived from 10 cotyledons) was analyzed. B, Void glucan elicitor. A buffer solution (100 μL of the same buffer as described in A) containing 50 ng of *P. megasperma* void glucan elicitor was applied to each of 10 wounded cotyledons. A 5-μL aliquot of the ethyl acetate extract (derived from 10 cotyledons) was analyzed. C, Bradyrhizobial cyclic β-1,6-1,3-glucan. A buffer solution (100 μL of the same buffer as described in A) containing 50 μg of *B. japonicum* cyclic β-1,6-1,3-glucan was applied to each of 10 wounded cotyledons. A 10-μL aliquot of the ethyl acetate extract (derived from 10 cotyledons) was analyzed.

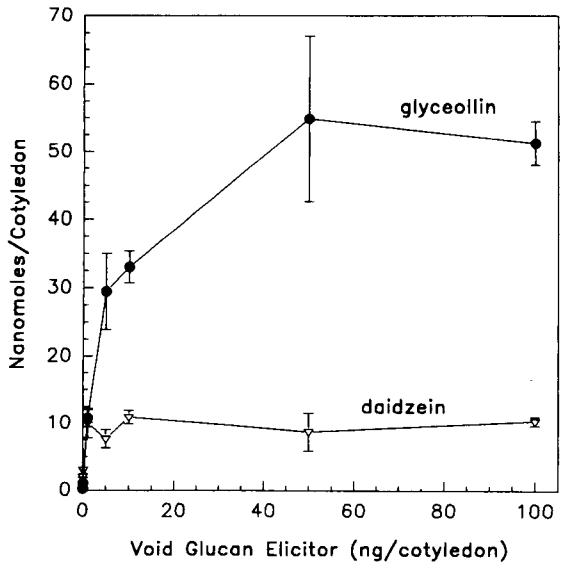

Figure 2. Accumulation of daidzein and glyceollin as a function of void glucan elicitor concentration. The amount of void glucan elicitor was varied between 0.1 and 100 ng per cotyledon. Daidzein and glyceollin levels within wound droplets were quantitated as described in "Materials and Methods." Glyceollin values represent the total amount of glyceollin I and II isomers. The results are expressed as nmol per cotyledon and represent the average of two determinations from a typical experiment. The error bars indicate the range.

ported previously for similar void glucan elicitor preparations (Hahn et al., 1992).

In addition to glyceollin accumulation within wound droplets, daidzein was also found to accumulate in response to the application of void glucan elicitor preparations to cotyledon wounds. Similar results have been reported previously for soybean cotyledon tissue (Graham and Graham, 1991). However, we are not aware of any previous report of daidzein accumulation within cotyledon wound droplets in response to fungal glucan preparations. It is interesting that low concentrations of daidzein were detected within wound droplets from control cotyledons to which only buffer solution had been applied (Fig. 1A). As shown in Figure 2, the accumulation of daidzein within wound droplets was also found to be a saturable response. However, maximum levels of daidzein within wound droplets were approximately 5- to 6-fold lower than levels of the glyceollin isomers.

Additional metabolites were also found to accumulate within cotyledon wound droplets upon exposure to void glucan elicitor. As shown in Figure 1B, two major metabolites were detected in HPLC profiles at approximately 16.5 and 18.7 min. The identity of these metabolites is unknown.

Bradyrhizobial Cyclic β-1,6-1,3-Glucan Preparations also Induce Elevated Levels of Daidzein and Glyceollin within Soybean Cotyledon Wound Droplets

HPLC analysis of ethyl acetate extracts revealed daidzein accumulation within wound droplets to be a major response to the application of bradyrhizobial cyclic β-1,6-1,3-glucan preparations (Fig. 1C). Like the response to void glucan elicitor preparations, the accumulation of daidzein in wound droplets exposed to bradyrhizobial glucan preparations was found to be saturable (Fig. 3). We noted, however, that higher concentrations of the bradyrhizobial cyclic β-1,6-1,3-glucan preparations (e.g. 5 μg/cotyledon) were required to elicit maximal accumulation of daidzein within wound droplets. It is interesting that the maximal level of daidzein accumulated within wound droplets (mean values approximately 15 to 20 nmol/cotyledon) in response to bradyrhizobial cyclic β-1,6-1,3-glucan preparations was similar to levels elicited by saturating concentrations of void glucan elicitor (Fig. 2).

Glyceollin isomers also accumulated within cotyledon wound droplets in response to bradyrhizobial cyclic β-1,6-1,3-glucan preparations (Figs. 1C and 3). Furthermore, the accumulation of glyceollin isomers was a saturable response, and microgram levels of glucans (e.g. 5 μg/cotyledon) were required to elicit maximal glyceollin accumulation within wound droplets.

As noted above, maximal levels of daidzein accumulated in response to void glucan elicitor, and bradyrhizobial cyclic β-1,6-1,3-glucan preparations were similar. This, however, was not true for glyceollin accumulation. Instead, higher

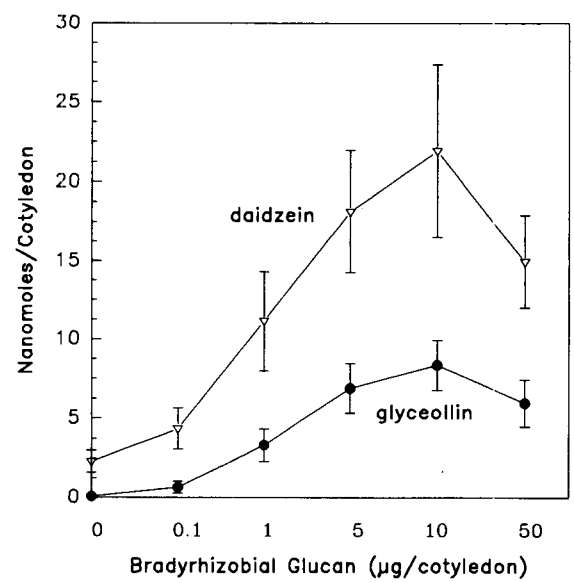

Figure 3. Accumulation of daidzein and glyceollin as a function of bradyrhizobial cyclic β-1,6-1,3-glucan concentration. The amount of bradyrhizobial cyclic β-1,6-1,3-glucan was varied between 0.1 and 50 μg per cotyledon. Daidzein and glyceollin levels within wound droplets were quantitated as described in "Materials and Methods." Glyceollin values represent the total amount of glyceollin I and II isomers. The results are expressed as nmol per cotyledon and represent the mean of at least nine determinations for each concentration of glucan tested (n = 14, 9, 10, 9, 10, 13 for 0, 0.1, 1, 5, 10, and 50 μg of glucan, respectively). The error bars represent the SE. Statistical differences between treatments were assessed by analysis of variance followed by Dunnett's multiple comparison test ($P < 0.05$). Significant increases in daidzein and glyceollin (compared with control cotyledons) were detected in response to the application of 5 μg or higher amounts of the bradyrhizobial cyclic β-1,6-1,3-glucan preparations.

levels (e.g. 6-fold higher) of glyceollin isomers accumulated within cotyledon wound droplets in response to the application of void glucan elicitor preparations. We found, however, that the relative proportion of glyceollin I and II isomers elicited by the two glucan preparations was similar (Fig. 1, B and C).

Because the bradyrhizobial cyclic β-1,6-1,3-glucan preparations were derived from cells cultured in a medium containing yeast extract, it was possible that the activity detected using the soybean cotyledon assay may have been attributed to a contaminant in the preparation derived from the yeast extract. Indeed, previous studies have shown that yeast extract contains glucan elicitors that induce glyceollin production within soybean cotyledons (Hahn and Albersheim, 1978). Therefore, to rule out this possibility, additional preparations of cyclic β-1,6-1,3-glucans were extracted from cells cultured in a defined growth medium (Gore and Miller, 1992) containing no added yeast extract. Subsequent assays revealed that the elicitor activity of these cell-associated cyclic β-1,6-1,3-glucan preparations was similar to that of glucans isolated from cells grown in media containing yeast extract (data not shown).

Additional experiments were performed using cyclic β-1,6-1,3-glucans extracted from *B. japonicum* AN279, a *nodB* mutant derived from strain USDA 110. Although strain AN279 does not synthesize lipo-oligosaccharide Nod metabolites (Sanjuan et al., 1992), the cyclic β-glucans purified from this mutant were found to have elicitor activity indistinguishable from that found for cyclic β-glucan preparations derived from strain USDA 110 (data not shown).

Glyceollin and Daidzein Accumulation within Wound Droplets Is Not Induced by Neutral Cyclic β-1,2-Glucan Preparations Derived from *R. leguminosarum* or *A. tumefaciens*

Consistent with previous studies that have shown glucan elicitors to contain β-1,6- and β-1,3-glycosidic bonds (reviewed by Darvill and Albersheim, 1984; Halverson and Stacey, 1986), no induction of glyceollin was detected when cyclic β-1,2-glucan preparations were applied to wounded soybean cotyledons. Neither glyceollin nor daidzein levels increased above those of controls when high concentrations (e.g. 50 μg/cotyledon) of neutral cyclic β-1,2-glucan preparations were applied to wounded soybean cotyledons (Table I).

Table I. *Daidzein and glyceollin accumulation within cotyledon wound droplets in response to different glucan preparations*

Glucans were extracted and purified as described in the text. Each preparation was tested at a concentration of 50 μg per cotyledon, and eight determinations were performed for each sample. The results are expressed as nmol per cotyledon and represent the mean ± SE. Statistical differences between treatments were assessed by analysis of variance, followed by Tukey's pairwise comparison test ($P < 0.05$). Only bradyrhizobial cyclic β-1,6-1,3 glucan preparations were shown to elicit a response significantly higher than the control response. "None" refers to wounded cotyledons treated with 100 μL of buffer solution (4 mM sodium acetate, 3 mM sodium) containing no added glucan preparation.

Glucan Preparation	Daidzein	Glyceollin
	nmol/cotyledon	*nmol/cotyledon*
None	1.3 ± 0.2	0.03 ± 0.03
Cyclic β-1,2-glucan from *A. tumefaciens* C58	2.1 ± 0.7	0.07 ± 0.07
Cyclic β-1,2-glucan from *R. leguminosarum* biovar *trifolii* TA-1	1.8 ± 0.5	0.12 ± 0.12
Cyclic β-1,6-1,3 glucan from *B. japonicum* USDA 110	11.9 ± 2.5	7.34 ± 2.08

DISCUSSION

We have previously shown that a variety of strains of *Bradyrhizobium* synthesize cyclic β-1,6-1,3-glucans (Miller et al., 1990). These glucans accumulate within the periplasmic compartment to relatively high concentrations, are released to the extracellular medium, and are preferentially synthesized when these bacteria are cultured at low osmolarity (Miller and Gore, 1992). Based on these properties, we have proposed that the cyclic β-1,6-1,3-glucans of *Bradyrhizobium* sp. may represent functional analogs of the cyclic β-1,2-glucans of *Rhizobium* sp. (Miller and Gore, 1992). The cyclic β-1,2-glucans of *Rhizobium* are believed to provide functions during hypo-osmotic adaptation as well as during legume nodulation.

Recently, we showed that bacteroids of *B. japonicum* USDA 110 isolated from mature soybean nodules (i.e. 6 to 9 weeks after inoculation) also contain high levels of cyclic β-1,6-1,3-glucans (Gore and Miller, 1993). This result suggests that the cyclic β-1,6-1,3-glucans may provide functions during the later stages of nodule development. It is interesting that this would appear to contrast with the roles for the high mol wt exopolysaccharides of these bacteria, whose levels have generally been shown to be reduced in the bacteroid state (Dixon, 1964; Roth and Stacey, 1989). It was noted, however, in recent studies by Streeter and co-workers (Streeter et al., 1992; Streeter and Salminen, 1993) that novel polysaccharides accumulate within symbiosomes of soybean nodules infected with certain strains of *B. japonicum*.

The functions for the cyclic β-1,6-1,3-glucans during legume nodule development remain unclear, but it is possible that they may act as signaling molecules. It is, therefore, intriguing that these glucans share structural features with glucan elicitors derived from the mycelial walls of fungal pathogens of the soybean plant such as *P. megasperma* (Darvill and Albersheim, 1984). Although the structure of the bradyrhizobial cyclic β-1,6-1,3-glucans has not yet been solved, the ratio of β-1,6 to β-1,3 linkages in these molecules is approximately 1.6:1, and these molecules are branched (Miller et al., 1990). It should be noted that Pfeffer and coworkers (Rolin et al., 1992) recently presented a possible backbone structure for the bradyrhizobial cyclic β-1,6-1,3-glucan, which contains 13 Glc residues.

The results of the present study reveal that the cyclic β-1,6-1,3-glucans of *B. japonicum* USDA 110, like the β-glucans derived from the mycelial cell walls of *P. megasperma*, are active elicitors of isoflavonoid biosynthesis in the soybean host. Although the bradyrhizobial glucans are not as potent

as the void glucan elicitor preparations from *P. megasperma* (i.e. 100-fold higher concentrations of bradyrhizobial glucan preparations are required to elicit a maximal response), significant accumulation of both glyceollin and daidzein was detected when micromolar concentrations of bradyrhizobial glucan preparations were applied to wounded cotyledons (e.g. 5 μg of bradyrhizobial glucan per cotyledon is equivalent to approximately 27 μM within the wound droplet, assuming an average size of 11.5 Glc residues; Miller et al., 1990).

It might seem inappropriate upon initial consideration that glucan preparations from a bacterial symbiont should elicit a response from the plant host that is similar to that elicited by glucan preparations derived from a fungal pathogen. In fact, it is possible that the cyclic β-1,6-1,3-glucans may be responsible in part for eliciting the accumulation of high levels of glyceollin within certain ineffective soybean nodules (Werner et al., 1985; Parniske et al., 1990, 1991b). Indeed, the levels of glyceollin detected within such nodules are of the same order of magnitude as that found within soybean root tissue infected with *P. megasperma* f. sp. *glycinea* (Werner et al., 1985; Parniske et al., 1990, 1991b). It is interesting that low levels of glyceollin may also accumulate within nodules infected with effective strains of *B. japonicum* (Karr et al., 1992).

In contrast to the above, it is also possible that the activity of the cyclic β-1,6-1,3-glucans as elicitors of glyceollin production may be advantageous to the bacterial symbiont during legume nodulation. For example, although glyceollin has been shown to be inhibitory to *B. japonicum*, recent studies have revealed that this bacterium is able to develop resistance to this isoflavonoid (Parniske et al., 1991a; Kape et al., 1992). In fact, prior exposure of *B. japonicum* to the isoflavones genistein and daidzein or the chalcone isoliquiritigenin has been shown to confer a high degree of tolerance to glyceollin. In this regard, it is of interest that cyclic β-1,6-1,3-glucan preparations also elicit the production of daidzein by the plant host. Thus, the induction of glyceollin and daidzein biosynthesis may be beneficial for this symbiosis, because it may lead to a shift in the microbial population in the vicinity of the root by selecting for bradyrhizobial strains.

Dakora and co-workers (1993) recently reached similar conclusions concerning the alfalfa-*Rhizobium meliloti* symbiosis. These researchers showed that exposure of alfalfa roots to *R. meliloti* leads to the production of the phytoalexin medicarpin as well as a flavonoid with *nod* gene-inducing activity.

The induction of daidzein biosynthesis by bradyrhizobial cyclic β-1,6-1,3-glucan preparations would appear to be beneficial for this symbiosis for a second reason. Specifically, daidzein has been shown to be a potent inducer of the *nod* genes of *B. japonicum* (Kosslak et al., 1987; Banfalvi et al., 1988; Göttfert et al., 1988). Thus, elevated levels of daidzein in the vicinity of the root should, in turn, lead to an increased synthesis of lipo-oligosaccharide Nod factors by *Bradyrhizobium*.

The present study has utilized the well-characterized soybean cotyledon bioassay to examine the possible plant signaling properties of the bradyrhizobial cyclic β-1,6-1,3-glucans. Additional studies aimed at examining the effects of these glucan preparations on isoflavonoid levels within root tissue and/or root exudate are clearly warranted. In this regard, we note a recent study by Werner and co-workers in which cultures of *B. japonicum* induced elevated levels of glyceollin within soybean root exudate (Schmidt et al., 1992). These researchers attempted to characterize the bradyrhizobial elicitor of glyceollin induction and found it to be a heat-stable, soluble factor. It is tempting to speculate that the cyclic β-1,6-1,3-glucans of *B. japonicum* may, in fact, be the source of the elicitor activity detected by Schmidt and co-workers. Future studies with mutants of *B. japonicum* defective for cyclic β-1,6-1,3-glucan biosynthesis should provide additional insight concerning the role(s) of these molecules throughout nodule development.

Received October 7, 1993; accepted November 26, 1993.

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