

Communication

Abscisic Acid Suppression of Phenylalanine Ammonia-Lyase Activity and mRNA, and Resistance of Soybeans to *Phytophthora megasperma* f.sp. *glycinea*¹

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ABSTRACT

Etiolated hypocotyls of the resistant soybean (*Glycine max* [L.] Merr.) cultivar Harosoy 63 became susceptible to *Phytophthora megasperma* (Drechs.) f.sp. *glycinea* (Hildeb.) Kuan and Erwin race 1 after treatment with abscisic acid. Susceptibility was expressed by increases in lesion size and a major decrease in accumulation of the isoflavonoid phytoalexin, glyceollin. In untreated hypocotyls, activity of phenylalanine ammonia-lyase and accumulation of mRNA for this enzyme increased rapidly after infection, but these increases were suppressed in abscisic acid-treated hypocotyls. The results suggest the possibility that biosynthesis of glyceollin in the resistance response of soybeans may be controlled at the transcriptional level by changes in abscisic acid concentrations caused by infection.

The accumulation of the phytoalexin, glyceollin (a mixture of three isomers), is believed to play a major role in resistance of soybean (*Glycine max* [L.] Merr.) cultivars to incompatible races of the fungal pathogen *Phytophthora megasperma* (Drechs.) f.sp. *glycinea* (Hildeb.) Kuan and Erwin. Glyceollin production can be promoted also by a variety of stimuli that include polysaccharide and glycoprotein elicitors from microbial or plant sources (1, 9, 11), salts of heavy metals (19, 23), and ultraviolet light (4). Perhaps the simplest explanation for this similarity in response to such diverse agents is that they indirectly exert their influence through a common control system. Plant growth hormones play a major, if not fully defined, role in the control of plant metabolic processes, and there appears to be no reason *a priori* why this should not include metabolic responses to infection.

In soybeans, it has been proposed that the plant growth hormone, ethylene, does not act as a messenger in the induction of glyceollin biosynthesis (16) but may influence the flow of carbon into the biosynthetic pathway (12). In beans (*Phaseolus vulgaris* L.), production of isoflavonoid phytoalexins in response to mercuric chloride or to cell wall material from *Rhizoctonia solani* was influenced by ABA treatment either positively or negatively, depending on the plant organ and

ABA concentration (8). Henfling *et al.* (10) reported that ABA treatment suppressed resistance and terpenoid phytoalexin accumulation in potato tubers infected with *P. infestans* or *Cladosporium cucumerinum*. Similarly, Salt *et al.* (18) found that ABA treatments increased susceptibility of tobacco to *Peronospora tabacina*.

In this paper, the effect of exogenously supplied ABA on the expression of resistance and the accumulation of the phytoalexin, glyceollin, in an incompatible interaction of soybeans with *Pmg* is described. Effects on activity of the enzyme PAL² (EC 4.3.1.5), which plays a key role in the synthesis of glyceollin and other phenylpropanoids, and on the production of mRNA for PAL are examined also.

MATERIALS AND METHODS

Pathogen and Host

The isolate of *Phytophthora megasperma* (Drechs.) f.sp. *glycinea* (Hildeb.) Kuan and Erwin race 1 was that used previously (20). It was maintained on V8 juice agar plates at 25°C in the dark, and zoospore suspensions (10⁵ mL⁻¹) were produced from the cultures as described previously (22). Seedlings of soybeans, cv Harosoy 63 (resistant to race 1), were grown in the dark for 6 d as described previously (22).

Abscisic Acid Treatment

Abscisic acid was obtained from Sigma as mixed isomers [(±)*cis-trans*]. The concentrations given in this paper refer to the biologically active (+) isomer only. Abscisic acid was dissolved in methanol and diluted with distilled water as required. Final methanol concentrations in ABA solutions and controls were adjusted to 0.2% v/v. To administer the ABA, the roots were removed from the seedlings and the cut ends of the hypocotyls were placed in ABA solutions and incubated overnight (approximately 14 h) in the dark under the conditions used for growth of seedlings. Controls were treated similarly with either 0.2% methanol or water.

Inoculation and Incubation

Treated hypocotyls were arranged horizontally in glass trays with the cut ends covered by moist cellucotton (22). They

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² Abbreviation: PAL, phenylalanine ammonia-lyase.

were inoculated by placing 10 μL drops of zoospore suspension or sterile distilled water on to the hypocotyl surface about 2 cm below the cotyledons. Where disease reaction, lesion size, and glyceollin content were measured, a single inoculum drop was used per hypocotyl. For the determination of PAL activity and isolation of mRNA, two drops placed approximately 5 mm apart were used. Trays of inoculated seedlings were closed with plastic film to maintain high humidity and incubated in the dark at 25°C.

Disease Response, Lesion Size, and Glyceollin Production

Hypocotyl responses to infection were evaluated after incubation for 24 h. Lesion lengths, along the hypocotyl axes, were measured, and data are presented as means and standard errors for lesions from 10 or 20 seedlings in each experiment.

For glyceollin determination, tissues of lesions were excised from at least 10 seedlings in each experiment, and glyceollin, a mixture of isomers, was extracted and quantified as described in detail previously (2). Glyceollin concentrations are expressed as $\mu\text{g g}^{-1}$ fresh weight of infected tissue.

Determination of Phenylalanine Ammonia-Lyase Activity

Sections, 1.0 to 1.5 cm long, containing two inoculated sites were excised from two replicates of ten hypocotyls in each experiment, weighed, frozen in liquid nitrogen, and stored at -70°C . The frozen sections were ground to a powder in liquid nitrogen with a mortar and pestle, suspended in 0.1 M sodium borate buffer (pH 8.8), containing 2 mM mercaptoethanol (13), and centrifuged at 15,600g in a microcentrifuge for 4 min.

Phenylalanine ammonia-lyase activity in the supernatant was determined as described previously (3). The enzyme activity was expressed as nmoles cinnamic acid produced in 1 h \cdot g^{-1} fresh weight of tissue, and data are means and standard errors from two experiments each with two replicates.

Preparation of Total RNA

The procedure was that of Prescott and Martin (17) with minor modifications. Powdered hypocotyl sections (see PAL activity) were suspended in 10 mL of extraction buffer (50 mM Tris-HCl, 150 mM LiCl, 5 mM EDTA, 5% SDS [pH 9.0]). The suspension was extracted three times with an equal volume of a mixture of phenol, saturated with Tris-HCl (100 mM, pH 8), and chloroform (1:1 v/v). After each extraction, the aqueous phase containing the nucleic acids was separated by centrifugation at 800g at 4°C. The aqueous phase was partitioned once with an equal volume of chloroform, and RNA was precipitated from the aqueous phase by adding LiCl (8 M), to give a concentration of 2 M, and then incubating at 4°C overnight. The suspension was centrifuged at 1100g for 15 min at 4°C to pellet the RNA. To remove contaminating DNA, the pellet was resuspended in 1.5 mL of 0.4 M LiCl, and the concentration was made to 2 M by adding LiCl (8 M). The suspension was incubated at 4°C overnight and centri-

fuged as above, and the resulting RNA pellet was resuspended in 200 μL of 0.4 M LiCl and stored at -70°C .

Northern Blot Analysis

The protocol for analysis of RNA samples was essentially that of Maniatis *et al.* (14). RNA samples (25 μg) were denatured for 15 min at 55°C in deionized formamide (50%, vol/vol), formaldehyde (6%, vol/vol) and 1 \times Mops buffer (20 mM Mops; 5 mM sodium acetate; 1 mM EDTA, pH 7.0). Samples were cooled immediately to 4°C in ice and mixed in a ratio of 10:1 with loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol). Agarose gels (1.2%) were cast in 1 \times Mops buffer and formaldehyde (7.4%, vol/vol) and samples in loading buffer were subjected to electrophoresis in 1 \times Mops as running buffer at 100 V for 4 to 5 h at room temperature. Following electrophoresis, RNAs were transferred to nitrocellulose filters by blotting using 10 \times SSC (SSC is 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0) (14).

RNA Blot Hybridization, Autoradiography and Densitometry

The RNA blots were hybridized with a heterologous probe prepared by nick-translation (14) of a 1775 bp cDNA fragment (kindly provided by C. J. Lamb), complementary to bean phenylalanine ammonia-lyase mRNA, generated by digestion of pPAL 5 (6) with *Pst*I and isolated by electroelution following gel electrophoresis. RNA blots were prehybridized for 30 min (65°C) with salmon sperm DNA (100 $\mu\text{g mL}^{-1}$) and polyadenylic acid (1 $\mu\text{g mL}^{-1}$) in a buffer comprised of 6 \times SSC, Ficoll (0.2%), PVP, (0.2%), SDS (0.1%). Hybridization with the ^{32}P -labeled probe was carried out at 65°C in a water bath shaker overnight, using the same buffer system except that 3 \times SSC was substituted for 6 \times SSC. The filter was washed twice for 30 min at 65°C in 2 \times SSC containing 0.5% SDS. Autoradiography was carried out for 72 h at -70°C with preflashed Fuji x-ray film and intensifying screens. Quantification of transcripts for PAL was achieved by microdensitometry (Joyce Loebel Chromoscan 3 microdensitometer). Standardization between treatments was based on the quantity of 18S rRNA that hybridized to a pea rDNA clone. The cosmid clone cDB107 containing pea rDNA cloned in the vector pHC79 was a gift from J. A. Gatehouse, Department of Botany, University of Durham, U.K.

RESULTS

Lesions on untreated hypocotyls and methanol controls were dark brown with necrotic tissue and generally restricted to the area of the hypocotyl surface covered by the inoculum drop (about 5 mm diameter) (Table I). Some increase in lesion size occurred in hypocotyls treated with 5×10^{-5} M ABA, but at this concentration the lesions were similar to controls in that they were dark brown with superficial necrosis. Lesions were much more extensive following treatment with higher concentrations of ABA, and those that developed at 2×10^{-3} M ABA were pale in color and resembled lesions that develop in hypocotyls of susceptible seedlings (22). Gly-

Table I. Changes in the Host Response, Lesion Size, and Glyceollin Accumulation in Soybean Hypocotyls Treated with Abscisic Acid and Inoculated with an Incompatible race of *P. megasperma f.sp. glycinea*

Treatment ^a	Host ^b Response	Lesion Length ^c mm	Glyceollin ^d $\mu\text{g g}^{-1}$ fresh wt
Water	R	5.6±0.9	1582±241
Methanol	R	6.2±1.2	1390±167
ABA			
5 × 10 ⁻⁵ M	R	9.1±1.2	1016
5 × 10 ⁻⁴ M	R-S	11.4±2.6	933
1 × 10 ⁻³ M	S	11.2±3.2	812±162
2 × 10 ⁻³ M	S	14.4±2.8	460±145

^a Seedlings were treated overnight with water, 0.2% methanol, or ABA in 0.2% methanol. ^b Hypocotyls were inoculated with a 10 μL drop of zoospore-suspension. Host responses after 24 h were rated resistant (R), when lesions were dark brown with superficial necrotic tissue and little spread from the inoculated area, or susceptible (S), when lesions were pale straw color, without necrosis of surface tissues, and spread both across (radially) and along the hypocotyl. ^c Lesion lengths were measured 24 h after inoculation along the axes of 10 or more hypocotyls per treatment. ^d Glyceollin was determined 24 h after inoculation in tissues excised from lesions on at least 10 hypocotyls per treatment. Data for lesion lengths and glyceollin are the means and standard errors from four separate experiments, except for 5 × 10⁻⁴ M and 5 × 10⁻⁵ M, which are from a single experiment.

ceollin concentrations were related inversely to lesion size and to ABA concentration (Table I). At 2 × 10⁻³ M ABA, glyceollin concentrations were reduced to about one-third of the concentrations in inoculated controls.

Phenylalanine ammonia-lyase activity increased rapidly in inoculated control hypocotyls from 2 h after inoculation (Fig. 1). In ABA-treated hypocotyls, PAL activity was lower than in controls at the time of inoculation. Activity increased from 4 h after inoculation but much more slowly than in treated hypocotyls. The Northern blot analysis for PAL mRNA indicated that, at 5 h after inoculation, the amount of transcript for PAL in infected hypocotyls treated with ABA was less than half that in untreated inoculated hypocotyls (Fig. 2).

DISCUSSION

Suppression of resistance and glyceollin accumulation by ABA treatments in soybean hypocotyls inoculated with an incompatible race of *Pmg* is consistent with the findings of Henfling *et al.* (10) for potato tubers inoculated with *P. infestans*. In soybeans, ABA treatment did not suppress glyceollin production to the level in uninoculated controls, but concentrations approached the range (100–250 $\mu\text{g mL}^{-1}$) reported previously for susceptible responses or for interactions in which susceptibility was induced by temperature or other treatments (20). Evidence from potatoes (10) and also from beans (8), that ABA applications regulate phytoalexin production following elicitor treatments, suggests that ABA influences the plant response directly and not the pathogen or its interaction with the host.

Phenylalanine ammonia-lyase is a key enzyme in the bio-

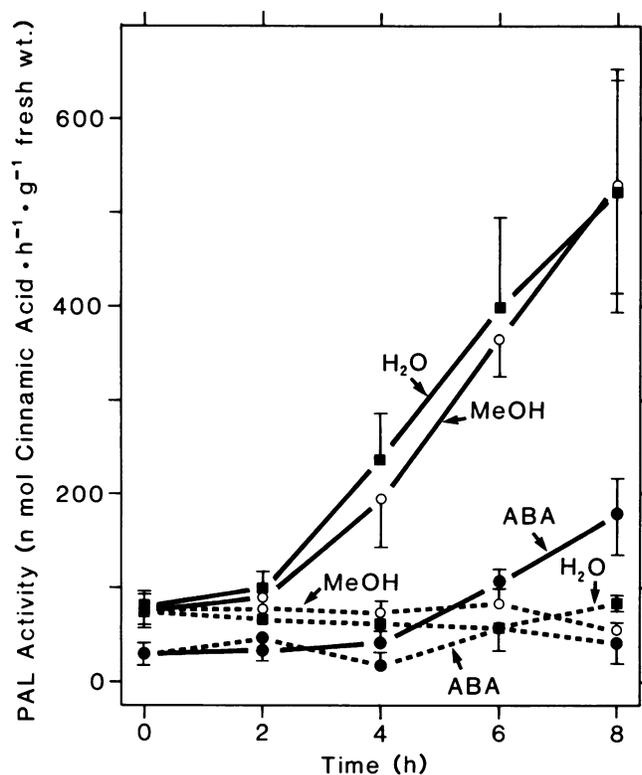


Figure 1. Influence of abscisic acid on phenylalanine ammonia-lyase activity in etiolated soybean hypocotyls inoculated with an incompatible race of *P. megasperma f.sp. glycinea*. Seedlings were treated with abscisic acid (2 × 10⁻³ M in 0.2% methanol) (●), 0.2% methanol (○), or water (■) overnight and then inoculated by placing drops of zoospore suspension (solid line) or water (broken line) on the hypocotyl surface. Points on the graph are the means of determinations from two experiments each with two replicates.

synthesis of phenylpropanoids, including glyceollin. It was not unexpected, therefore, that since ABA treatment suppressed glyceollin accumulation it should also suppress PAL activity. Cytologically, resistant and susceptible responses are clearly distinguishable 2 to 3 h following inoculation (21), at which time increases in PAL activity can be detected in inoculated untreated hypocotyls (Fig. 1; refs. 2 and 3). One interpretation of these results is that ABA induces susceptibility by suppressing PAL activity and consequent production of glyceollin and other phenylpropanoids during this critical period in the development of the host-pathogen interaction.

Inoculation of control hypocotyls (water or methanol treated) induced the synthesis of PAL mRNA (Fig. 2, lanes E and F) consistent with a previous study using intact etiolated soybean hypocotyls (7). Treatment of hypocotyls with ABA prior to inoculation caused a major decrease in the accumulation of PAL mRNA, consistent with the suppression of PAL activity. If the steady state level of PAL mRNA is primarily a function of the rate of PAL gene transcription, as demonstrated for a related system in bean cell suspensions (6), ABA may regulate PAL activity at the transcriptional level. This possibility is consistent with evidence from other systems that ABA regulates enzyme activity by interfering with transcription (*e.g.* 5, 15).

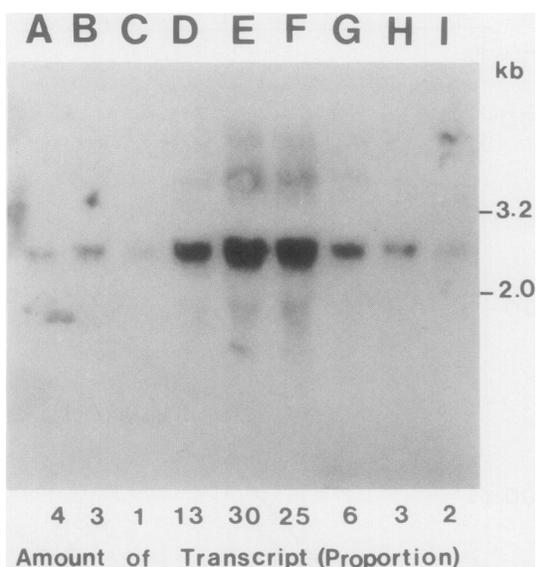


Figure 2. Influence of abscisic acid on the steady state level of phenylalanine ammonia-lyase mRNA in etiolated soybean hypocotyls inoculated with an incompatible race of *P. megasperma* f.sp. *glycinea*. Seedlings were treated with abscisic acid (2×10^{-3} M in 0.2% methanol), 0.2% methanol, or water overnight and then inoculated by placing drops of zoospore suspension or water on the hypocotyl surface. Total cellular RNA was isolated at the time of inoculation (0 h) or 5 h after inoculation and fractionated by agarose gel electrophoresis. Blots on nitrocellulose were hybridized with a 32 P-labeled cDNA for phenylalanine ammonia-lyase from beans, and phenylalanine ammonia-lyase mRNA was detected by autoradiography. A, 0 h, abscisic acid; B, 0 h, methanol; C, 0 h, water; D, 5 h, abscisic acid, inoculated with zoospores; E, 5 h, methanol, inoculated with zoospores; F, 5 h, water, inoculated with zoospores; G, H, I, as D, E, and F, respectively, but treated with water drops. The markers on the right-hand side are 3.2 kb and 2.0 kb for 25s and 18s rRNA, respectively. Proportion of transcript for phenylalanine ammonia-lyase was calculated after standardization of phenylalanine ammonia-lyase mRNAs in individual treatments with the respective amount of 18s rRNAs hybridized to a pea rDNA probe.

The results extend those of others who have reported that treatment of plants with high concentrations of ABA suppresses disease resistance (10, 18). They demonstrate that resistance is suppressed in soybeans also and in addition that this is accompanied by suppression of glyceollin accumulation, PAL activity, and transcription of mRNA for PAL. In terms of the interaction of soybeans with *P. megasperma* f.sp. *glycinea*, these findings suggest that resistance-related responses, that lead to the synthesis of glyceollin, may be controlled at the level of gene transcription by changes in ABA concentration caused by infection. This possibility will be examined in subsequent studies.

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