Specific binding of a fungal glucan phytoalexin elicitor to membrane fractions from soybean *Glycine max* (β-1,3-[3H]glucan elicitor/*Phytophthora megasperma* glycinea/ binding sites/soybean plasma membrane/phytoalexin synthesis)

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ABSTRACT

Treatment of soybean tissues with elicitors results in the production of phytoalexins, one of a number of inducible plant defense reactions against microbial infections. The present study uses a β-1,3-[3H]glucan elicitor fraction from *Phytophthora megasperma* f. sp. *glycinea*, a fungal pathogen of soybean, to identify putative elicitor target sites in soybean tissues. Use of the radiolabeled elicitor disclosed saturable high-affinity elicitor binding site(s) in membrane fractions of soybean roots. Highest binding activity is associated with a plasma membrane-enriched fraction. The apparent *K*ₐ value for β-glucan elicitor binding is ~0.2 × 10⁻⁶ M and the maximum number of binding sites is 0.5 pmol per mg of protein. Competition studies with the [3H]glucan elicitor and a number of polysaccharides demonstrate that only polysaccharides of a branched β-glucan type effectively displace the radiolabeled ligand from membrane binding. Differential displacing activity of the glucans on *P. megasperma* elicitor binding corresponds closely to their respective ability to elicit phytoalexin production in a cotyledon bioassay.

Plants possess numerous defense mechanisms conferring disease resistance against potential pathogens. Among these, several inducible biochemical responses, including the production of antimicrobial phytoalexins, the reinforcement of the plant cell wall through the synthesis of lignin-like materials, the deposition of callose, the accumulation of hydroxyproline-rich glycoproteins, or the enhancement of the activity of certain hydrolytic enzymes, appear to play an important role (1–3). The defense responses can be induced not only by infection but also by exposure of different plant tissues to biotic or abiotic elicitors (3–6). The term elicitor has been commonly used to refer to compounds that induce phytoalexin synthesis in plants.

Previously it was shown that soybean (*Glycine max*) tissues produce and accumulate isoflavonoid phytoalexins following either inoculation with a soybean pathogen, the fungus *Phytophthora megasperma* f. sp. *glycinea*, or treatment with a β-glucan elicitor isolated from the fungal cell walls (3). Phytoalexin accumulation was correlated with transient increases in the activities of the phytoalexin biosynthetic enzymes (3). The transient increase in chalcone synthase activity, the first enzyme specific for flavonoid/iso-flavonoid biosynthesis, was preceded by a large and rapid enhancement in chalcone synthase mRNA activity and amount. This suggests that the phytoalexin defense response in soybean, as in other systems (7, 8), is controlled at the transcriptional level (3).

The key event in the elicitor-mediated phytoalexin response is the interaction of the elicitor with the presumed target site(s). The site(s) mode of action, and ultimate fate of an elicitor have not been characterized for any elicitor-active molecule. Various sites, including the cell wall, the plasmalemma, and some intracellular components have been suggested as putative targets for the primary action of elicitors. Using [14C]mycolaminaran, a branched β-1,3-glucan from *Phytophthora* spp. as ligand, saturable glucan-binding sites have been reported to exist in membrane fractions isolated from soybean cotyledons (9). Mycolaminaran has, however, relatively low phytoalexin elicitor activity when compared to the *P. megasperma* β-glucan (10), and the structural relationship of mycolaminaran to the β-glucan elicitor isolated from *P. megasperma* f. sp. *glycinea* cell walls (5, 11) has not been fully established.

Here we report on the identification of saturable and high-affinity elicitor binding in microsomal soybean membrane fractions using as ligand a biologically highly active β-1,3-glucan elicitor fraction from *P. megasperma* that had been tritiated by reduction with sodium boro[3H]hydride. Highest elicitor binding activity was found in a plasma membrane-enriched fraction. Furthermore, a close correlation existed between the effectiveness of a number of β-glucan-type polysaccharides as competitors to the β-[3H]glucan elicitor for binding and the ability of these substances to enhance phytoalexin synthesis in a bioassay.

MATERIALS AND METHODS

Chemicals. Sodium boro[3H]hydride (806 GBq/ mmol) and uridine diphospho-[U-14C]glucose (10.4 GBq/ mmol) were obtained from Amersham Buchler (Braunschweig, F.R.G.). Various polysaccharides were obtained from the following sources: dextran T500 (Pharmacia); laminarin from *Laminaria digitata*, pustulan from *Umibucillaria papulosa* (Calbiochem); lichenan from *Cladophora islandica*, nigeran from *Aspergillus niger* (Koch-Light Laboratories, Bucks, England); arabinogalactan from larch wood, chitosan from crab shells, glucon and mannan from *Saccharomyces cerevisiae*, pullulan from *Aureobasidium pullulans*, xylan from larch wood (Sigma). Mycolaminaran was a gift from M. Yoshikawa (Kyoto), an oligogalacturonide fraction from soybean cell walls was a gift from M. Hahn (Athens), and a cell wall fraction from *Alternaria carthami* was a gift from H. Strasser and H. Wendorff (Freiburg, F.R.G.).

Buffers. The following buffers were used: buffer A, 25 mM Tris-HCl, pH 7.0/10 mM MgCl₂/2 mM dithiothreitol; buffer B, 10 mM Tris-HCl, pH 7.2/1 mM EDTA/0.1 M KCl/2 mM dithiothreitol; buffer C, 50 mM Tris-HCl, pH 7.8/1 mM EDTA/25 mM KCl/0.25 M sucrose/2 mM dithiothreitol; buffer D, 10 mM Tris-HCl, pH 7.0/10 mM MgCl₂/1 M NaCl.

Soybean Seedlings. Seeds of soybean *G. max (L.)* Merr. cv. Effi were obtained from Kleinwanzlebener Saatzucht (Einbeck, F.R.G.). Seedlings were grown in vermiculite as described (12). A photoperiodic regime of 14 hr of illumination (Osram HQI; 23,000 lx) and 10 hr of darkness was used. The temperatures during the light and dark cycles were 28°C and 26°C, respectively. The relative humidity was 75%. Seven-day-old seedlings were used for experiments.

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Fungal Cultures. *P. megasperma* Drechs f. sp. glycinea Kuan and Erwin race 1 was obtained from E. Ziegler (Aachen, F.R.G.) and grown as described (12). Large batches of mycelium were a gift from M. Liersch (CIBA-Geigy, Basel, Switzerland).

Elicitor Preparation. *P. megasperma* mycelial walls were prepared and partially hydrolyzed as described (13) with the modifications outlined below. Lyophilized mycelial walls (5 g) were suspended in 500 ml of 2 M trifluoroacetic acid in a 500-ml Pyrex bottle fitted with a Teflon-sealed screwcap. Partial acid hydrolysis was carried out at 85°C for 2 hr. Solubilized elicitor-active fragments were partially purified by successive low- and high-resolution gel filtration on Bio-Gel P-4 equilibrated with distilled water at room temperature and 55°C, respectively. A glucan elicitor fraction with an average degree of polymerization of 22 and consisting of 100% glucose was used as ligand in the binding studies.

The elicitor was titrated by reduction with sodium borohydride. The crude radioactively labeled product was purified by high-resolution gel filtration on Bio-Gel P-4 at 55°C to yield a specific radioactivity of ≈2 Ci (74 GBq)/mmol. Reduced unlabeled elicitor was prepared by the same method.

Cell-Free Extracts. Soybean seedling tissue (30 g) was homogenized twice for 3 sec each at 4°C in 120 ml of buffer A in a blender (Moulinex). The slurry was filtered through a nylon screen (pore size, 75 μm) and then centrifuged for 10 min at 8000 × g. A membrane fraction was collected by centrifugation of the supernatant for 40 min at 43,000 × g. The pellet was frozen in liquid nitrogen and stored at −70°C.

Sucrose Gradient Centrifugation. A 16-ml linear 15–45% (wt/vol) sucrose gradient in buffer B was layered over a 45% sucrose solution (1.5 ml) in buffer B. A 15% sucrose solution (1.5 ml in buffer B) was layered onto the top of the gradient. Crude cell-free extracts for density gradient centrifugation were prepared from soybean roots using buffer C. The precentrifuged (10 min, 6000 × g) extract (18 ml) was applied to the sucrose gradient. The gradient was centrifuged at 100,000 × g for 150 min at 2°C using a Beckman (model L2-65B) ultracentrifuge equipped with an SW 27 rotor. After centrifugation, fractions (1 ml each) were collected and the positions of the marker enzymes, glucose-6-phosphate dehydrogenase (EC 1.1.1.49), NADH-cytochrome reductase, 1,3-β-glucan synthase (EC 2.4.1.34), and cytochrome-c oxidase (EC 1.9.3.1) were determined by standard assays (14–16).

For the demonstration of elicitor binding by density gradient sedimentation, fractions (each over a range of 4% changes in sucrose concentration) of the sucrose gradient were combined. The combined fractions were diluted by addition of buffer D, and the membranes were collected by centrifugation for 50 min at 70,000 × g.

Binding Assay. Pellets of soybean membrane fractions were suspended in buffer D using a Potter–Elvehejm-type homogenizer. Membrane fractions containing 500–800 μg of protein were incubated for 2 hr at 4°C in a total vol of 400 μl of buffer D, containing 80 nM [3H]glucan elicitor and 7.5 mM β-d-thiogluco and 1,5,6-glucanolactone. Nonspecific binding was determined in the presence of 57 mM μg glucan elicitor. Incubations were carried out at 30°C for 45 min in 1 ml of a 0.3 M sucrose solution in buffer D and centrifuging for 45 min at 45,000 × g. The supernatant was discarded and the pellet was carefully washed twice with 500 μl each of buffer D. The pellet was extracted with 1 ml of water for 20 min at 96°C and the water-soluble radioactivity was measured by scintillation spectrometry.

Assay for Elicitor Activity. Elicitor activity was measured by using a described soybean cotyledon assay (12, 17). The data are expressed as the A285 at the sample, relative to the A285 of a *P. megasperma* "large extracellular elicitor" (12), which induced maximum phytoalexin accumulation at an appropriate concentration (A285/A285max). The A at 285 nm is directly proportional to the amount of pterocarpan phytoalexins in the wound droplets (12, 17).

Analytical Procedures. Protein was measured by a modification of the method according to Lowry (18), with bovine serum albumin as reference. Hexose content was determined by the anthrone method (19), with glucose as standard. Reducing sugars were measured by the method of Nelson (20) as modified by Somogyi (21) using glucose as reference. The glycosyl-linkage composition of oligoglycans was determined by permethylation of the oligomers (22) followed by gas chromatographic/mass spectrometric analysis of the mixed acetoxy/methoxy derivatives (23). Radioactivity was determined by scintillation spectrometry in Rotiszint 22 (Roth, Karlsruhe, F.R.G.), which consists of a mixture of toluene and Triton X-100 containing 2,5-diphenyloxazole and 2,2'-phénylène bis-(5-phenyloxazole).

**RESULTS**

Radioactive Elicitor Ligand. It has been demonstrated (13) that partial acid hydrolysis of *P. megasperma* cell walls releases elicitor-active glucan fragments. Low- and high-resolution gel filtration on Bio-Gel P-4 of a mixture of acid-solubilized material resulted in a partial separation of glucan fragments. Fragments with an average degree of polymerization of 20–25 were collected. These fragments were composed entirely of glucose and possessed a glycosyl-linkage composition typical of the *P. megasperma* branched β-glucan consisting of terminal, 3-, 6-, and 3,6-linked glucosyl residues (13, 24, 25). The relative proportion of the differently linked glucosyl residues, as determined by methylation analysis (22, 23), was 23 mol % of terminal, 32 mol % of 3-linked, 3.7 mol % of 4-linked, 18 mol % of 6-linked, and 23 mol % of 3,6-linked residues. The fragments showed high phytoalexin elicitor activity when tested in the cotyledon bioassay (Table 1). The radioactive elicitor ligand used in the binding studies was obtained by reduction of the oligoglycan fraction with sodium borohydride to yield [3H]oligoglucosyl glucitols. Reduction does not affect the elicitor activity of the *P. megasperma* glucan (13), as demonstrated for the oligoglycan fraction, which was reduced with unlabeled sodium borohydride and purified in the same way as the radioactive elicitor (Table 1).

Elicitor Binding. Measurement of binding of the glucan elicitor was demonstrated by incubating soybean microsomal fractions with 80 nM [3H]glucan elicitor and separating free from bound elicitor by centrifugation of the reaction mixture through a 0.3 M sucrose layer. Elicitor binding obeyed the criterion for specific (saturable) binding (27) in that competition for the binding site(s) could be demonstrated in the presence of a large excess (57 μM) of unlabeled elicitor (Fig. 1). When microsomal fractions from 7-day-old cotyledons, hypocotyls, and roots were compared, highest specific binding activity (≈0.2 pmol per mg of protein) was found with membranes from soybean roots, whereas membranes from hypocotyls and cotyledons, specific binding activity was lower (0.06 and 0.1 pmol per mg of protein, respectively). Soybean roots were therefore used as the source of microsomal fractions throughout the studies. Initial trials showed that the incubation for 19 hr of [3H]glucan elicitor with root membranes produced a series of oligoglycans with a degree of polymerization considerably lower (≤6) than that of the original elicitor fraction (≈22) (Fig. 2). To avoid elicitor degradation by endogenous β-glucosidases during binding studies that might have obscured analysis of binding data, a series of compounds was tested for their ability to inhibit elicitor degradation. As shown in Fig. 2, addition of either 5 mM β-d-thioglucose or 5 mM 1,5,6-d-glucanolactone prevented elicitor degradation to fragments smaller than degree of...
Table 1. Comparison of various polysaccharides for their displacing ability of [3H]glucan elicitor binding and their phytoalexin elicitor activity.

<table>
<thead>
<tr>
<th>Substance*</th>
<th>Chemical characteristics (preparation)†</th>
<th>Replacement of β-glucan IC50,μg/ml (μM)</th>
<th>Phytoalexin synthesis EC50,μg/ml (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. megasperma</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucan elicitor</td>
<td>β-1,3(55%)-, 1,6(41%)-glucan (partially acid hydrolyzed)</td>
<td>1 (0.3)</td>
<td>0.3 (0.1)</td>
</tr>
<tr>
<td>Glucan elicitor</td>
<td>30% carbohydrate (water extracted)</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td><em>Phytophthora</em> sp.</td>
<td>Mycolaminaran</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elicitor</td>
<td>β-1,3(94%)-, 1,6(6%)-glucan</td>
<td>200 (40)</td>
<td>200 (40)</td>
</tr>
<tr>
<td><em>A. carthami</em></td>
<td>50% carbohydrate (water extracted)</td>
<td>300</td>
<td>180</td>
</tr>
<tr>
<td>L. digitata</td>
<td>Laminarin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Reduced)</td>
<td>β-1,3(90%)-, 1,6-glucan</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>(Reduced)</td>
<td>50</td>
<td>80</td>
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</table>

*No replacement and no phytoalexin synthesis found with mannan, glucan (S. cerevisiae), dextran, chitosan, xylan, arabinogalactan, pullulan, pustulan, lichenan, nigeran, and oligogalacturonide.
†The partially acid-hydrolyzed *P. megasperma* glucan elicitor fraction was prepared according to ref. 13 with the modifications described in Materials and Methods. A crude *P. megasperma* glucan elicitor fraction containing 30% carbohydrate and a crude *A. carthami* elicitor was obtained by extracting mycelial walls with water (24). The glycosyl-linkage composition for mycolaminaran was calculated as described (26).
‡IC50, 50% inhibitory concentration as calculated from the dose-response curves of competitors on specific [3H]glucan binding; EC50, 50% stimulatory concentration as calculated from the dose-response curves of substances for phytoalexin production in the cotyledon bioassay.

polymerization 6. Therefore, 7.5 mM β-D-thioglucose and 7.5 mM 1,5-β-D-glucosolactone were added to the standard binding assay throughout the experiments. The effect of increasing the concentration of unlabeled glucan elicitor on the binding of [3H]glucan elicitor is shown in Fig. 1. A 50% displacement (IC50) of the labeled ligand was attained with ≈0.3 μM unlabeled ligand. Scatchard analysis of the data (Fig. 3) gave an apparent Kd value of 0.2 × 10⁻⁶ M and a maximal concentration of binding site(s) of 0.5 pmol per mg of protein.

**Kinetic Analysis.** Kinetic analysis of [3H]glucan elicitor binding to membrane fractions demonstrated that, at 4°C, half-maximal saturation was attained in ≈25 min (Fig. 4). Nonspecific glucan binding remained almost constant throughout the period investigated. When dissociation of the labeled ligand was induced by an excess of unlabeled glucan elicitor after 120 min of initial incubation with the labeled ligand (Fig. 4, arrow), partial displacement of ≈45% of the labeled by the unlabeled glucan was observed. When the excess of unlabeled ligand was added at various times, beginning at the onset of incubation (Fig. 4, 0 min) and the assay mixture was analyzed after a further 120 min, most of the labeled ligand was replaced after 120 min. Thus, the relative amount of apparently nondisplaceable ligand increased over a period of 250 min. In the standard assay procedure, binding and dissociation were, therefore, studied

![Fig. 1](https://example.com/image1.png)

**Fig. 1.** Displacement of [3H]glucan elicitor by unlabeled glucan elicitor. Soybean root microsomes were incubated at 4°C in the assay medium containing 80 mM labeled ligand, in the absence or presence of various concentrations of unlabeled ligand. After 120 min, the samples were centrifuged through a 0.3 M sucrose layer, and the pellets were analyzed for radioactivity.

![Fig. 2](https://example.com/image2.png)

**Fig. 2.** Degradation and its inhibition of [3H]glucan elicitor after incubation with a soybean root membrane fraction. Microsomes were incubated for 19 hr in the absence (curve A) or presence of either 5 mM β-D-thioglucose (curve B) or 5 mM 1,5-β-D-glucosolactone (curve C), the reaction products subjected to thin-layer chromatography on silica gel 60 (solvent: toluene/dioxane/water/isopropanol, 20:46:20:20, vol/vol) and analyzed by a radiochromatogram scanner. Oligosaccharides with a defined degree of polymerization (DP) were used for calibration; curve D, untreated [3H]glucan elicitor sample used as reference.
in parallel samples at 4°C for 120 min so as to reach binding equilibrium and to obtain maximal displacement.

**Displacement by Various Saccharides.** The displacing ability of a number of polysaccharides was studied (Table 1). IC₅₀ (50% inhibitory concentration) values for a number of polysaccharides were calculated from the dose–response curves of their displacement of [³H]glucan binding. The displacing ability was compared with EC₅₀ (50% stimulatory concentration) values for phytoalexin elicitor activity in a cotyledon bioassay. Of the polysaccharides tested, only the branched β-glucan type effectively competed with the [³H]glucan elicitor for binding. These included mycelaminaran from *Phytophthora* spp. (9), a cell wall–released fraction from *A. cartilagi* (28), and laminarin from *L. digitata*. The differential effects of the various glucans on radiolabeled ligand binding (IC₅₀ values) corresponded closely with their respective ability to enhance phytoalexin production in the cotyledon bioassay (EC₅₀ value; Table 1). A larger number of polysaccharides that were unable to compete for binding or to elicit phytoalexin production in the concentration range tested included dextran, pustulan from *U. papillosa*, lichenan from *C. islandica*, nigeran from *A. niger*, arabinogalactan and xylan from larch wood, chitosan from crab shells, glucan and mannan from *S. cerevisiae*, pullulan from *A. pullulans*, and an oligogalacturonide fraction from soybean (17). The disaccharides maltose, gentiobiose, saccharose, and laminariobiose at concentrations of 0.1 mM did not affect radiolabeled ligand binding. At 1 mM laminariobiose inhibited specific [³H]glucan elicitor binding by 78%.

**Localization of Specific Elicitor Binding Sites.** Binding of the [³H]glucan elicitor to different subcellular fractions was examined after separating the membranes of cell-free root extracts by centrifugation on a linear sucrose density gradient. For elicitor binding assays, successive gradient fractions representing ~4% changes each in sucrose concentration were combined. The membranes were collected by centrifugation, and the membrane pellets were immediately resuspended in assay buffer. The separation of mitochondria from plasma membrane vesicles and endoplasmic reticulum was analyzed by measuring the distribution in the gradient fractions of the marker enzyme activities cytochrome-c oxidase (mitochondria), 1,3-β-glucan synthase (putative plasma membrane), and cytochrome reductase (endoplasmic reticulum). As shown in Fig. 5, the marker enzyme activities were clearly separated on the sucrose gradient and there was little contamination by the soluble enzyme glucose-6-phosphate dehydrogenase. Specific elicitor binding activity correlated well with the distribution of 1,3-β-glucan synthase activity (Fig. 5), thus indicating an association of the [³H]glucan elicitor binding sites with a plasma membrane–enriched fraction.

**DISCUSSION**

One important aspect of the elicitor–mediated activation of the phytoalexin defense response is the identification of the elicitor target in the receptive plant cell. To help in this identification, a high-specific-activity β-glucan elicitor fraction from *P. megasperma* was prepared by reduction of a
phytoalexin
soybean
seed;
physiological significance
unpublished
parsley
of
unpublished
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example, mycolaminaran,
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stringent
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[3H]glucan
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1,5-D-gluconolactone
(30),
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is
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assume that
and specific binding
partially
purified glucan fraction
We have shown that this radioactively labeled
ligand can be successfully used to demonstrate saturable,
high-affinity, and specific binding site(s) for the glucan
elicitor in a microsomal fraction of soybean roots. One must
assume that the elictor fraction used in these binding studies
is composed of a mixture of oligoglucans of different sizes
and glucose-linkage compositions (11, 13, 25). Nevertheless,
[3H]glucan elictor binding, as a whole, yielded a linear
Scatchard plot (Fig. 3). It is not known, at present, whether
linearity in the plot results from competitive binding of two
or more oligoglucans to a single class of identical and
independent sites (29) or whether it reflects binding of only
each of the oligoglucans.

Indirect evidence indicates that the glucan elictor binds to
a protein(s), as binding is abolished by Pronase treatment
of the microsomal fraction and stabilized in the presence of
dithiothreitol. Microsomal fractions contain β-glucosidases
(ref. 9; unpublished data), which are potentially capable
of binding the glucan elictor. However, the presence in
the binding assay of the competitive inhibitor of β-glucosidase,
1,5-D-gluconolactone (30), and of β-D-thiglucose, both of
which inhibit glucan elictor degradation (Fig. 2), very
probably rule out the possibility that the observed high-affinity
binding is associated with glucan hydrolyzing enzymes.

One remarkable result is the high ligand specificity of the
binding. Although only a limited variety of polysaccharides
were available, it is quite clear that displacement of the
[3H]glucan elictor from binding requires the competitor
able to fulfill stringent structural requirements (Table 1). Further-
more, the differential effects of a number of glucans on
radiolabeled ligand binding corresponded well with their
phytoalexin elictor activity in the cotyledon bioassay. For
example, mycolaminaran, which has been used as ligand in
binding studies by Yoshikawa et al. (9), shows similarly weak
displacing and phytoalexin elictor activities in our studies,
whereas laminaran exhibits intermediate activities. Further
experiments are required to establish whether the [3H]glucan
elictor merely shows superior binding to the same site(s) as
that described by Yoshikawa et al. (9), or whether different
binding sites have been investigated in the two studies.

Further important is the distribution of [3H]-
glucan elictor binding in subcellular fractions from soybean
roots. Analysis of glucan binding in sucrose gradient fractions
(Fig. 5) strongly suggests that elictor binding is associated
with a plasma membrane-enriched fraction.

The results of both the binding and localization studies
make it conceivable that the elictor binding site(s) identified
in vitro is related to or identical with the putative elictor
target in vivo. Evidence that elictor-induced phytoalexin
synthesis in plant cells might be a membrane-mediated
process has also been obtained by the demonstration that
elictor activity in soybean cell suspension cultures is
enhanced by the presence of Ca^{2+} ions (ref. 31; M. Stäb
and J.E., unpublished data) and that elictors rapidly inhibit
uptake of both inorganic phosphate and amino acids by
parsley (28) and soybean (M. Stäb, D. Grab, and J.E.,
unpublished data) cell suspension cultures. In conclusion,
our results suggest that the binding described here is of
physiological significance in the glucan elictor-mediated
phytoalexin response in soybean tissues.

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