De Novo Messenger RNA and Protein Synthesis Are Required for Phytoalexin-mediated Disease Resistance in Soybean Hypocotyls

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MASAHIKI YOSHIKAWA, KAZUMA YAMAUCHI, AND HAJIME MASAGO
Laboratory of Plant Pathology, Faculty of Agriculture, Kyoto Prefectural University, Kyoto 606, Japan

ABSTRACT
Actinomycin D inhibited the synthesis of poly(A)-containing messenger RNA in healthy soybean (Glycine max [L.] Merr. cv. Harosoy 63) hypocotyls and in hypocotyls inoculated with the pathogenic fungus Phytophthora megasperma var. sojae A. A. Hildb., but had little effect on protein synthesis within 6 hours. Blasticidin S, conversely, inhibited protein synthesis in the hypocotyls without exhibiting significant effects on messenger RNA synthesis. The normal cultivar-specific resistance of the Harosoy 63 soybean hypocotyls to the fungus was completely diminished by actinomycin D or blasticidin S. The fungus grew as well in hypocotyls treated with either inhibitor as it did in the near isogenic susceptible cultivar Harosoy, and production of the phytoalexin glyceolin was concomitantly reduced. The effects of actinomycin D and blasticidin S were pronounced when the treatments were made at the time of fungus inoculation or within 2 to 4 hours after inoculation, but not after longer times. These results indicated that the normal expression of resistance to the fungus and production of glyceolin both required de novo messenger RNA and protein synthesis early after infection. Furthermore, actinomycin D and blasticidin S also were effective in suppressing resistance expression and glyceolin production in soybean hypocotyls when inoculated with various Phytophthora species that were normally nonpathogenic to the plants. This indicated that the mechanism of general resistance to these normally nonpathogenic fungi also involves de novo messenger RNA and protein synthesis and production of glyceolin.

Disease resistance in several plant-fungal pathogen interactions has been suggested as being due to the inducible production of antibiotic molecules, phytoalexins (1, 12). The initial biochemical events which may occur soon after infection and lead to the subsequent expression of resistance or production of phytoalexins are not, however, currently well characterized. It has been proposed (2, 6) that the initiation of resistance responses is mediated by de novo gene activation resulting in the synthesis of new species of messenger RNAs and proteins possibly required for the production of phytoalexins.

Quantitative and qualitative enhancements in protein metabolism appear to occur in some resistant-responding plants at early stages of infection (19, 21), but a more direct indication of the importance of de novo protein synthesis in disease resistance is the fact that certain protein synthesis inhibitors diminish plant resistance, thereby resulting in considerable pathogen growth (15, 17). Although possible alterations in messenger RNA metabolism in diseased plants have not been critically evaluated, a recent study (Tani et al., personal communication) using an RNA synthesis inhibitor indicated that de novo RNA synthesis is also necessary for resistance expression in the oat crown rust disease. Hadwiger and co-workers (5, 7, 14) found that pisatin, a phytoalexin produced in pea plants, could be induced by various chemicals which were presumed to affect the conformation of DNA; based on this and inhibitor experiments they (7, 14) suggested that pisatin production depended upon both de novo RNA and protein synthesis. To date, however, no systematic study that would link de novo RNA and protein synthesis, phytoalexin production, and disease resistance has been conducted.

The expression of monogenic resistance in soybean (Glycine max [L.] Merr.) to a fungal pathogen Phytophthora megasperma var. sojae A. A. Hildb. has been attributed to the production of a phytoalexin (8–11, 23), glyceolin (13). Yoshikawa et al. (22) recently found that soybean hypocotyl resistant to P. megasperma var. sojae synthesized poly(A)-containing RNA about six times more rapidly than uninoculated plants early after infection and preceding the time of occurrence of resistant expression or phytoalexin production. The poly(A)-containing RNA was later characterized to be in fact messenger RNA in the in vitro wheat germ translation system (Yoshikawa, unpublished data) and this provided the first evidence that the expression of disease resistance in plants is accompanied by activation of messenger RNA synthesis. The present experiments were designed to elucidate further whether de novo messenger RNA synthesis and protein synthesis are causally linked with the expression of resistance and the production of glyceolin by soybean hypocotyls.

MATERIALS AND METHODS

Chemicals. [G-3H]Uridine and [U-14C]lysine were obtained from The Radiochemical Centre, Amersham. Poly(U)-Sepharose (4B) was purchased from Pharmacia Fine Chemicals and actinomycin D from Schwarz/Mann. Blasticidin S was donated by H. Sumi.

Plant and Fungus. The soybean cultivar Harosoy 63 (resistant to race 1 of P. megasperma var. sojae) was grown as described previously (22) and used throughout the experiments unless otherwise specified. Race 1 of P. megasperma var. sojae was maintained on V-8 juice agar. For inoculum production, the fungus was grown for 3 to 5 days in a pea broth medium (10) and rinsed with deionized H2O prior to inoculation.

Seven- to 8-day-old plants were cut at the base of the hypocotyls and the cut ends placed in deionized H2O or aqueous solutions of either blasticidin S, a protein synthesis inhibitor (4), or actinomycin D at various concentrations. A longitudinal slash wound (about 1 cm long) was made with a razor blade on each hypocotyl, approximately 1 cm below the cotyledonary node, and inoculated with a small piece of mycelium.

Plants were maintained at a relative humidity of 100% after

1 This research was supported in part by Grant 256040 from the Ministry of Education of Japan to M.Y.
inoculation in a growth chamber at 25 C under fluorescent lighting (about 2,000 lux).

**Messenger RNA and Protein Synthesis.** At various times after inoculation or antibiotic treatment, the fungal inoculum was removed from the hypocotyl wounds prior to isotope feeding. Ten μl of [3H]uridine solution (50 μCi/ml, 6.5 Ci/mmole) for measurement of messenger RNA synthesis or L-[U-14C]lysine solution (1 μCi/ml, 348 μCi/mmole) for measurement of protein synthesis were placed on each wound of the hypocotyls and the feeding was continued for 2 hr. Twenty plants were used for each measurement. After feeding, wounded portions (1 cm long) of the hypocotyls were harvested and then immediately pulverized in a mortar under liquid N2.

For measurement of protein synthesis, the resulting fine powder was suspended in 20 ml of ice-cold 5% (w/v) trichloroacetic acid containing unlabeled L-lysine at 5 mM and kept for 1 hr at 0 C. Three-ml aliquots of the suspension were filtered through fiberglass filters (Whatman GF/C, 2.4 cm diameter). The filters were washed three times with 10 ml of ice-cold 5% (w/v) trichloroacetic acid containing unlabeled L-lysine at 5 mM and then dried. The radioactivities on the filters was directly counted in 10 ml of a toluene scintillation mixture (22) and referred to as the protein fraction.

Rates of messenger RNA synthesis was determined by extracting [3H]uridine-labeled total RNA from the pulverized powder by phenol-chloroform-isomyl alcohol method and isolating poly(A)-containing messenger RNA from the total RNA by poly(U)-Sepharose affinity chromatography as described previously (22).

**Glycoollin Analysis.** Wounded portions (1 cm long) of the 20 hypocotyls were harvested at 2 days after inoculation at which glycocollin concentrations in the inoculated hypocotyls reached about the maximum (8, 23), and glycocollin was extracted and quantitated as described elsewhere (23).

**Microscopic Observation.** Fungal growth in soybean hypocotyls was determined after making hand sections and staining them with Rose Bengal as described previously (22). At least five hypocotyls were randomly sampled and about five penetration points/hypocotyl were observed in each experiment.

### RESULTS

**Effects of Actinomycin D and Blasticidin S on Messenger RNA and Protein Synthesis.** Actinomycin D and blasticidin S at 10 μg/ml and 0.5 μg/ml, respectively, the minimal concentrations needed to block completely the expression of resistance by soybean hypocotyls as described below, efficiently inhibited the synthesis of messenger RNA or protein in both uninoculated and inoculated soybean hypocotyls (Table I). Messenger RNA synthesis in inoculated plants was about five times greater than that in uninoculated plants at 4 hr after inoculation, but this increase was inhibited by actinomycin D. Rates of protein synthesis were not significantly affected by infection at the same time after inoculation, but protein synthesis was inhibited by blasticidin S in both inoculated and un inoculated plants. The effect of actinomycin D on protein synthesis or of blasticidin S on messenger RNA synthesis was minimal until at least 6 hr after the antibiotic treatments (Fig. 1). Thus, it appeared that actinomycin D and blasticidin S could be used as selective inhibitors of messenger RNA and protein synthesis, respectively, in soybean hypocotyls.

**Effects of Actinomycin D and Blasticidin S on the Expression of Resistance and the Production of Glycoollin.** Harosoy 63 soybean hypocotyls were normally resistant to race 1 of *P. megasperma* var. *sojae* as evidenced by suppression of fungal growth, and this was accompanied by high levels of glycoollin accumulation in the infected plants (Table II). Resistance was diminished by relatively low concentrations of actinomycin D and blasticidin S when the treatments were initiated simultaneously with inoculation (Table II). Actinomycin D at 10 μg/ml or greater and blasticidin S at 0.5 μg/ml or more completely negated resistance.

![Figure 1](https://www.plantphysiol.org/figure/1)

**FIG. 1.** Time course of inhibitions of messenger RNA (A) and protein (B) synthesis by actinomycin (AMD) and blasticidin S (BCS) in Harosoy 63 soybean hypocotyls. Plants (uninoculated) were treated with the antibiotics for various periods as indicated on the abscissa and then fed with the isotopes for subsequent 2 hr. Messenger RNA and protein analysis are the same as described in Table I.

<table>
<thead>
<tr>
<th>Antibiotics1</th>
<th>Concentration of antibiotic (μg/ml)</th>
<th>Visible plant symptom</th>
<th>Hypobacterial growth (in mm)</th>
<th>Concentration of glycoollin2</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMD</td>
<td>0.5</td>
<td>85</td>
<td>576</td>
<td>1180</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>75</td>
<td>640</td>
<td>1294</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>17</td>
<td>1127</td>
<td>392</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0</td>
<td>&lt;1500</td>
<td>36</td>
</tr>
<tr>
<td>Blasticidin S</td>
<td>0.05</td>
<td>80</td>
<td>601</td>
<td>1247</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>49</td>
<td>651</td>
<td>390</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>7</td>
<td>1212</td>
<td>264</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0</td>
<td>&lt;1500</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0</td>
<td>&lt;1500</td>
<td>88</td>
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<tr>
<td>Susceptible control (R0)</td>
<td>0</td>
<td>&lt;1500</td>
<td>176</td>
<td></td>
</tr>
</tbody>
</table>

1 Tissue treatments were done on four separate experiments.  
2 Tissue treatments were done on four separate experiments.  
3 Visible plant symptom: resistant: no rot; susceptible: extensive rot and water soaking.  
4 Hypobacterial growth in the hypocotyl measured at 1 day after inoculation by making two hand sections and staining them with Rose Bengal.  
5 Glycoollin concentrations measured at 2 days after inoculation.  
6 Tissue treatments were done on four separate experiments.  
7 Visible plant symptom: resistant: no rot; susceptible: extensive rot and water soaking.  
8 Hypobacterial growth in the hypocotyl measured at 1 day after inoculation by making two hand sections and staining them with Rose Bengal.  
9 Glycoollin concentrations measured at 2 days after inoculation.  
10 Tissue treatments were done on four separate experiments.  
11 Visible plant symptom: resistant: no rot; susceptible: extensive rot and water soaking.  
12 Hypobacterial growth in the hypocotyl measured at 1 day after inoculation by making two hand sections and staining them with Rose Bengal.  
13 Glycoollin concentrations measured at 2 days after inoculation.  
14 Tissue treatments were done on four separate experiments.  
15 Visible plant symptom: resistant: no rot; susceptible: extensive rot and water soaking.  
16 Hypobacterial growth in the hypocotyl measured at 1 day after inoculation by making two hand sections and staining them with Rose Bengal.  
17 Glycoollin concentrations measured at 2 days after inoculation.
and fungal growth in the hypocotyls was identical to that occurring in the near isogenic susceptible soybean cultivar Harosoy. Concomitantly with the suppression of disease resistance, levels of glyceollin accumulated in the infected and inhibitor-treated hypocotyls were reduced to those normally found in the susceptible genotype. These results indicated that de novo messenger RNA and protein synthesis were required for the expression of resistance in soybean hypocotyls to P. megasperma var. sojae.

Fungal growth in Harosoy 63 hypocotyls normally ceased at 8 to 10 hr after inoculation (Fig. 2), but actinomycin D and blasticidin S completely blocked this expression of resistance. It was therefore assumed that de novo messenger RNA and protein synthesis, if causally linked with the expression of resistance, should occur early after infection. This was tested by applying the antibiotics at various times after inoculation. Actinomycin D suppressed resistance and glyceollin accumulation when supplied until 2 hr after inoculation, but the effects were greatly diminished when the treatment was made at 4 hr or more after inoculation (Table III). Similarly, the inhibitory effects of blasticidin S were pronounced until 4 hr, but the later initiation of the treatment was ineffective. The results therefore indicate that de novo messenger RNA and protein synthesis required for resistance expression must occur within about 3 to 5 hr after inoculation. The results suggest that the messenger RNA synthesis required for resistance occurs before the corresponding protein synthesis, since later treatments with blasticidin S than with actinomycin D were still effective in suppressing resistance (Table III).

The general resistance of soybean hypocotyls to various Phytophthora species which are normally nonpathogenic was characterized by high levels of glyceollin accumulation (Table IV). This resistance to normal nonpathogens was also reduced by actinomycin D and blasticidin S, and again was accompanied by repressed glyceollin production (Table IV). These results suggest that the resistance of soybean hypocotyls to various nonpathogens also involves de novo messenger RNA and protein synthesis-mediated processes which ultimately result in glyceollin biosynthesis.

**DISCUSSION**

The present study clearly indicated that the normal expression of disease resistance in soybean hypocotyls requires de novo messenger RNA and protein synthesis. This seemed to be true not only for the cultivar-specific resistance of the Harosoy 63 soybean cultivar to P. megasperma var. sojae (Table II), but also for the general resistance of soybeans to various pathogens (Table IV).

**Fig. 2. Time course of growth of race 1 of P. megasperma var. sojae in Harosoy 63 soybean hypocotyls treated with H2O (control), actinomycin D (AMD, 10 μg/ml), or blasticidin S (BCS, 0.5 μg/ml), and in the susceptible hypocotyls (susceptible control) of the near isogenic Harosoy soybean cultivar. Antibiotic treatments were initiated simultaneously with inoculation.**

**Table III. Effects of actinomycin D and blasticidin S that were applied from various times after the inoculation on growth of race 1 of Phytophthora megasperma var. sojae in soybean hypocotyls and on phytoalexin (glyceollin) production by the plants.**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Antibiotics</th>
<th>Visible plant symptom</th>
<th>Mycelial growth in host</th>
<th>Glyceollin (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0.45</td>
</tr>
<tr>
<td>Actinomycin D (10 μg/ml)</td>
<td>0</td>
<td>0</td>
<td>&gt;1500</td>
<td>74</td>
</tr>
<tr>
<td>Blasticidin S (0.5 μg/ml)</td>
<td>0</td>
<td>0</td>
<td>&gt;1500</td>
<td>101</td>
</tr>
</tbody>
</table>

**Table IV. Effects of actinomycin D (10 μg/ml) and blasticidin S (0.5 μg/ml) on growth of various nonpathogenic Phytophthora species in soybean hypocotyls and on phytoalexins (glyceollin) production by the plants.**

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Antibiotics</th>
<th>Visible plant symptom</th>
<th>Mycelial growth in host</th>
<th>Glyceollin (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytophthora strains</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0.45</td>
</tr>
<tr>
<td>Actinomycin D (10 μg/ml)</td>
<td>0</td>
<td>0</td>
<td>&gt;1500</td>
<td>74</td>
</tr>
<tr>
<td>Blasticidin S (0.5 μg/ml)</td>
<td>0</td>
<td>0</td>
<td>&gt;1500</td>
<td>101</td>
</tr>
</tbody>
</table>

**FIG. 2.** Time course of growth of race 1 of P. megasperma var. sojae in Harosoy 63 soybean hypocotyls treated with H2O (control), actinomycin D (AMD, 10 μg/ml), or blasticidin S (BCS, 0.5 μg/ml), and in the susceptible hypocotyls (susceptible control) of the near isogenic Harosoy soybean cultivar. Antibiotic treatments were initiated simultaneously with inoculation.

The de novo messenger RNA and protein synthesis associated with the expression of resistance appeared to occur relatively early after infection since fungal growth was inhibited about 8 to 10 hr after inoculation (Fig. 2). This was corroborated by the fact that actinomycin D and blasticidin S were effective in reversing resistance only when their treatments were initiated soon after inoculation (Table III). This is consistent with previous observations (22) which indicated that messenger RNA synthesis in resistant-responding soybean hypocotyls was about six times greater than in uninoculated plants at early stages of infection (about 4–8 hr after inoculation). The present results also indicate that the observed synthesis of messenger RNA is directly involved in the expression of resistance and phytoalexin production, since disease resistance and glyceollin production were concomitantly diminished by the inhibitors. This and the fact (23) that glyceollin accumulates in infected portions of soybean hypocotyls at concentrations sufficient to cause the observed inhibition of fungal growth occurring in the resistant host at 8 to 10 hr after inoculation further indicate that glyceollin accumulation is the mechanism responsible for the resistance of soybean to P. megasperma var. sojae and possibly also to various nonpathogens.

The use of selective antibiotics for evaluating resistance mechanisms in plant-fungal pathogen interactions has been relatively limited (3, 5, 16–18, 20). The major reason for this probably arises from the difficulty in finding suitable inhibitors. Only those chemicals to which some aspect of plant but not pathogen metabolism is sensitive will be successful inhibitors. As found here, actinomycin D and blasticidin S did not inhibit the growth of Phytophthora species in the host when they were applied through the hypocotyls. On the other hand, however, the author tested several RNA inhibitors for their ability to suppress resistance of soybean to P. megasperma var. sojae by the same methods employed with actinomycin D but found that only actinomycin D was effective. Among the ineffective inhibitors were cordycepin, chromomycin, ethidium bromide, proflavine, 8-azauridine, and

**Table V.**
6-methylmercaptopurine. The precise reason for ineffectiveness of these inhibitors is not known except that cordycepin did not inhibit messenger RNA synthesis in the soybean hypocotyls as strongly as did actinomycin D even after prolonged periods of incubation (30-40% inhibition of messenger RNA synthesis after 15 hr incubation).

It seems difficult to prove unequivocally that the observed suppression of resistance expression and glyceollin production in soybean hypocotyls by actinomycin D and blasticidin S resulted solely from specific inhibition of messenger RNA and protein synthesis, or whether the results were due to nonspecific side effects of the inhibitors. In the present study, however, the latter possibility appeared to be excluded, based on the combined facts that: (a) actinomycin D and blasticidin S had only minor effects on nontarget metabolism such as protein and messenger RNA synthesis, respectively, in the soybean hypocotyls (Fig. 1); and (b) the inhibitor effects on resistance occurred within 4 to 6 hr after the initiation of the treatments (Fig. 2 and Table III).

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