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A Structural Model for the Mechanisms of Elicitor Release from Fungal Cell Walls by Plant β-1,3-Endoglucanase[1,2]

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The release of elicitor-active carbohydrates from fungal cell walls by β-1,3-endoglucanase contained in host tissues has been implicated as one of the earliest processes in the interaction between soybean (*Glycine max*) and the fungal pathogen *Phytophthora megasperma* f. sp. *glycinea* leading to host defense responses such as phytoalexin production. The present study was conducted to evaluate the primary structure of the glucanase-released elicitor (RE). Gel-filtration chromatography of carbohydrates released from mycelial walls by purified soybean β-1,3-endoglucanase resolved them into the four fractions (elicitor-active RE-I, -II, and -III and elicitor-inactive RE-IV). Sugar composition analysis indicated that all of the fractions were composed almost entirely of glucose. ^{1}H- and ^{13}C-nuclear magnetic resonance analysis indicated the presence of both β-1,3- and β-1,6-linkages for the elicitor-active RE-I, -II, and -III fractions and only β-1,3 linkage for the elicitor-inactive RE-IV fraction. Methylation analysis and degradation studies employing β-1,3-endo- and β-1,3-exoglucanase further suggested that the basic structure of elicitor-active RE consists of β-1,6-linked glucan backbone chains of various lengths with frequent side branches composed of β-1,3-linked one or two glucose moieties. From these structural analyses of RE, a structural model of how RE is originally present in fungal cell walls and released by host β-1,3-endoglucanase is also proposed.

Phytoalexins, which are low-molecular-weight antibiotic compounds that are inducibly formed in higher plants in response to microbial pathogen infection, appear to constitute one of the important defense mechanisms in plants (Keen, 1981). The induction of phytoalexins in infected plants is presumed to be mediated by an initial recognition process between plants and pathogens that involves detection by plant cells of certain unique molecules termed elicitors that are derived from pathogens (Yoshikawa, 1983). Elicitor preparations with phytoalexin-inducing activity have been obtained from several fungal pathogens and used for investigations of the molecular basis of phytoalexin production or disease-resistance expression. It therefore appears important to identify and use for these investigations the natural elicitors that function in vivo for the induction of phytoalexins in fungus-infected plant tissues. However, approaches to evaluating how such elicitors are generated during the natural infection processes as well as their structure have been limited (Yoshikawa, 1983).

The interaction between soybean (*Glycine max*) and the fungal pathogen *Phytophthora megasperma* f. sp. *glycinea* is one of the best-understood gene-for-gene plant-pathogen systems in which the processes leading to production of the phytoalexin glyceollin have been analyzed (Yoshikawa, 1983; Yoshikawa and Takeuchi, 1991). Mycelial walls of the fungus have been shown to be potent elicitors of glyceollin accumulation, and their possible role in the induction of phytoalexin accumulation in infected plants has been suggested (Yoshikawa, 1983). Isolated cell-wall preparations are, however, insoluble in water. Although harsh treatments such as autoclaving or exposure to acid or alkali can solubilize active elicitor moieties from mycelial walls (Yoshikawa, 1983) as exemplified by the smallest elicitor-active oligosaccharide, hepta-β-D-glucopyranoside (Sharp et al., 1984), these conditions are unlikely to exist in biological environments. Thus, the question remains as to how insoluble elicitor molecules on or in mycelial walls are solubilized and recognized by plant cells during natural infection processes.

Yoshikawa et al. (1981) found that soybean tissues contained a factor capable of releasing soluble and highly active carbohydrate elicitors from mycelial walls of *P. megasperma* f. sp. *glycinea*. The factor was later shown to be β-1,3-endoglucanase, which is constitutively present in soybean tissues (Keen and Yoshikawa, 1983; Takeuchi et al., 1990a, 1990b). Further studies indicated that the release of elicitors likely occurred in the in vivo soybean-fungus interaction, and RE was suggested to be responsible for the initiation of phytoalexin production in the infected soybean tissues (Takeuchi et al., 1990a; Yoshikawa et al., 1990). The results of an analysis of the primary structure of RE are presented in this communication, and a structural model of how RE is originally present in fungal cell walls and is

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Abbreviation: RE, β-1,3-endoglucanase-released elicitor.

released by host β-1,3-endoglucanase upon infection of plant cells is proposed.

MATERIALS AND METHODS

Preparation and Fractionation of RE

RE was prepared from cell walls isolated from mycelia of *Phytophthora megasperma* f. sp. *glycinea* race 1 by incubating them with purified soybean (*Glycine max*) β-1,3-endoglucanase as described by Yoshikawa and Sugimoto (1993), except that the final preparation of RE after CM Bio-Gel A column chromatography was further fractionated on a Sephadex G-75 gel-filtration chromatography column that was equilibrated and eluted with deionized water. Four different molecular weight fractions (RE-I, -II, -III, and -IV) were pooled separately, and after concentration by lyophilization, each pool was rechromatographed on the same column. The peaks corresponding to the original fractions were collected and used for structural analysis. Molecular weight estimates for the gel-filtration fractions were made by comparison of their elution volumes with those of standard dextrans from Pharmacia.

Chemical Techniques and Structural Analysis

Total hexose was determined with the anthrone reagent (Bartnicki-Garcia and Nickerson, 1962) with D-Glc as the standard. Monosaccharide compositions of different RE fractions were determined as alditol acetates by GLC as described by Slonker (1972) after hydrolysis of RE with 2.0 M TFA for 3 h at 100°C. The GLC was performed using a Shimadzu (Kyoto, Japan) GC-8A chromatograph equipped with a flame-ionization detector and glass column (3 mm × 2 m) packed with 3% Silicone OV-225 on Chromosorb W (80–100 mesh) operated at 193°C. Monosaccharide peaks were identified by parallel chromatography using alditol acetates of authentic rhamnose, Fuc, Rib, Ara, Xyl, Man, Gal, and Glc with retention times of 10.8, 11.2, 13.8, 14.5, 19.0, 39.8, 43.9, and 49.6 min, respectively.

For methylation analysis, RE was methylated successively according to the methods described by Ciucanu and Kerek (1984). The permethylated polysaccharides were hydrolyzed and converted to alditol acetates using the same procedures as described for the monosaccharide analysis. The methylated alditol acetates were analyzed and quantified using GLC. The GLC was performed using a Shimadzu GC-14A chromatograph equipped with a capillary column (DB-225, 0.25 mm × 25 m, J&W Scientific Co., Folson, CA). The oven temperature was held at 195°C. The peak areas were measured with a Shimadzu C-R6A Chromatopac. The methylated alditol acetate sample was also analyzed by GLC-MS using a Hewlett-Packard 5890 gas chromatograph equipped with the same capillary column and interfaced with a JEOL mass spectrometer (SX-102A) in the electron-impact mode. The identification of each of the methylated alditol acetates was determined by comparison of the mass spectra obtained with those of known standards reported by Jansson et al. (1976).

^{1}H- and ^{13}C-NMR spectra were recorded using Hitachi FX-90 or JEOL GX 200 and 400 instruments using penta-deuterio-acetone and sodium 3-trimethylsilylpropionate, respectively, as the internal standards for chemical shift assessment.

The smallest elicitor-active oligosaccharide, hepta-β-D-glucopyranoside, reported by Sharp et al. (1984), was chemically synthesized according to the procedure described by Fugedi et al. (1987). The structure of the synthesized glucopyranoside was confirmed by the complete agreement of its ^{1}H- and ^{13}C-NMR spectra with the reported spectra (Fugedi et al., 1987). Enzymatic hydrolysis of RE was performed by incubation of each RE fraction (approximately 1 mg of Glc equivalent) in 20 mM sodium acetate buffer (pH 5.5) for 8 h at 35°C with 100 μg of either soybean β-1,3-endoglucanase purified according the described procedure (Takeuchi et al., 1990a) or β-1,3-exoglucanase purified from cell walls of soybean cotyledons according to the procedure described by Cline and Albersheim (1981) in a total volume of 1 mL. Hydrolyzed RE fractions were rechromatographed on a Sephadex G-75 gel-filtration column as described above. As reported previously (Keen and Yoshikawa, 1983; Takeuchi et al., 1990a) the purified β-1,3-endo- and exo-glucanase gave a single major band on SDS-PAGE, and degraded β-1,3-linked glucans (laminarin, mycolaminaran, chrysolaminarin, and carboxymethyl-pachyman) but did not attack β-1,4-linked glucan (carboxymethylcellulose), β-1,6-linked glucan (pustulan), pectin, starch, or yeast mannan. When incubated with mycolaminaran, the endoglucanase gave predominantly tetra-, tri-, and disaccharides with no monosaccharide, and the exoglucanase gave only monomeric Glc as the degradation products.

Bioassay for Phytoalexin Elicitor Activity

Elicitor activity of RE fractions and other elicitors was assayed on wounded soybean cotyledons excised from 8- to 10-d-old seedlings, and the amounts of glyceollin accumulated were quantitated as described previously (Yoshikawa, 1978).

RESULTS

Incubation of cell walls isolated from *P. megasperma* f. sp. *glycinea* with the purified soybean β-1,3-endoglucanase resulted in the release of soluble carbohydrates highly active in inducing the phytoalexin glyceollin on soybean tissues (Yoshikawa et al., 1981; Keen and Yoshikawa, 1983; Keen et al., 1983). The REs were composed of carbohydrates with different molecular weights ranging from >50,000 to several hundred when fractionated on a Sephadex G-75 gel-filtration column and were separately pooled into the four fractions (RE-I, -II, -III, and -IV, with average molecular weights of 40,000, 15,000, 4,000, and <500, respectively) according to their molecular sizes (Fig. 1a). Phytoalexin elicitor activity of the RE fractions in comparison to the chemically synthesized hepta-β-glucopyranoside, the smallest elicitor-active oligosaccharide, previously reported by Sharp et al. (1984), was measured using the soybean cotyledon assay. The results showed that all of the RE fractions except RE-IV were highly elicitor active, being

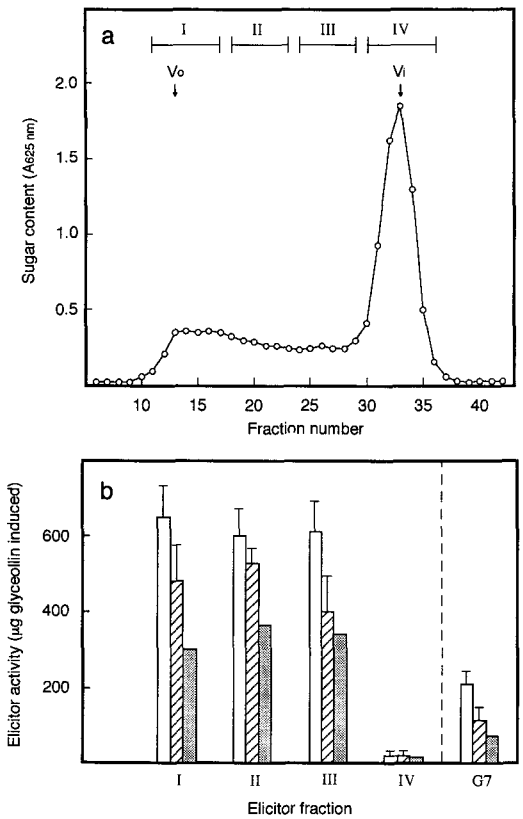

Figure 1. Sephadex G-75 gel filtration profile of carbohydrates released from mycelial walls of *P. megasperma* f. sp. *glycinea* by soybean β-1,3-endoglucanase (a) and phytoalexin-inducing activity of the different released-elicitor fractions (b) in comparison to chemically synthesized hepta-β-D-glucopyranoside (G7). Four elicitor fractions (RE-I through RE-IV) eluted from the gel-filtration column were separately pooled, and their glyceollin-inducing activity was assayed on soybean cotyledons (10 cotyledons per assay) at the concentrations of 1 (□), 0.1 (▨), and 0.01 (▩) μM (1 μM corresponds to 40, 15, 4, 0.5, and 1.25 μg/mL for RE-I, -II, -III, -IV, and G7, respectively, based on the estimated average molecular weights of each fraction). Means and SE values are indicated where three separate experiments were run, and other values are means of two separate experiments.

at least 100 times more active than the hepta-glucopyranoside based on molar concentrations, since the highest concentration (1 μM) of the glucopyranoside tested induced smaller amounts of glyceollin than did the lowest concentration (0.01 μM) of RE-I to -III (Fig. 1b). Although these comparisons of elicitor activity among different RE fractions and the glucopyranoside may give only rough estimates due to various factors, at least the possibility that a single, large RE molecule is processed into and generates many smaller elicitor-active molecules in plant tissues is unlikely, since ^{14}C-labeled RE-I (Yoshikawa and Sugimoto, 1993) incubated with wounded soybean cotyledons for prolonged periods (4–8 h) did not appear to be further degraded or processed into smaller fragments when the ^{14}C recovered from the soybean tissue was analyzed on Sephadex G-75 gel filtration chromatography (data not shown). RE-IV, which was found to be composed mainly of di- and trisaccharide when analyzed on a Bio-Gel P-10 gel-filtration column (data not shown), was completely devoid of elicitor activity. All of the RE fractions were composed almost entirely of Glc, with only traces of Man (Table I), although a low percentage of unidentified substances was also present, especially in the RE-III and -IV fractions.

The anomeric proton signal region of the ^1H-NMR spectrum of RE-I (Fig. 2a) established that all of the glucopyranosyl residues of RE-I were β-linked because there were no signals between 5.0 and 5.5 ppm, the α-anomeric region of the spectrum (Sharp et al., 1984). The rather broad proton signal with a chemical shift centered at 4.39 ppm was assigned to the anomeric protons of residues linked to *O*-6 of another residue, whereas the signal at 4.74 ppm was assigned to the anomeric protons of residues linked to *O*-3 of another residue. These assignments were made possible by examination of the ^1H-NMR spectrum of chemically synthesized hepta-β-D-glucopyranoside (Fig. 2b), in which the anomeric proton signals of glucosyl residues β-linked to *O*-6 and *O*-3 glucoside residues were 4.37 and 4.73 ppm, respectively (Sharp et al., 1984). These assignments from the ^1H-NMR spectra were confirmed by ^{13}C-NMR spectra (Fig. 3). The ^{13}C-NMR spectra of RE-I through RE-III, but not RE-IV, were essentially similar to that of hepta-β-D-glucopyranoside, showing signals of C1 anomeric carbons β-linked to C3 and C6 carbons with chemical shifts centered at 103.4, 85.1, and 69.4 ppm, respectively (Fugedi et al., 1987; Birberg et al., 1989). The assignment of the peak at 69.4 ppm to C6-linked anomeric carbons was also confirmed by the Inept spectrum of ^{13}C-NMR (Fig. 3c), in which the peak was reversed. In contrast to the spectra of RE-I through RE-III, the spectrum of RE-IV lacked the peak of C6-linked carbon (Fig. 3f) and was highly similar to that of the β-1,3-linked Glc dimer, laminaribiose (data not shown). These NMR data in conjunction with monosaccharide composition and molecular size evaluation data suggest that RE-I through RE-III, like hepta-β-D-glucopyranoside, are composed mainly of β-1,3- and β-1,6-linked glucans of different chain lengths and that RE-IV is composed mainly of β-1,3-linked Glc dimer and trimer.

Although it is unlikely that long β-1,3-linked glucan homo-oligomer chains exist in different RE structures, since RE is the product liberated by β-1,3-endoglucanase attack on fungal cell walls, this possibility was further examined by re-incubation of RE-I through RE-III with soybean β-1,3-endoglucanase for an extensive time period

Table I. *Monosaccharide composition of different RE fractions (RE-I through RE-IV) analyzed by GLC*

Sugar or Unidentified Substance	Retention Time on GLC	Composition of RE Fraction			
		I	II	III	IV
	min	%			
Glc	49.59	98.8	98.8	94.3	92.9
Man	39.75	0.9	0.5	3.3	1.2
Unidentified 1	9.87	0.3	0.5	0.6	1.4
Unidentified 2	10.52	0	0.2	0.3	0
Unidentified 3	28.95	0	0	1.5	4.5

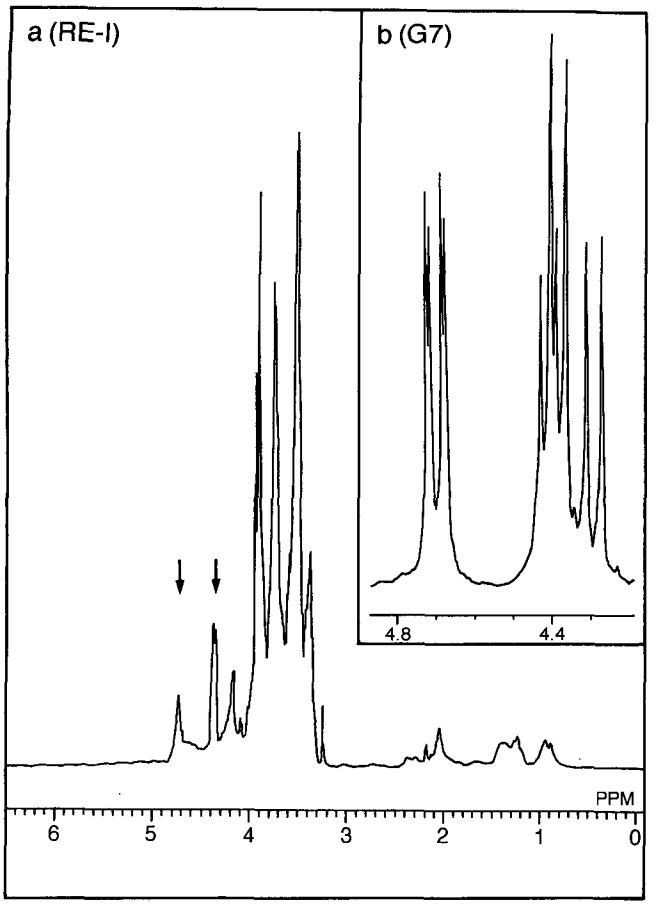

Figure 2. ^{1}H-NMR spectra of glucanase-released elicitor fraction I (RE-I, a) in comparison to hepta-β-D-glucopyranoside (G7, b) using 200- and 400-MHz NMR instruments, respectively. Anomeric proton signals at the chemical shifts of approximately 4.75 and 4.35 ppm, which were assigned to those of residues β-linked to *O*-3 and *O*-6, respectively, of another residue, are indicated by arrows.

(Fig. 4). There was indeed no sign of further degradation of RE-I through RE-III, since the re-incubation with the endoglucanase enzyme did not affect the gel-filtration patterns of the different RE fractions or their phytoalexin elicitor activity, indicating that long β-1,3-linked glucan chains do not exist in the RE structures. This in turn suggests that the main backbone chains of RE-I through RE-III could be β-1,6-linked glucans. This assignment was further supported by the ^{13}C-NMR spectrum of RE-I, in which the signal (chemical shift centered at 85.1 ppm) corresponding to C3, but not to C1 and C6, β-linked carbon disappeared after extensive β-1,3-exoglucanase treatment of RE-I described below (Fig. 3b).

In contrast to β-1,3-endoglucanase treatment, RE-I through RE-III were partially degraded by β-1,3-exoglucanase, resulting in the shift of gel-filtration patterns to slightly lower-molecular-weight fraction, liberating Glc monomer, as well as complete loss of elicitor activity (Fig. 4). These results suggest that one or two β-1,3-linked Glc moieties are attached to numerous Glc residues of the β-1,6-linked main chain as side branches, and that they are attacked by β-1,3-exoglucanase but not by β-1,3-endoglucanase. The results also indicate that the presence of the β-1,3-linked side branches is required for elicitor activity of RE-I through RE-III. In addition, incubation of the different RE fractions with commercial α-mannosidase, α-glucosidase, or β-glucuronidase affected neither the elution profiles nor their phytoalexin elicitor activity (data not shown).

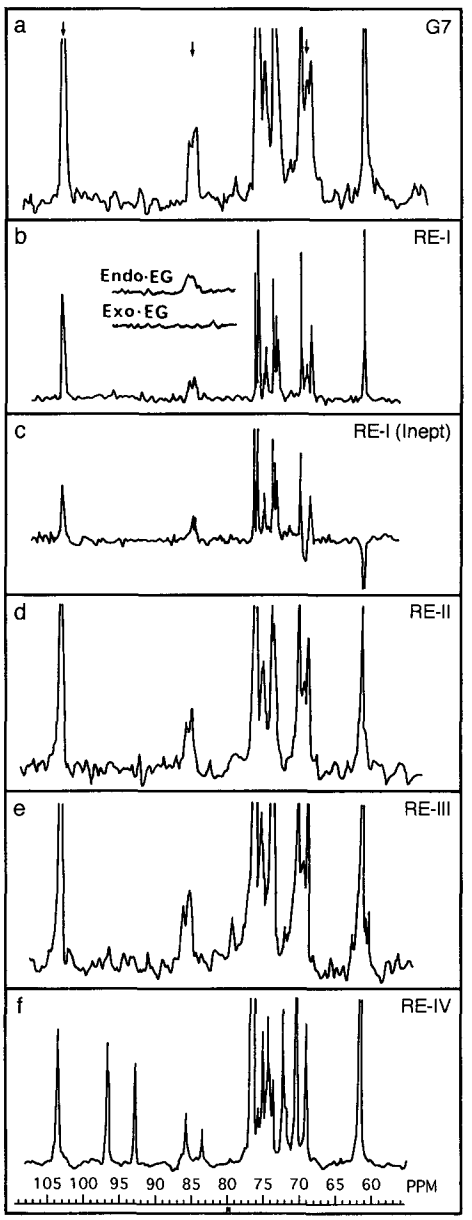

Figure 3. Ninety-megahertz ^{13}C-NMR spectra of different glucanase-released elicitor fractions (RE-I through RE-IV) in comparison to hepta-β-D-glucopyranoside (G7). Signals assigned to β-linked C1, C3, and C6 carbons are indicated by arrows in the top panel at chemical shifts of approximately 103.5, 85.5, and 69.5 ppm, respectively. In b, spectra are inserted that correspond to the area indicating the signal of β-linked C3 carbons after treatment of RE-I with β-1,3-endoglucanase (Endo·EG) and β-1,3-exoglucanase (Exo·EG) as described in Figure 4.

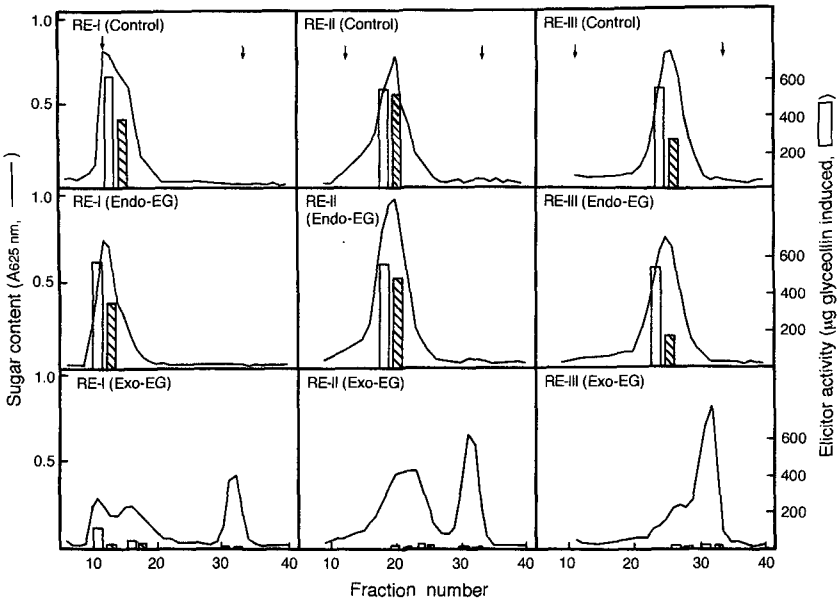

Figure 4. Effects of β-1,3-endoglucanase (Endo-EG) and β-1,3-exoglucanase (Exo-EG) on Sephadex G-75 gel-filtration profiles and phytoalexin elicitor activity of different glucanase-released elicitor fractions (RE-I through RE-IV). After incubation of the RE fractions with the respective enzymes, the reaction mixtures were heated to 100°C for 10 min to inactivate the enzymes. The reaction mixtures cleared by centrifugation were then applied to a Sephadex G-75 gel-filtration column. Phytoalexin elicitor activity of major eluted peaks was assayed on soybean cotyledons (10 cotyledons per assay) after diluting to 1 (□) and 0.1 (▨) μM based on the estimated average molecular weights of each fraction.

Methylation analysis of RE-I confirmed the presence of 1,6-linked glucans with frequent 1,3-linked side branches (Table II). Molar ratios of the branching glucosyl residues (1,3,6-linked) nearly coincided with those of the terminal glucosyl residues (1-linked). The number of 1,6-linked glucosyl residue with branches (1,3,6-linked) was about 3 times higher than that of glucosyl residues without branches (1,6-linked), indicating that approximately three out of four 1,6-linked glucosyl residues have 3-linked branches. Furthermore, the number of 1,3-linked glucosyl residues was about two-thirds that of the terminal glucosyl residues, which suggested that approximately two-thirds of 1,3-linked branches have two glucosyl moieties. The presence of longer (more than three glucosyl moieties) 1,3-linked branches is not likely, since they should be hydrolyzed by β-1,3-endoglucanase (Keen and Yoshikawa, 1983) and the results (Fig. 4) indicate that the endoglucanase does not further hydrolyze RE. These results, in combination with the results of NMR and β-1,3-endo- or -exoglucanase treatment, lead to a proposed average structural unit for elicitor-active RE-I through -III (Fig. 5), in which these REs are composed of β-1,6-linked backbone glucans of different chain lengths with highly frequent side branches of one or two β-1,3-linked Glc moieties.

DISCUSSION

Although different components from diverse groups of fungi have been reported to induce phytoalexin accumulation and/or hypersensitive reactions in plants, evaluations of whether such elicitors function in vivo during natural host-parasite interactions are limited (Yoshikawa, 1983). RE, the subject of this report, is an elicitor released by an enzyme contained in host tissues and, although RE may not completely represent a complex mixture of elicitors generated in planta, the results of the earlier works (Takeuchi et al., 1990a; Yoshikawa et al., 1990) nonetheless suggest the possibility that RE is generated in the in vivo soybean-*P. megasperma* interaction and may be responsible for the elicitation of phytoalexin production in the fungus-infected soybean tissues. Structural elucidation of such an elicitor, possibly involved in the in vivo interaction, thus appears to be of primary importance to understanding the initial

Table II. *Methylation analysis for glycosyl-linkage composition of RE-I by GLC-MS*

Position of *O*-Methyl Groups	Glucosyl Residue[a]	Relative Retention Time on GLC[b]	Molar Ratio[c]	Major Mass Fragments (*m/z*)
2,3,4,6	1-Linked (terminal)—A	1.00	1.0 (3)	43, 45, 71, 87, 101, 129, 145, 161, 205
2,4,6	1,3-Linked—B	1.54	0.64 (2)	43, 45, 87, 101, 117, 129, 161, 203, 233
2,3,4	1,6-Linked—C	1.80	0.35 (1)	43, 87, 101, 117, 161, 189, 233
2,4	1,3,6-Linked—D	3.03	1.1 (3)	43, 87, 117, 129, 159, 189, 233

[a] The letters A, B, C, and D designate the different glucosyl residues used in the illustration of Figure 5. [b] Relative retention time with respect to that of authentic 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [c] The numbers in parentheses are estimates of the number of each differently linked glucosyl residue present in an average structural unit of RE as illustrated in Figure 5.

events of the host-parasite interactions leading to the phytoalexin production and plant defense responses.

NMR and sugar constituent analysis of RE indicated that elicitor-active RE fractions with different molecular sizes (RE-I through RE-III) were almost entirely composed of glucans linked by β-1,3 and β-1,6 linkages. Since further extensive incubation of the RE fractions with β-1,3-endoglucanase did not degrade RE, the major backbone chain structure was proposed to be a β-1,6-linked glucan. Although this assignment was not tested by use of β-1,6-glucan-degrading enzymes due to its unavailability, the presence of β-1,6-linked backbone chain was further supported by the ^{13}C-NMR spectrum in which the signal indicating C3, but not C1 or C6, β-linked carbon disappeared after extensive digestion of RE-I by β-1,3-exoglucanase.

In contrast to β-1,3-endoglucanase, RE was partially degraded by β-1,3-exoglucanase and its elicitor activity was completely eliminated (Fig. 4). The results therefore suggest that branches with one or two β-1,3-linked Glc moieties, which cannot be further hydrolyzed by β-1,3-endoglucanase but can be hydrolyzed by β-1,3-exoglucanase, are attached to the backbone chain of the β-1,6-glucan. This presumed structure was also supported by methylation analysis (Table II), which indicated that RE is a 1,6-linked glucan with frequent 1,3-linked side branches as proposed in Figure 5. The presence of frequent β-1,3-linked side branches was also supported by the fact that β-1,3-exoglucanase treatment of RE fractions liberated high amounts of Glc monomer that were almost equivalent to undegraded, β-1,6-linked glucans (Fig. 4). In addition, the similarity among different-sized fractions of RE (RE-I through RE-III) in NMR spectra and responses to β-1,3-endo- and exoglucanase suggests that RE-I through RE-III have basically common structures as described above, but the lengths of the β-1,6-linked backbone chains are different.

The structural evaluation of RE also made possible a deduction as to how RE was originally present in fungal cell walls and how it was released by host β-1,3-endoglucanase (Fig. 6). β-1,6-Linked glucans of various chain lengths with frequent β-1,3-linked glucan side chains are presumed to be present as constituents in the fungal cell wall. Upon contact of fungal cells with host tissues during infection processes, the β-1,3-linked glucan side chains are presumed to be attacked by β-1,3-endoglucanase constitutively present in soybean tissues, resulting in the release of elicitor-active β-1,6-linked glucans of various chain lengths with frequent side branches composed of one or two β-1,3-linked Glc moieties (RE-I through RE-III). The attack of β-1,3-side chains by the host endoglucanase also results in the release of elicitor-inactive β-1,3-linked Glc dimer and trimer (RE-IV), which are the major degradation products of β-1,3-linked glucans generated by treatment of the walls with the soybean glucanase (Keen and Yoshikawa, 1983). These considerations well explain the release of different elicitor-active RE fractions (RE-I through RE-III) with different molecular sizes and of relatively large amounts of elicitor-inactive RE-IV from fungal cell walls following the attack by β-1,3-endoglucanase as shown in Figure 1a. The deduced structures of cell-wall-bound elicitors are also consistent with the previously evaluated structural features of fungal cell-wall glucans (Ayers et al., 1976), the hepta-β-D-glucopyranoside derived from fungal cell walls (Sharp et al., 1984) or its related oligo-β-glucosides (Cheong et al., 1991), and elicitor-active glucans released from germinating zoospores of the fungus (Waldmuller et al., 1992). In these cases the major cell-wall glucans are also thought to be β-1,3- and β-1,6-linked; however, except for the hepta-glucopyranoside, the detailed structures of the wall glucans have not been assigned in the previous studies. Although the present study deduced the average structural units of various RE fractions, the exact primary structure of a single RE molecule could not be elucidated, due to the heterogeneity in molecular sizes among the various RE fractions, which are also of relatively large molecular size.

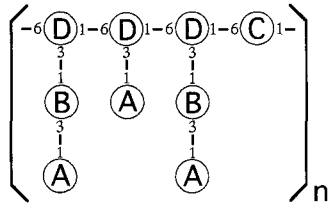

Figure 5. An average structural unit of RE deduced from NMR, treatment with specific β-1,3-endo- or β-1,3-exoglucanases, and methylation analysis. Each circled letter represents the differently linked glucosyl residue shown in Table II, and the small numbers at the side of the circles indicate the positions of the linkages. The illustrated basic structure is not meant to signify a repeating unit, and the positions of β-1,3-linked side branches may be at random in the RE structures, but the composition ratios of each differently linked glucosyl residue (A:B:C:D = 3:2:1:3) are those obtained from methylation analysis as shown in Table II. The length of β-1,3-linked side branches should be no more than two glucosyl residues, since RE was not further degraded by β-1,3-endoglucanase, which cannot hydrolyze either a terminal or branching β-1,3-linked glucosyl residue (Keen and Yoshikawa, 1983).

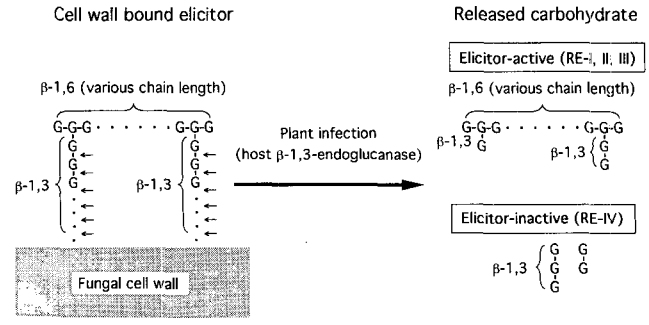

Figure 6. Tentatively proposed structures of elicitors in cell walls of *P. megasperma* f. sp. *glycinea* and their released forms due to attack by soybean β-1,3-endoglucanase. β-1,6-Glucans of various chain lengths with frequent β-1,3 side chains could originally exist in fungal cell walls. Upon infection, β-1,3 side chains are attacked by the endo-type soybean β-1,3-glucanase, resulting in the release of elicitor-active β-1,6-glucans of various chain lengths with frequent side branches of one or two β-1,3-linked Glc moieties (corresponding to RE-I, -II, and -III) and elicitor-inactive β-1,3-linked Glc dimer or trimer (corresponding to RE-IV) derived from the endoglucanase attack of β-1,3 side chains of the glucans. Small arrows indicate the possible sites for glucanase attack. G indicates a Glc moiety.

We are now attempting to isolate several elicitor-active and relatively small RE molecules in order to determine their exact structures.

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