Elicitation of Diterpene Biosynthesis in Rice (*Oryza sativa* L.) by Chitin[1]

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ABSTRACT

Cell-free extracts of UV-irradiated rice (*Oryza sativa* L.) leaves have a much greater capacity for the synthesis from geranylgeranyl pyrophosphate of diterpene hydrocarbons, including the putative precursors of rice phytoalexins, than extracts of unstressed leaves (KA Wickham, CA West [1992] Arch Biochem Biophys 293: 320–332). An elicitor bioassay was developed on the basis of these observations in which 6-day-old rice cell suspension cultures were incubated for 40 hours with the substance to be tested, and an enzyme extract of the treated cells was assayed for its diterpene hydrocarbon synthesis activity as a measure of the response to elicitor. Four types of cell wall polysaccharides and oligosaccharide fragments that have elicitor activity for other plants were tested. Of these, polymeric chitin was the most active; a suspension concentration of approximately 7 micrograms per milliliter gave 50% of the maximum response in the bioassay. Chitosan and a branched β-1,3-glucan fraction from *Phytophthora megasperma* f. sp. *glycinea* cell walls were only weakly active, and a mixture of oligogalacturonides was only slightly active. A crude mycelial cell wall preparation from the rice pathogen, *Fusarium moniliforme*, gave a response comparable to that of chitin, and this activity was sensitive to predigestion of the cell wall material with chitinase before the elicitor assay. *N*-Acetylglucosamine, chitobiose, chitotriose, and chitotetrose were inactive as elicitors, whereas a mixture of chitin fragments solubilized from insoluble chitin by partial acid hydrolysis was highly active. Constitutive chitinase activity was detected in the culture filtrate and enzyme extract of cells from a 6-day-old rice cell culture; the amount of chitinase activity increased markedly in both the culture filtrate and cell extracts after treatment of the culture with chitin. We propose on the basis of these results that soluble chitin fragments released from fungal cell walls through the action of constitutive rice chitinases serve as biotic elicitors of defense-related responses in rice.

Two groups of diterpenoid metabolites that are proposed to serve as phytoalexins have been identified in rice (*Oryza sativa* L.) plants infected with *Magnaporthe grisea* or irradiated with UV light. Momilactones A and B, which were initially isolated as dormancy factors from rice husks by Kato *et al.* (13), were subsequently found by Cartwright *et al.* (7) to be produced in infected or UV-irradiated rice leaves. Independently, a group of Japanese investigators (1, 15, 26) isolated and characterized oryzalexins A–D from rice plants infected

with the rice leaf blast fungus. The momilactones are oxygenated derivatives of $9\beta H$-pimara-7,15-diene-19,6-β-olide (31), whereas the oryzalexins are oxygenated derivatives of a sterically different pimaradiene, *ent*-sandaracopimara-8(14),15-diene (15). The structures of momilactone A and oryzalexin A are indicated in Figure 1. Both groups of substances are effective antifungal agents. In addition, other uncharacterized antifungal agents have been reported to occur in infected rice leaves (14, 17, 18). The precise role of these phytoalexins in the resistance of rice plants to infection by microbial pathogens is not well understood at present.

A proposed pathway for the biosynthesis of the momilactones and oryzalexins is outlined in Figure 1. In this scheme, the momilactones are formed by oxygenation of $9\beta H$-pimara-7,15-diene, which is generated by a two-step cyclization of geranylgeranyl pyrophosphate[2] via the bicyclic intermediate, 9,10-*syn*-copalyl pyrophosphate. The oryzalexins are formed by oxygenation of *ent*-sandaracopimaradiene, which originates in a two-step cyclization with *ent*-copalyl pyrophosphate as the bicyclic intermediate. Wickham and West (33) have provided support for this proposed scheme. $9\beta H$-Pimara-7,15-diene and *ent*-sandaracopimara-8(14),15-diene are synthesized in cell-free enzyme preparations from UV-elicited rice leaves as predicted, along with *ent*-kaurene, a gibberellin precursor, and two other unidentified pimaradiene-like diterpene hydrocarbons. *ent*-Sandaracopimara-8(14),15-diene and *ent*-kaurene, but not $9\beta H$-pimara-7,15-diene, are also synthesized from *ent*-copalyl pyrophosphate as predicted. Only *ent*-kaurene is synthesized in non-elicited leaf extracts. Recently, a synthetic sample of 9,10-*syn*-copalyl pyrophosphate has been shown to be convertible to $9\beta H$-pimaradiene in UV-elicited leaf tissue in agreement with the scheme (our unpublished data). Thus, the diterpene hydrocarbons synthesized in UV-elicited tissues include those that are putative precursors of the momilactone and oryzalexin families of rice phytoalexins.

The work reported in this paper was undertaken to provide information about the biotic elicitors responsible for stimulating the synthesis of the diterpene hydrocarbons that serve

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[2] Abbreviations: geranylgeranyl pyrophosphate, pyrophosphate ester of *trans,trans*-3,7,11,15-tetramethylhexadec-2,6,10,14-tetraen-1-ol; *ent*-copalyl pyrophosphate, pyrophosphate ester of *trans-enan-tio*-lambda-8(17),13-dien-15-ol; 9,10-*syn*-copalyl pyrophosphate, pyrophosphate ester of *trans*-9,10-*syn*-lambda-8(17),13-dien-15-ol; cyclase activity, collective diterpene hydrocarbon synthase activities with geranylgeranyl pyrophosphate as the substrate.

MVA

geranylgeranyl-PP

9βH-labdadienyl-PP   copalyl-PP

9βH-pimara-7,15-diene   sandaracopimaradiene

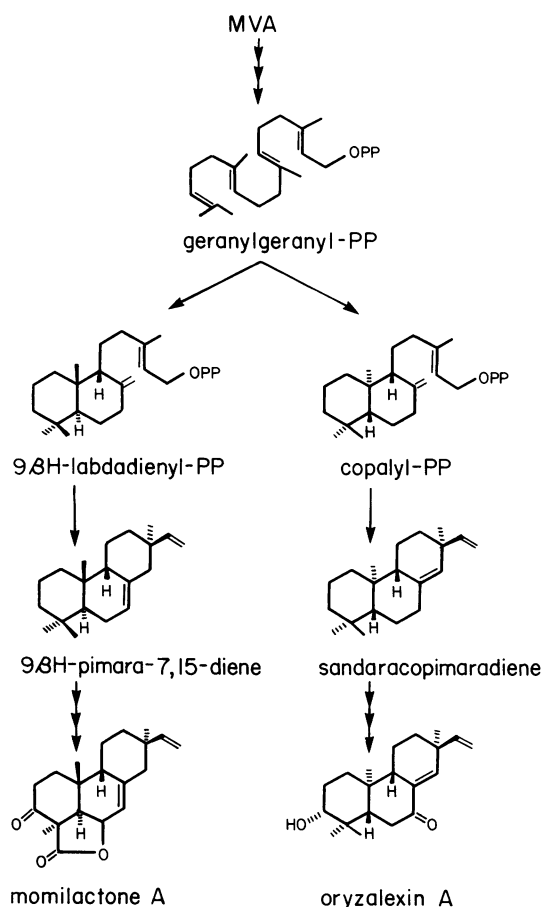momilactone A   oryzalexin A

Figure 1. Proposed pathways of diterpene phytoalexin biosynthesis in rice.

as the putative precursors of the rice phytoalexins. An elicitor bioassay was developed involving exposure of rice cells in liquid suspension to substances to be tested for elicitor activity, and subsequent examination of enzyme extracts from treated cells for their diterpene hydrocarbon synthase (cyclase) activity as a measure of elicitor activity. Among the substances examined with this assay were several cell wall oligosaccharide components that have been reasonably well characterized as elicitors of defense responses in other plants. These included branched β-1,3-glucans, chitosan and chitin (fungal cell wall components), and oligogalacturonides (a plant cell wall fragment). The branched β-1,3-glucan fragments from *Phytophthora megasperma* f. sp. *glycinea* cell walls have been well characterized as elicitors of glyceollins in soybean (27, 28). Chitosan, a β-1,4-linked D-glucosaminoglycan, is an active elicitor of the phytoalexin, pisatin, in pea (11), whereas chitin, a β-1,4-linked N-acetylglucosaminoglycan, has been reported to elicit lignin production in wheat (2, 22), phenolic acids in carrot cells (16), and chitinase activity in melon (*Cucumis melo* L.) seedlings (24). Oligo-α-1,4-D-galacturonides have been investigated as elicitors of phytoalexins in castor beans (5) and soybean (21), of lignin in castor bean cells (6) and cucumber hypocotyls (23), and of proteinase inhibitors in tomato (4). The studies reported here identify chitin as the most active elicitor for rice cells.

MATERIALS AND METHODS

Biological Materials

The rice (*Oryza sativa* L. cv Shesheniski) callus culture employed in this research was kindly made available by J. Mundy, H. Trinh, and N.H. Chua, Laboratory of Plant Molecular Biology, Rockefeller University. Cell suspension cultures were grown in 250-mL flasks in 50 mL of B-5 medium (9) supplemented with 0.45 μM 2,4-D, pH 5.6, at 25°C in the dark with shaking at 90 to 100 rpm. Cells were passaged every 8 d into fresh, sterilized B-5 medium supplemented as indicated above. Callus stock cultures were maintained on the same medium in 1.0% purified agar.

Materials Tested for Elicitor Activity

Chitin (poly-β-1,4-N-acetyl-D-glucosaminoglycan, purified from crab shells), N-acetylglucosamine monomer, chitobiose, chitotriose, and chitotetrose were obtained from Sigma. Chitin fragments were prepared by the acid hydrolysis method described by Barber *et al.* (2). Pectic fragment elicitor (a mixture of α-1,4-D-galacturonide oligomers with an average degree of polymerization of 15.5) was prepared by R. Bruce by the partial digestion of polygalacturonic acid (Sigma, grade II) with pure *Rhizopus stolonifer* endopolygalacturonase as described elsewhere (6). The sample of nitrous acid-treated chitosan elicitor was supplied by L. A. Hadwiger, Washington State University, Pullman. The sample of branched β-1-3-glucan elicitor was supplied by M. Hahn of the Complex Carbohydrate Research Center, University of Georgia, Athens. This sample, which was purified from a partial acid hydrolysate of *Phytophthora megasperma* f. sp. *glycinea* (hereafter referred to as *P. megasperma*) cell walls, corresponded to the oligoglucosides recovered from the low resolution P-2 column in the procedure of Sharp *et al.* (28).

Radioactive Substrates

[1-³H]Geranylgeranyl pyrophosphate (20 μCi/μmol) was synthesized by I. Mendez in our laboratory. Unlabeled geranylgeraniol was prepared from all-*trans*-farnesylacetone (K & K Laboratories) as previously described (32). The all-*trans* isomer of geranylgeraniol was purified by silicic acid chromatography and oxidized to geranylgeraniol with MnO₂. The aldehyde was then reduced with tritium-labeled NaBH₄ to [1-³H]geranylgeraniol. The product was diluted to the desired specific radioactivity by the addition of unlabeled geranylgeraniol. Pyrophosphorylation of the [1-³H]geranylgeraniol was performed by the procedure developed by Poulter and his associates (8). ³H-Chitin was prepared from ³H-acetic anhydride (500 mCi/mmol; Amersham) and chitosan as described by Molano *et al.* (20).

Other Materials

Chitinase (from *Streptomyces griseus*) and laminarinase (from a *Penicillium* species) were obtained from Sigma. One unit of chitinase activity is defined as that amount capable of solubilizing 1.0 mg of chitin in 48 h at pH 6.0 and 25°C. One

unit of laminarinase is defined as that amount capable of solubilizing 1.0 mg of reducing sugar (measured as glucose) from laminarin per min at pH 5.0 and 37°C.

Fusarium moniliforme Mycelial Cell Wall Preparation

F. moniliforme (*Gibberella fujikuroi* in the perfect stage), strain 1-a, cultures were grown for 5 d in a 2-L Fernbach flask with 1 L of 10% ICI-glucose medium on a shaker as described by Geissman *et al.* (10). The mycelia (about 17 g wet weight) were harvested by suction filtration on a coarse sintered-glass funnel and washed on the funnel with 1 L of distilled water. The mycelia suspended in 250 mL of distilled water were homogenized in a Waring Blendor, and the homogenate was suction filtered through Whatman No. 4 filter paper. The residual material on the filter was washed with 500 mL of chloroform:methanol (1:1; v/v), followed by 500 mL of acetone, and was air-dried to yield 1.5 g of crude mycelial cell wall preparation.

Elicitor Bioassay

Stock solutions or suspensions of materials to be tested for elicitor activity were prepared by transferring a weighed amount of the substance to a sterile container and dissolving or suspending the material in an appropriate amount of deionized water. The resulting solutions or suspensions were sterilized either by autoclaving for 5 min or by passage through a 0.20 μm filter. Aseptic transfer of different volumes of stock solutions or suspensions of the elicitor were made to a series of flasks of rice suspension cells in their 6th day of growth after transfer. The final concentration of elicitor in each flask was estimated on the assumption that the final volume after the addition was 50 mL. Control flasks received a volume of sterile water equivalent to that of the elicitor solution. Each concentration of the elicitor was tested in duplicate flasks. The cell cultures were incubated at 25°C in the dark with shaking for an additional 40 h, or a time indicated in individual experiments, after addition of the test solution.

The preparation of cell-free enzyme extracts from the cells in each flask and measurement of the diterpene hydrocarbon synthase activity of that extract were carried out according to the general procedure of Wickham and West (33) as follows. The cells from each flask were harvested by filtration through Whatman No. 3 filter paper on a Büchner funnel. Packed cells (3 g) were mixed with 1 g of Polyclar AT and ground to a powder in a mortar with pestle in the presence of liquid N₂. The liquid N₂ was allowed to boil off and 6 mL of cold Hepes buffer (50 mM Hepes, 150 mM sucrose, 10 mM Na₂S₂O₅, 10 mM ascorbate, 10 mM DTT, 10 μM leupeptin, 10% glycerol [v/v], 1 mM EDTA, and 1 mM PMSF) was added. The resulting suspension was squeezed through four layers of cheesecloth and then centrifuged at 39,000g for 20 min at 4°C. The supernatant solution was used immediately for enzyme assay. The cyclization reaction was initiated by adding 100 μL [³H]geranylgeranyl pyrophosphate (1 × 10⁵ dpm; 20 mCi/mmol) to a mixture of 100 μL water, 150 μL of enzyme extract, and 150 μL of incubation buffer containing 5 mM MgCl₂ and 10 mM KH₂PO₄, pH 7.0 (final incubation volume

= 0.50 mL). The mixture was incubated in a 30°C water bath for 40 min and the reaction was stopped by the addition of 1 mL of ethanol:petroleum ether (1:3; v/v). Three successive 1.5 mL extractions with petroleum ether were employed to transfer diterpene hydrocarbons and alcohols from the aqueous reaction mixture into an organic phase. After the volume was concentrated to 0.5 mL in a stream of N₂, the organic phase was transferred to a 1 × 10 cm Econo-Column (Bio-Rad) slurry-packed to a depth of 3 cm with silicic acid suspended in hexane. Hexane was employed as the eluting solvent; the first 7 mL of hexane eluant, which contained only the diterpene hydrocarbons, was collected. (The diterpene alcohols, *e.g.* geranylgeraniol, are retained by the column under these conditions.) The radioactivity associated with the hydrocarbon fraction was assayed by adding the hexane eluant to Scintiverse LC liquid scintillation cocktail and measuring the radioactivity in a Beckman model LS-8100 Spectrometer. The cpm associated with the diterpene hydrocarbon fraction from a test sample were converted to pmol of diterpene hydrocarbons formed by dividing them by 22.2 cpm pmol⁻¹, which takes into account the specific radioactivity of the substrate and the observed counting efficiency for ³H under the conditions of measurement. Elicitor activity is expressed in terms of units of cyclase activity where 1 unit is defined as that amount of enzyme that catalyzes the formation of 1 pmol of diterpene hydrocarbon under the standard assay conditions.

A comparison of elicitor activities of test samples within an experiment should be valid because a standard set of conditions was employed, including the inocula for the flasks, the growth conditions, the cell mass used for preparing the enzyme extract, the extraction conditions, and the amount of extract employed in the cyclase assay. The variability in elicitor activities for duplicate tests receiving the same treatment is indicated by plotting the results from both in the figures. Some variation in the sensitivity to elicitor was experienced with cells grown at different times for use in bioassays. Therefore, the results reported in a given table or figure were from bioassays performed at the same time with cells grown from the same inoculum.

Assays for Rice Cell Suspension Chitinase Activities

Cell suspensions were separated into a cell fraction and a culture filtrate fraction by filtration on a Büchner funnel with Whatman No. 3 filter paper. To prepare an enzyme extract for assay, cells (4 g fresh weight) frozen in liquid N₂ were ground into a powder in a mortar with pestle in the presence of 2 g of Polyclar AT. After the liquid N₂ was allowed to boil off, 8 mL of sodium acetate buffer (0.2 M sodium acetate and 0.1% 2-mercaptoethanol, pH 5.2) were mixed with the powder. The homogenate suspension was squeezed through four layers of cheesecloth, and the expressed suspension was centrifuged at 20,000g for 20 min at 4°C. The resulting supernatant solution was dialyzed against 1 L of the sodium acetate buffer overnight to yield the extract for assay.

The culture filtrate was prepared for the chitinase assay as follows. The filtrate was centrifuged at 20,000g for 10 min at 4°C. Ammonium sulfate was added to the supernatant solution to 95% of saturation at 4°C. The resulting precipitate was collected by centrifugation, and the pellet was redis-

solved in the sodium acetate buffer. The resulting solution was dialyzed against 1 L of the same buffer overnight to yield the enzyme fraction for assay. The volumes of the enzyme extracts from both the cells and culture medium were adjusted so that a given volume of each represented the same amount of original cell suspension culture.

Chitinase activity was assayed with ^{3}H-chitin as the substrate as previously described (20). Enzyme extract from either the cells or the culture filtrate (150 μL) was mixed with 50 μL of 100 μM Na$_2$HPO$_4$, pH 6.5, and 50 μL of a 1-mg suspension of ^{3}H-chitin in water. After 2 h of incubation at 37°C, the reaction was stopped by the addition of 250 μL of 1 M TCA. The incubation mixture was subjected to centrifugation at 2,000g for 5 min, and a 200-μL aliquot of the supernatant solution was transferred to Scintiverse BD liquid cocktail for determination of the associated radioactivity by liquid scintillation spectrometry. Control incubations in which 150 μL of water replaced the enzyme extract were also run. Chitinase activity is expressed as the cpm of radioactivity solubilized from the substrate in the 200-μL aliquot of the incubation mixture minus the cpm of radioactivity detected in the 200-μL aliquot of the control incubation.

RESULTS

Characteristics of the Elicitor Bioassay

The elicitor bioassay described in "Materials and Methods" is based on the measurement of diterpene hydrocarbon synthase (cyclase) activities in extracts of rice that have been exposed to a substance to be tested for elicitor activity. Suspension cell cultures were employed as the test material after initial efforts to develop an assay with wounded rice leaves were abandoned because of difficulties in getting proper exposure of the leaf cells to the potential elicitor.

Under the conditions employed in the elicitor bioassay, the cell density in the cultures began to increase in the fourth day after transfer, and the growth rate reached its maximum on day 6. After that the growth rate declined, and by d 10 the cells began to discolor. An experiment was performed in which cells at different stages of growth were tested in the elicitor bioassay as described in "Materials and Methods" with a suspension of chitin as the elicitor (data not shown). The response of the cells to elicitor increased from the second day to a maximum at the sixth day, after which it decreased. Thus, the cell's responsiveness was positively correlated with the observed growth rate. In all the experiments reported here, elicitor was added to cell suspension cultures during the 6th day after passage of the cells.

The time course for the appearance of cyclase activity after treatment of 6-d-old suspension cell cultures with elicitor was also determined (Fig. 2). The experimental conditions were the same as those described for the elicitor bioassay in "Materials and Methods" except that various times of exposure after elicitor addition were employed before the enzyme extract was prepared. Chitin (25 μg mL^{-1}) served as the elicitor. The cyclase activity was first detected at significantly elevated levels 28 h after treatment with chitin and reached a maximum at about 40 h. At times longer than 40 h, the activity was progressively more reduced. This experiment
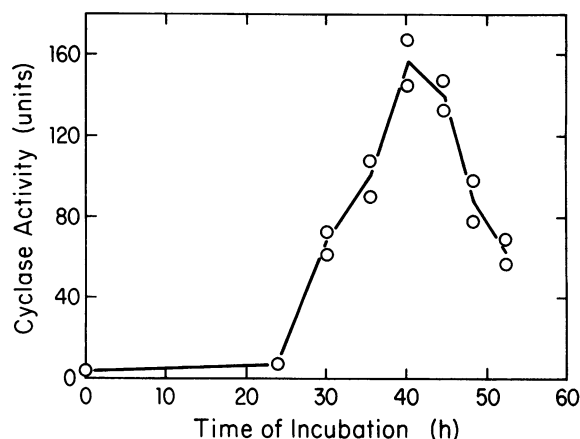

Figure 2. Cyclase activity of rice cells as a function of the time of incubation after treatment with chitin. A suspension of 1.25 mg of chitin was added to each of 16 6-d-old, 50-mL rice cell suspension cultures at zero time. The cells were incubated at 25°C with shaking in the dark for the times indicated. At each time point, two flasks were removed, the cells were harvested, and an enzyme extract was prepared from each as described in "Materials and Methods." A 150-μL aliquot of each enzyme extract was assayed for cyclase activity under the conditions described in "Materials and Methods." The units of cyclase activity produced in each assay is plotted as a function of the time of incubation in hours after the addition of chitin. One unit of cyclase activity is defined as that amount of enzyme required to produce 1 pmol of diterpene hydrocarbon under the standard assay conditions.

was performed three times with similar results each time. Exposures to elicitor of 40 h were routinely used in the elicitor bioassay to achieve the maximum response.

Tests of Oligosaccharide Components of Cell Walls for Elicitor Activity

Tests for elicitor activity were carried out in rice cells for four fungal and plant cell components that have been reported to have elicitor activity in other assay plants. A dose-response curve for suspensions of chitin of up to 200 μg mL^{-1} is illustrated in Figure 3. The nonlinear response curve to increasing doses of chitin is characteristic for this substance. A steep increase in the response is seen at low doses of chitin, with a progressively lower increase at higher doses. It is difficult to discern the nature of the response curve at doses below 10 μg mL^{-1} from the plot in Figure 3A. The nature of the response to low levels of chitin is more evident from the plot in Figure 3B (inset). It is clear from these results that chitin is an effective elicitor of cyclase activity. We have estimated that approximately 7 μg of chitin in suspension mL^{-1} give 50% of the maximum response in this assay.

Chitosan is structurally equivalent to chitin except for the absence of an N-acetyl group at the 2-amino position of each glucosaminyl moiety. A chitosan sample that had been prepared by nitrous acid treatment of chitosan was tested in the elicitor bioassay. As can be seen from the dose-response curve for chitosan in Figure 3A, it is much less active than chitin, especially at low concentrations. The chitosan preparation, unlike the chitin utilized, is water-soluble. This may
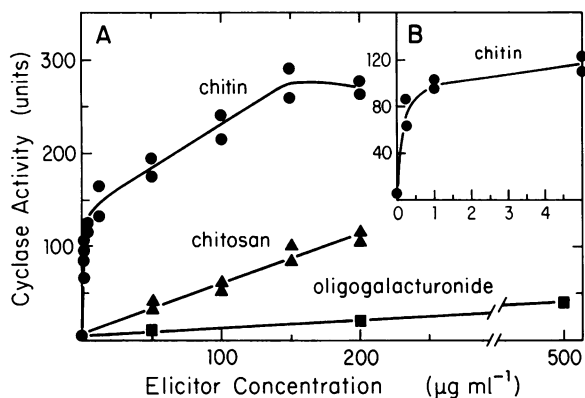
Figure 3. Elicitor activities of chitin, chitosan, and oligo-α-1,4-D-galacturonides for rice suspension cells. Differing amounts of sterile elicitor suspension (chitin) or solution (chitosan and oligogalacturonides) were added to a series of 6-d-old 50-mL rice cell suspension cultures to the final concentrations indicated. Duplicate tests were run at each elicitor concentration. The cells were incubated at 25°C with shaking in the dark. After 40 h, the cells from each flask were harvested, and an enzyme extract was prepared as described in "Materials and Methods." A 150-μL aliquot of each enzyme extract was assayed for cyclase activity under the conditions described in "Materials and Methods." A, The units of cyclase activity produced in each assay are plotted as a function of elicitor concentration. One unit of cyclase activity is defined as that amount of enzyme required to produce 1 pmol of diterpene hydrocarbon under the standard assay conditions. Elicitors: chitin (●); chitosan (▲); oligogalacturonides (■). B (inset), The cyclase activities elicited by 0, 0.2, 1.0, and 5.0 μg mL⁻¹ of chitin are replotted on a different scale to give a clearer indication of the relationship of the elicitor response to chitin concentration in this range.

account for the linear dose-response curve seen with the chitosan sample. It appears that a concentration of chitosan greater than 200 μg mL⁻¹ would be required to give 50% of the maximum response in this assay.

A mixture of α-1,4-D-galacturonide oligomers with an average degree of polymerization of 15.5 was tested (Fig. 3A). Very little elicitor activity was detected, even at the relatively high concentration of 1,000 μg mL⁻¹. Even though higher concentrations are typically required for pectic fragment elicitors than for fungal cell wall elicitors in other assay systems, we conclude that the oligogalacturonides are weak elicitors at best in the rice cell assay.

A preparation of branched β-1,3-glucan fragments purified from a partial acid hydrolysate of *P. megasperma* cell walls was tested. A dose-response curve for the branched β-glucan sample is shown in Figure 4, along with a reference sample of chitin for comparison. It can be seen that the glucan is a relatively weak elicitor in comparison with chitin and is somewhat comparable to chitosan in its activity.

Test of a Crude Fungal Cell Wall Preparation for Elicitor Activity

Chitin is a major component of many fungal cell walls, including those of fungal pathogens (3). Therefore, if chitin is an elicitor of defense responses in rice cells, one might also expect a cell wall preparation from a fungal pathogen to be an active elicitor of diterpene cyclase activity in rice cells. To

test this, a crude mycelial cell wall preparation was obtained from *F. moniliforme* (*G. fujikuroi*), a fungal pathogen of rice seedlings. A suspension of this cell wall material was tested for its activity in the rice cell elicitor bioassay. Figure 5 illustrates the response of rice cells to various amounts of cell wall preparation. It was active as an elicitor, and the general shape of the response curve was very similar to that obtained with chitin. It can be estimated from the results in Figure 5 that the amount of crude cell wall material required to give 50% of the maximum response was about 15 μg mL⁻¹. This compares with an estimated value of 7 μg mL⁻¹ for chitin alone.

To learn whether the elicitor activity of the cell wall preparation was due to its chitin content, a series of predigestions of suspensions of cell wall material were performed with chitinase or laminarinase, or both together, to determine what effect they had on elicitor activity of the digested mixture. Chitinase and laminarinase act as endohydrolases for chitin and β-1,3-glucans, respectively; these two glycans are the major polysaccharide components of most fungal cell walls (3). In the experiments whose results are summarized in Table I, 1 mg of *F. moniliforme* mycelial wall preparation was incubated with chitinase (2 units) and/or laminarinase (2 units), as indicated, at pH 5.2 and 40°C for 30 h with stirring. At the end of the incubation period, each mixture was heated for 10 min in a boiling bath, cooled, and the entire incubation mixture was tested as an elicitor. Preliminary experiments not presented here showed that very little elicitor activity is released into a solubilized form by these enzymes under
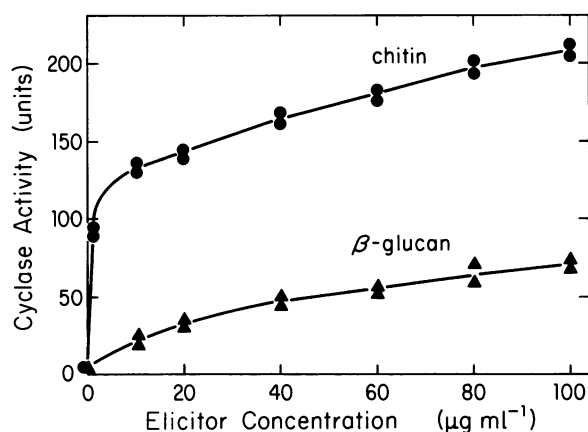

Figure 4. Elicitor activities of chitin and a *P. megasperma* β-glucan preparation for rice suspension cells. Differing amounts of a sterile suspension (chitin) or solution (β-glucan) were added to a series of 6-d-old, 50-mL rice cell suspension cultures to the final concentrations indicated. Duplicate tests were run at each elicitor concentration. The cells were incubated at 25°C with shaking in the dark. After 40 h, the cells from each flask were harvested, and an enzyme extract was prepared as described in "Materials and Methods." A 150-μL aliquot of each enzyme extract was assayed for cyclase activity under the conditions described in "Materials and Methods." The units of cyclase activity produced in each assay are plotted as a function of elicitor concentration. One unit of cyclase activity is defined as that amount of enzyme required to produce 1 pmol of diterpene hydrocarbon under the standard assay conditions. Elicitors: chitin (●); *P. megasperma* β-glucan preparation (▲).
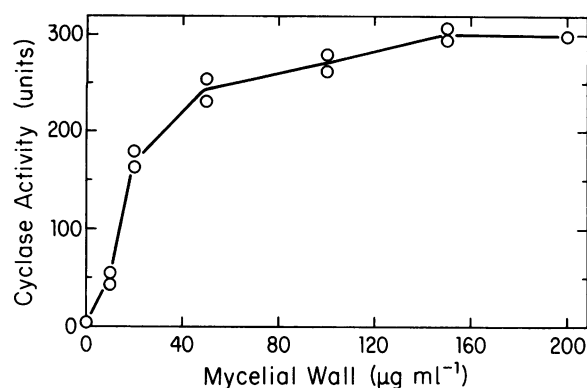
Figure 5. Elicitor activity of F. moniliforme cell wall preparation for rice suspension cells. The crude F. moniliforme cell wall material was prepared as described in "Materials and Methods." Differing amounts of this wall material were added to a series of 6-d-old, 50-mL rice cell suspensions to the final suspension concentration indicated. Duplicate tests were run at each elicitor concentration. The cells were incubated at 25°C with shaking in the dark. After 40 h, the cells from each flask were harvested, and an enzyme extract was prepared as described in "Materials and Methods." A 150-μL aliquot of each enzyme extract was assayed for cyclase activity under the conditions described in "Materials and Methods." The units of cyclase activity produced in each assay are plotted as a function of elicitor concentration. One unit of cyclase activity is defined as that amount of enzyme required to produce 1 pmol of diterpene hydrocarbon under the standard assay conditions.

these incubation conditions. Therefore, any activity observed in the elicitor bioassay is presumably due to undigested cell wall material remaining at the end of the digestion. Test 1 served as a positive control in which the mycelial wall was not pretreated with wall-digesting enzymes. Pretreatment of mycelial wall preparation with chitinase alone (test 2) caused a significant reduction of elicitor activity, although considerable insoluble wall material and residual elicitor activity still remained. Pretreatment with laminarinase alone (test 3) caused a visible reduction in insoluble wall material but no change in elicitor activity. Finally, pretreatment with a mixture of chitinase and laminarinase (test 4) led to an almost complete absence of insoluble wall material and residual elicitor activity. This overall experiment was repeated two more times with similar results. These results taken together indicate that chitin is the major elicitor of the F. moniliforme wall preparation. The simultaneous presence of laminarinase with added chitinase facilitated the digestion of the wall material, presumably by exposing more of each type of substrate to its respective enzyme. There is no indication from these results that the β-1,3-glucan component of the mycelial wall preparation has elicitor activity for rice cells.

Tests of Chitin Fragments for Elicitor Activity

N-Acetyl-D-glucosamine monomer and its β-1,4-linked oligomers, chitobiose, chitotriose, and chitotetrose, were tested in the elicitor bioassay to learn something about the structural requirements of chitin fragments for activity. None of these small, soluble chitin fragments at concentrations of 20 μg

mL^{-1} showed elicitor activity that was above the untreated control value in the assay (data not shown). Insoluble polymeric chitin served as a positive control in this experiment.

Attempts were made to prepare soluble chitin fragments that were active as elicitors by digestion of insoluble polymeric chitin with chitinase preparations from either S. griseus or from rice cell suspension cultures (see "Materials and Methods" and below). Chitin was digested for various periods of time with chitinase, and the soluble supernatant fractions free of insoluble material were recovered, concentrated by lyophilization, and tested in the elicitor bioassay. However, the soluble material generated in this way had only weak elicitor activity. We believe that the following explanation might rationalize these results. The soluble endochitinases would be expected to release soluble chitin fragments only slowly from the highly insoluble chitin substrate. Once released, however, the soluble fragments become very good substrates for the chitinases and are rapidly degraded to small, inactive oligomers. Thus, the accumulation of significant levels of intermediate size soluble fragments that are active as elicitors may not be possible with this type of in vitro experiment.

Partial acid hydrolysis of insoluble chitin by the method of Barber et al. (2) was successful in producing soluble chitin fragments that were elicitor-active in our assay. Figure 6 illustrates a response curve for cyclase activity as a function of the chitin fragment concentration in the elicitor bioassay. It is estimated from these results that approximately 5 μg mL^{-1} of chitin fragments is sufficient to give 50% of the maximum response in the bioassay. We tentatively conclude that N-acetylglucosamine oligomers of a size larger than four are responsible for the activity of these preparations that were solubilized by partial acid hydrolysis. Attempts to characterize this fraction and better define the chemical nature of the active components are in progress.

Table I. Effects of Pretreatments with Wall-Degrading Enzymes on the Elicitor Activity for Rice Cells of F. moniliforme Mycelial Walls

One milligram of F. moniliforme mycelial wall preparation was pretreated by incubation at pH 5.2 and 40°C for 30 h without enzyme (test 1) or with chitinase (2 units) and/or laminarinase (2 units) as indicated (tests 2–4). Each test was run in duplicate. At the end of the pretreatment, the incubation mixture was heated in a boiling water bath for 10 min prior to testing the entire mixture for elicitor activity with rice cells by the standard bioassay described in "Materials and Methods." The elicitor activity of a control incubation mixture containing 2 units each of chitinase and laminarinase, but lacking mycelial wall, was also determined. The control value of 8.0 pmol per assay was subtracted from the test samples. Elicitor activities of the test samples are expressed as pmol of cyclase activity per assay ± the deviation from the mean of the duplicate samples.

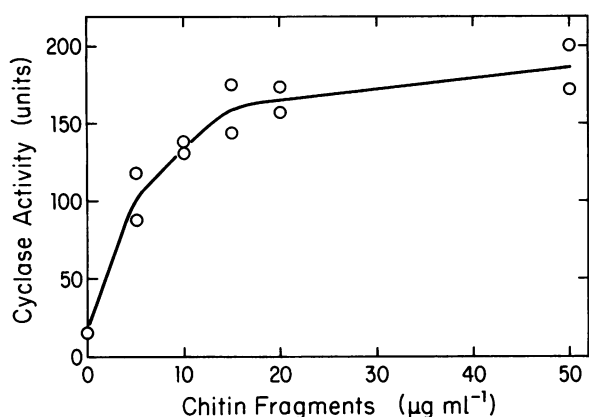| Test No.	Pretreatment Mixtures			Cyclase Activity
	Mycelial wall	Chitinase	Laminarinase	
				pmol/assay
1	+	−	−	79.7 ± 0.1
2	+	+	−	53.2 ± 1.9
3	+	−	+	78.8 ± 3.1
4	+	+	+	12.4 ± 1.2

Figure 6. Elicitor activity of chitin oligomers for rice suspension cells. A mixture of chitin oligomers was prepared by partial acid hydrolysis by the procedure of Barber et al. (2). Differing amounts of a sterile solution of these oligomers were added to a series of 6-d-old, 50-mL rice cell suspension cultures. Duplicate tests were performed at each concentration. The cells were then incubated at 25°C with shaking in the dark. After 40 h of incubation, the cells from each flask were harvested, and an enzyme extract was prepared as described in "Materials and Methods." A 150-μL aliquot of each enzyme extract was assayed for cyclase activity as described in "Materials and Methods." The units of cyclase activity produced in each assay are plotted as a function of elicitor concentration. One unit of cyclase activity is defined as that amount of enzyme required to produce 1 pmol of diterpene hydrocarbon under the standard assay conditions.

Chitinase Activity in Rice Cell Suspension Cultures

We propose that chitinases produced by the rice cells may be responsible for degrading the insoluble chitin polymer to soluble fragments that are responsible for elicitor activity. Therefore, the chitinase activity of both the cells and the medium were examined as a function of time after treatment of 6-d-old rice cell suspension cultures with 20 μg mL^{-1} of chitin. The assay of Molano et al. (20) was employed. This assay utilizes ^{3}H-acetyl-labeled chitin as the substrate and measures the radioactivity released into soluble products at the end of an incubation period as an indication of the chitinase activity. This assay detects both exo- and endo-chitinase activity and would also detect chitin deacetylase activity if present; however, the rice extracts utilized for chitinase assays in this research were examined for chitin deacetylase activity by the selective procedure of Siegrist and Kauss (29), and none was detected (data not included). Enzyme extracts of both the cells and culture medium were assayed at 0, 12, 24, 36, and 48 h after addition of chitin, with the results shown in Figure 7. The activity from comparable amounts of medium and cell extracts was greater in the medium throughout the entire time course. Both the culture medium and the cells showed constitutive chitinase activity at zero time, and chitinase activity was induced in both the cells and the medium by the presence of chitin. However, the induced activity of chitinase in cells tended to level off after 12 h, whereas the induced activity in the medium continued to increase to 36 h. The dashed line shows that the amount of chitinase activity of the cells remains

essentially unchanged throughout the 48-h period in control cells incubated in the absence of chitin.

DISCUSSION

The elicitor bioassay is based on the evidence of Wickham and West (33) that UV-irradiated rice leaves are induced to produce ent-sandaracopimara-8(14),15-diene and 9βH-pimara-7,15-diene, the putative precursors of rice phytoalexins, and at least two other pimaradiene-like diterpene hydrocarbons along with ent-kaurene, whereas nonirradiated leaves make only ent-kaurene, a known precursor of the gibberellins, among these diterpene hydrocarbons. Because of technical difficulties in developing a suitable elicitor bioassay with wounded leaves, the more convenient assay utilizing a rice cell suspension was employed for the studies described in this report. It has been amply demonstrated in this report that the suspension cultures of rice cells in liquid medium respond to elicitors to produce a greatly increased capacity for the synthesis of metabolites with the general chromatographic properties of pimaradiene hydrocarbons. Our recent unpublished data have further shown 9βH-pimara-7,15-diene and sandaracopimara-8(14),15-diene to be among these elicited, diterpenoid products formed from geranylgeranyl pyrophosphate. Thus, we feel that the induced cyclase activity for a mixture of diterpene hydrocarbons serves as a valid measure of elicitor activity even though it does not measure the oxygenated diterpenoid phytoalexins directly.
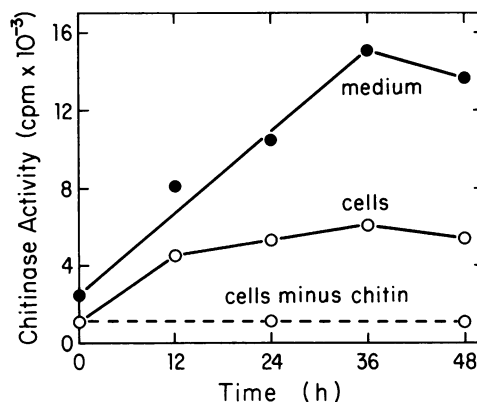

Figure 7. Chitinase activities of rice cells and the culture filtrate at various times after treatment of rice cell suspension cultures with chitin. Enzyme extracts were prepared by procedures described in "Materials and Methods" from the cells and culture filtrate of suspension cultures at 12-h intervals between 0 and 48 h after treatment of the culture at zero time with chitin at a suspension concentration of 20 μg mL^{-1}. As controls, enzyme extracts were also prepared at 24 h and 48 h from cells that were not treated with chitin. The enzyme extracts were adjusted so that equal volumes of each represented the same amount of suspension culture. Chitinase activity was measured by the procedure of Molano et al. (20) with ^{3}H-chitin as the substrate. Chitinase activity is expressed as the cpm released in soluble form from ^{3}H-chitin by 150 μL of enzyme extract in 2 h at 37°C after correction for the cpm released in soluble form in a control incubation of ^{3}H-chitin lacking enzyme. Sources of enzyme extract: culture filtrate from chitin-treated suspensions (●——●), cells from chitin-treated suspensions (O——O), and cells without chitin treatment (O - - - - -O).

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Chitin is the most active of the four oligosaccharides with known elicitor activity for other plants that were tested in the present study. It seems reasonable that chitin might serve as an elicitor in rice because chitin is a major component of the cell walls of many fungal pathogens (3), including fungal pathogens of rice. Chitin has been reported to elicit lignin accumulation in wheat (2, 22), phenolic acids in carrot (16), and chitinase activity in melon (24); however, the role of chitin as an elicitor has not been so extensively investigated as for other types of elicitors. This report extends the types of defense-related responses that chitin has been reported to stimulate in plants to include phytoalexin biosynthesis and calls attention to this possibility that chitin may be a more common elicitor than has been previously recognized.

Chitosan is much less effective than chitin as an elicitor of cyclase activity in rice cells. It can be estimated from the data of Figure 3A that 300 μg mL^{-1} of soluble chitosan elicitor is required to give the same response as 10 μg mL^{-1} of insoluble chitin. This finding makes it seem unlikely that the activity of chitin in the rice cell elicitor bioassay can be due solely to contamination of the chitin preparations with chitosan or the presence of intramolecular stretches of chitin that have become deacetylated. On the other hand, it is conceivable that the weak activity of the chitosan preparation could be due to the presence of N-acetylglucosamine residues in the chitosan sample tested. Alternatively, chitosan might have a weak intrinsic activity of its own.

The branched β-1,3-glucan preparation from P. megasperma was not very effective as an elicitor in the rice bioassay. A solution of the glucan containing 100 μg mL^{-1} gave a response equivalent to 2 to 4 μg mL^{-1} of chitin in the rice elicitor bioassay. According to information supplied by M. Hahn, the branched β-1,3-glucan sample employed in these tests was from the void volume of the low resolution P2 column, a stage of purification of a partial acid hydrolysate of P. megasperma cell walls according to Sharp et al. (28); about 20 μg mL^{-1} of this fraction is sufficient to saturate the glyceollin response of wounded soybean cotyledons. The highest concentration of this glucan fraction tested in the rice cell elicitor assay (100 μg mL^{-1}) gave approximately 27% of the maximum response. Thus, the P. megasperma fraction is considerably less active in rice than it is in soybean, and it is much less active as an elicitor in rice than chitin. It is possible that a fungal cell wall glucan fraction from a rice pathogen might be more active in the rice bioassay than the glucan from P. megasperma. However, treatment of F. moniliforme cell walls with the β-1,3-glucanase, laminarinase, did not reduce the elicitor activity of the cell wall preparation (Table I) even though it clearly solubilized some of the cell wall. Glucan and chitin are the two major components of most fungal cell walls. Our results suggest that fungal cell wall chitin, rather than fungal cell wall glucan, is a major source of elicitor for activation of defense-related responses in rice cells.

A mixture of oligogalacturonides released from polygalacturonic acid by partial digestion with an endopolygalacturonase (average degree of polymerization = 15.5) was the weakest elicitor of those tested. Even at the relatively high concentration of 1,000 μg mL^{-1}, this elicitor gave less than 20% of the maximum response in the rice bioassay. Also,

there was no indication of a synergistic interaction between those oligogalacturonides and chitin in the rice cell elicitor bioassay (data not shown). Perhaps it should not be surprising that pectic fragments are not good elicitors in rice because rice cell walls, in common with many other monocots, do not have a very high content of pectic substances (12).

The positive response of the rice cell elicitor bioassay to a crude cell wall preparation from the fungal pathogen, F. moniliforme, is consistent with the role of chitin as a biotic elicitor in rice. Fusarium species have been shown to have chitin as a major component of their cell walls (30). Furthermore, the ability of chitinase to reduce significantly the elicitor activity of the crude cell wall preparation (Table I) supports the view that chitin is at least one of the active elicitors in the crude cell wall preparation.

One feature of the elicitation of diterpene hydrocarbon synthase activity in rice that is somewhat surprising is the long period of time that elapses between the addition of the exogenous chitin to the cell culture and the appearance of enzyme activity. Activity is first detectable after about 24 h, and the maximum activity is seen at about 40 h (Fig. 2). A similar time course is seen for the appearance of diterpene hydrocarbon synthase activity in rice leaves that have been elicited by UV irradiation (33). The existence of this lag suggests the possibility that the induction of diterpene hydrocarbon synthase activity might be a secondary event dependent on some primary action of these two types of elicitor treatment. In this connection, it is interesting to note that a significant increase in chitinase activity is already evident 12 h after the addition of chitin to the rice culture (Fig. 7). Thus, these two responses to chitin elicitation appear to have different time courses in rice, with the production of chitinase activity being an early event and phytoalexin biosynthesis coming on later.

It is difficult to envision how a water-insoluble polymer such as chitin could function effectively as an elicitor without modification. It is our working hypothesis (a) that an endochitinase(s) produced by the plant is responsible for catalyzing the hydrolysis of insoluble chitin to release water-soluble chitin fragments, and (b) that the released chitin fragments of appropriate size then interact with rice cells to elicit defense-related responses. We further propose that the plant chitinase responsible for the release of chitin fragments is an extracellular endochitinase that is produced constitutively and, thus, is already present at the outset of a rice cell/chitin interaction or during a rice plant/fungal pathogen interaction.

Experiments have been performed to test the general validity of this hypothesis. The most direct approach would be to demonstrate the release of elicitor-active chitin fragments from chitin through the action of chitinase. However, as described in "Results," we were able to detect only low amounts of solubilized elicitor activity from in vitro digestions of chitin with chitinase preparations. This could be because of overdigestion of the released fragments to inactive oligomers under the conditions that prevail in these in vitro digestions. Thus, the negative result does not rule out the possibility that chitin fragments are being produced by chitinase action in vivo. The fact that elicitor-active chitin fragments were generated by the partial hydrolysis of chitin in acid solution (Fig. 6) supports the idea that such fragments

are obligate intermediates in the elicitation process. The concentration of soluble chitin fragments prepared in this way that gave 50% of the maximum response in the elicitor bioassay was approximately 5 μg mL^{-1}, which is very close to that for polymeric chitin (7 μg mL^{-1}). Presumably, the active soluble elicitors in these chitin fragment preparations are oligomers composed of more than four N-acetylglucosamine units because the tetramer and smaller oligomers are inactive. The characterization of these fragments and determination of the structural features necessary for their elicitor activity will require further work.

The elicitor activities of size-fractionated chitin oligomers have been investigated in other plants. Roby et al. (24) reported that the hexamer through the nonamer were the most efficient elicitors of chitinase activity in melon (Cucumis melo L.), whereas the monomer through the tetramer, and an unfractionated mixture of soluble oligomers containing 13 to 20 monomer units, had little or no activity. Barber et al. (2) tested the elicitor activities of chitin monomer through hexamer for lignification in wounded wheat leaves. The monomer through trimer had little or no activity, whereas the tetramer through hexamer were all effective elicitors. The corresponding chitosan oligomers in this size range were not active as elicitors. These studies also support a role for soluble chitin oligomers in a specific size range as elicitors of defense-related responses in sensitive plants.

Constitutive extracellular chitinase activity is detected in the culture filtrate of 6-d-old rice cell suspensions (see the activity at zero time in Fig. 7). Although the constitutive activity is relatively low in comparison with the induced activity after chitin treatment, it may nonetheless be sufficient to act as a sensor for the presence of chitin in the extracellular space. It has not yet been determined whether the constitutive activity is due to an endochitinase, an exochitinase, or both. The properties of the rice chitinases are currently under investigation in our laboratory.

Chitinases are commonly found in plants among the array of pathogenesis-related proteins that are induced as a result of a plant-pathogen interaction. Roby et al. (24) demonstrated that chitin and chitin oligomers elicit chitinase production in C. melo seedlings. It is evident from the results presented in Figure 7 that chitin also elicits increased chitinase activity in rice cell cultures. In addition, β-1,3-glucanase activity is elicited by chitin in the rice cell cultures (data not shown). A function for the induced chitinase and β-1,3-glucanase as defense proteins has been inferred from the finding that chitinase (25), or chitinase in combination with β-1,3-glucanase (19), serves directly as a potent anti-fungal growth inhibitor. In addition to this function, we propose that the constitutive extracellular chitinases of plant cells may serve a second function in the defense of plants that detect chitin fragments as an elicitor, namely to produce chitin oligomers from fungal cell wall chitin to serve as a signal for the induction of synthesis of a variety of defensive components in the plant. This appears to be an important aspect of regulation of a defense response in rice, and we suspect that the role of chitin oligomers as elicitors may be more widespread in other plants than is presently appreciated.

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