Induction of Defense Reactions in Sugar Beet and Wheat by Treatment with Cell Wall Protein Fractions from the Mycoparasite *Pythium oligandrum*

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ABSTRACT

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To detect molecules with elicitor properties from *Pythium oligandrum*, cell wall protein fractions (CWPs) were extracted from 10 *P. oligandrum* isolates and examined for elicitor activity in sugar beet and wheat. *P. oligandrum* isolates were divided into two groups based on the number of major proteins in CWP: isolates with two major proteins (D-type) and isolates with one major protein (S-type). Sugar beet seedlings treated with both types of CWP through their roots showed enhanced activities of phenylalanine ammonia lyase and chitinase, and D-type-treated seed-

lings also showed significantly higher cell wall-bound phenolic compounds, mainly ferulic acid, compared with the distilled-water-treatment control. Damping-off severity was significantly reduced on seedlings treated with both types of CWP compared with the control, following challenge with *Rhizoctonia solani* AG2-2. Both types of CWP significantly reduced the number of infected spikelets developed from the injected spikelet compared with the control, following challenge with *Fusarium graminearum*. Neither type of CWP resulted in any reduction in pathogen growth rate in plate tests. These results demonstrate that CWPs of *P. oligandrum* have elicitor properties in sugar beet and wheat.

Additional keywords: *Beta vulgaris*, biocontrol mechanism, *Fusarium head blight*, *Triticum aestivum*.

Pythium oligandrum Drechs. has received considerable attention as a potential biocontrol agent of a number of soilborne plant pathogens. *P. oligandrum* has been reported to have an antagonistic activity via mycoparasitism (5,6,15), antibiosis (5,8,15), enzyme production (15), and competition for nutrients (17). *P. oligandrum* has been shown to colonize the rhizosphere of a wide range of agriculturally important crops (2). However, there is no direct evidence that *P. oligandrum* is plant pathogenic: Martin and Hancock (17) have shown that *P. oligandrum* was not pathogenic to 12 species of agriculturally important plants. Furthermore, recent investigations indicate additional advantages of this fungus as a biocontrol agent, including growth promotion of pepper (3). Light and electron microscope studies have shown that *P. oligandrum* penetrates the tomato root system without inducing extensive cell damage (23), and triggers an array of structural defense-related reactions upon challenge with *Fusarium oxysporum* f. sp. *radicis-lycopersici* (4).

The induction of plant defense reactions is attractive because induced disease resistance is nonspecific in affording enhanced protection against different types of pathogens (32) and utilizes the plant's own defense mechanisms, which can be a natural, safe, effective, persistent, and durable alternative to pesticides in controlling plant diseases (31). It was anticipated that detecting molecules with elicitor properties from *P. oligandrum* would serve as a basis for further evaluation of its potential as a biocontrol agent of root pathogens. To date, a number of fungal elicitors have been isolated and characterized from the culture medium of fungi grown in vitro and from intercellular washing fluids of infected

plants. Among them, a family of small extracellular proteins produced by *Phytophthora* spp., called elicitins (24,34), have been studied extensively for their biochemical and molecular characterizations and their biological activities (22). They have the interesting property of inducing necrosis and systemic acquired resistance on nonhost plants, including tobacco, and are readily translocated through the vascular system. Similar elicitin-like proteins have been detected from the culture filtrate of some *Pythium* spp. (11,20). Recently, Picard et al. (21) purified a 10-kDa elicitin-like protein termed oligandrin from the culture filtrate of *P. oligandrum*. This protein displayed the ability to induce plant defense reactions upon challenge with *Phytophthora parasitica* in tomato plants. Protein and peptide elicitors have a potential application for controlling plant diseases via plant genetic transformation.

The most critical events underlying attachment and recognition between microorganisms and plants require cell surface molecules; therefore, the objective of this study was to detect the molecules with elicitor properties from the cell wall of *P. oligandrum*. Takenaka et al. (29,30) already found that 13 *Pythium* spp., not including *P. oligandrum*, had species-specific proteins with distinctive and conserved structure in cell wall fragment, named alanine-rich hydroxyproline-containing wall proteins (AHWPs), whose biological functions have not been defined. Therefore, in this study, we attempted the extraction and the partial characterization of cell wall protein fractions (CWPs) from *P. oligandrum* isolates, which were distinct from oligandrin, and examined their ability to trigger induction of the defense reactions in sugar beet and wheat.

MATERIALS AND METHODS

Fungal cultures. Isolates of *P. oligandrum* used in this study are listed in Table 1. These isolates were grown in flasks con-

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taining 120 ml of 10% V-8 broth at 25°C for 3 weeks. Mycelial mats, collected by filtration, washed with distilled water, and blotted dry with a paper, were used for the extraction of CWPs.

For the production of inoculum of the sugar beet pathogen, *Rhizoctonia solani* AG2-2 isolate Pf-28 (isolated from sugar beet roots in Hokkaido prefecture; 18) was grown in flasks containing 25 g of vermiculite–wheat bran–Difco potato dextrose broth (PDB) medium (30 ml of vermiculite, 30 ml of wheat bran, 0.18 g of PDB, and 15 ml of distilled water) at 25°C for 13 to 15 days. For the production of inoculum of the wheat pathogen, *Fusarium graminearum* isolate S1 (provided by Tokachi Agricultural Experiment Station, Japan) was cultured for 7 days on a potato dextrose agar medium, and conidia were produced in mung bean medium (20 g of mung beans in 1 liter of distilled water) through suspension culture.

Plant material. For the bioassay with sugar beet, sugar beet plants (cv. Megumi) were seeded in quartz sand in glass tubes and fertilized each days with 5 ml of a nutrient solution as described by Abe (1). The plants (one plant/tube) were grown for 3 weeks in a growth chamber at 23°C with a 16-h photoperiod. For the bioassay with wheat, Japanese spring wheat plants (cv. Harunoakebono) were cultivated in the greenhouse at 18 to 22°C with natural light until the flowering stage.

Electrophoretic analysis of CWPs. CWPs from nine isolates of *P. oligandrum* listed in Table 1 were extracted as described previously (29). The samples were fractionated by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in the discontinuous buffer system described by Laemmli (14), followed by Coomassie brilliant blue staining.

N-terminal amino acid sequence analysis. Major cell wall proteins from CWPs of *P. oligandrum* isolates MMR2 and MMR3 separated by SDS–PAGE were electroblotted onto polyvinylidene difluoride membranes (Immobilon-P[®]; Millipore, Bedford, MA) as described by Hirano (10). Each blotted protein was sequenced by a gas-phase protein sequencer (Applied Biosystems, Foster, CA).

Enzyme assays. Three-week-old sugar beet seedlings grown under the above conditions were removed from the glass tubes and allowed to absorb 350 µl of solutions containing 750 µg of CWP of *P. oligandrum* isolates MMR2 or MMR3 through their root system. Control seedlings were treated with the same volume of distilled water (DW). The root and hypocotyl tissues from each of three seedlings collected at the indicated times after treatment were ground in liquid nitrogen. The resulting powder was suspended in 25 mM sodium tetraborate buffer, pH 8.8, containing 1% polyvinylpyrrolidone, 2 mM dithiothreitol (DTT) and 0.5 mM phenylmethanesulphonylfluoride (PMSF) at 5 ml/g for phenylalanine ammonia-lyase (PAL) assay; and in 0.1 M sodium citrate buffer, pH 5.0, containing 10% polyvinyl polypyrrolidone, 2 mM DTT, and 0.5 mM PMSF at 4 ml/g for chitinase assay. The homogenate was centrifuged at 18,800 × g for 20 min at 4°C. Solid ammonium sulfate was added to the supernatant to 80% saturation and proteins were precipitated at 4°C for 60 min. The pellet collected by centrifugation at 18,800 × g for 60 min at 4°C was redissolved in 25 mM sodium tetraborate buffer, pH 8.8, 2 mM DTT for PAL assay, and in 10 mM sodium acetate buffer, pH 5.0, for chitinase assay to final volume of 2.5 ml. After desalting using Column PD-10 (Amersham Pharmacia Biotech AB, Uppsala, Sweden), the protein fraction was used for enzyme assays. The protein content was determined by the method of Lowry et al. (16). PAL activity was determined using L-phenylalanine as substrate, by the method of Huges and Dickerson (12). Chitinase activity was determined using colloidal chitin as substrate, as described by Boller and Mauch (7). The experiment was conducted three times for each enzyme assay.

Quantification of cell wall-bound phenolics. Cell wall-bound phenolics were extracted from seedlings using a modified procedure described by Stadnik and Buchenauer (27). For this, 200 mg of the root and hypocotyl tissues from each of three seedlings

collected at the indicated times after CWP or DW treatment were ground in liquid nitrogen and extracted three times with 2 ml of 80% methanol. The residue was suspended in 2 ml of 0.5 M NaOH and incubated on a shaker at room temperature for 16 h under nitrogen atmosphere. After centrifugation at 18,800 × g for 20 min, the supernatants were acidified to approximately pH 3 with HCl. The phenolic acids were extracted twice by addition of 1 ml of ethyl acetate and evaporated to dryness. The resulting pellet was resolved in 1 ml of methanol, and 1/10-diluted samples with DW were used for determination of phenolics and for high-performance liquid chromatography (HPLC) analysis. The determination of phenolics was performed by the method described by Swain and Hills (28). Phenolic content was determined using a standard curve prepared with *p*-coumaric acid. HPLC was carried out with a liquid chromatograph (Intelligent HPLC system; JASCO Co., Tokyo) equipped with a TSK-GEL ODS-80T (4.6 by 250 mm, Tosoh Co., Tokyo), according to the method of Shibuya (25). Quantification was performed by external standard mode and ferulic and coumaric acids were injected as standard because these two phenolic acids are major wall-bound phenolic compounds in the case of wheat (27). The experiment was conducted four and three times for determination of phenolics and for HPLC analysis, respectively.

Sugar beet bioassay. After 12 h of absorption of CWP or DW as described above, each seedling was returned to quartz sand in a glass tube, inoculated by placing inoculum of *R. solani* (2 g/plant) around the root, and left in the growth chamber under the same condition for seedling growth. Disease severity of each treatment was estimated 14 days after inoculation as the index based on a 0-to-5 scale where 0 = no symptoms on hypocotyl, 1 = necrosis of <50% area of hypocotyl without constriction, 2 = necrosis of >50% area of hypocotyl without constriction or necrosis of <50% area of hypocotyl with slight constriction, 3 = necrosis of >50% area of hypocotyl with slight constriction, 4 = severe stunting of hypocotyl, and 5 = seedling dead. Each treatment consisted of six plants and the experiment was conducted four times.

Wheat bioassay. At the anthesis day of wheat plants grown in the greenhouse under the conditions described above, each spike was treated with 10 µl of solution containing 75 µg of CWP from *P. oligandrum* isolates MMR2 or MMR3 or the same volume of DW by injection into a single spikelet located at the center of spike using a micropipette. The spike was detached 24 h after treatment, and 10 µl of conidial suspension (5 × 10³ conidia) of *F. graminearum* was injected into the same spikelet injected with CWP or DW. After inoculation, incubation continued at 22°C and 90% humidity with 10 h of daylight. Disease severity was scored with the number of infected spikelets developed around the injected spikelet 12 days after inoculation. Each treatment consisted of six plants (one spike/plant) and the experiment was conducted three times.

Statistical analysis. The effect of CWP on plant enzyme activities, plant cell wall-bound phenolic content, and disease suppression was determined by analysis of variance with treatment of experiments as blocks. The Dunnett's one-sided *t* test was used to

TABLE 1. Isolates of *Pythium oligandrum* and their sources

Isolate number	Habitat	Origin
MMR2	Soybean soil	Memuro, Hokkaido prefecture
MMR3	Wheat soil	Biei, Hokkaido prefecture
MMR7	Wheat soil	Kunneppu, Hokkaido prefecture
MMR10	Wheat soil	Memambetsu, Hokkaido prefecture
MMR14	Alfalfa soil	Kunneppu, Hokkaido prefecture
MMR15	Timothy soil	Kunneppu, Hokkaido prefecture
MMR19	Wheat soil	Kamikawa, Hokkaido prefecture
MMR21	Wheat soil	Kamikawa, Hokkaido prefecture
IFO32559 ^a	Vegetable soil	Sakai, Osaka prefecture
OPU425 ^a	Vegetable soil	Sakai, Osaka prefecture

^a Provided by M. Tojo, University of Osaka prefecture, Japan.

determine significant differences between each type of CWP and the control ($P < 0.05$).

RESULTS

Partial characterization of CWP. When CWPs extracted from 10 isolates of *P. oligandrum* listed in Table 1 were applied to SDS-PAGE, two major proteins with molecular mass of ≈ 28 kDa and 24 kDa or one major protein of ≈ 27 kDa were detected from these isolates (Fig. 1). That is, *P. oligandrum* isolates were divided into two classes based on the number of major protein. The CWPs, including two major proteins and only one major protein, were termed D-type and S-type of CWPs, respectively (Fig. 1D and S).

The N-terminal sequences of these three proteins from *P. oligandrum* isolates MMR2 (D-type) and MMR3 (S-type) were determined up to 20th amino acid residue (Fig. 2). The N-termini of the 28-kDa protein of D-type and 27-kDa protein of S-type were identical, and these two proteins and the 24-kDa protein of D-type showed 65% sequence similarity. However, differences in the N-termini were observed between these three proteins and oligandrin described by Picard et al. (21).

Biological activity of CWP. PAL activity was measured in extracts of root and hypocotyl tissues from the CWP-treated and control sugar beet seedlings. Sugar beet seedlings treated with DW alone in the absence of CWP showed low PAL activity and no significant change throughout the assay (Fig. 3). In contrast, a rapid and transient increase in PAL activity was observed in seedlings treated with both types of CWP. Maximum activity was reached 4 h after treatment, and D-type and S-type caused

approximately 20- and 13-fold increase in PAL activity compared with DW treatment, respectively.

Next, accumulation of cell wall-bound phenolics was analyzed in the CWP-treated and control plants. The difference in content between D-type and DW was statistically significant 12 h after treatment (Fig. 4). HPLC analysis of the above phenolics indicated that the major component of cell wall-bound phenolics of sugar beet was ferulic acid; coumaric acid was not detected from any analyzed samples in sugar beet (Table 2). Similarly to the cell wall-bound phenolics 12 h after treatment, the content of ferulic acid in D-type treatment was significantly higher than that in DW treatment. However, the significant differences were not detected between the contents of S-type and DW.

Chitinase activity also was compared between the CWP-treated and control plants. Chitinase activity of DW-treated seedlings showed no significant change from 8 to 24 h after treatment (Fig. 5). A transient, but significant, increase in chitinase activity was found in the seedlings treated with D- and S-types of CWP 12 h after treatment (Fig. 5).

Disease suppression in sugar beet and wheat. To determine the ability of D- and T-types of CWP to induce resistance to *R. solani* in sugar beet, each type of CWP or DW-treated plants were

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cell wall protein fractions (CWPs) of 10 isolates of *Pythium oligandrum*. Each lane contained 4 μ g of protein and was stained with Coomassie brilliant blue. Arrows indicate major proteins, and D and S refer to D- and S-types of CWP, respectively. Lane M, molecular weight standards; lane 1, MMR2; lane 2, MMR3; lane 3, MMR7; lane 4, MMR10; lane 5, MMR14; lane 6, MMR15; lane 7, MMR19; lane 8, MMR21; lane 9, IFO32559; and lane 10, OPU425.

Fig. 2. The N-terminal amino acid sequence of three major proteins of cell wall protein fractions from *Pythium oligandrum* isolates MMR2 (D-type) and MMR3 (S-type). Standard one-letter amino acid code is used and X denotes an unidentified amino acid. Homologous amino acid residues are underlined among these three proteins. The N-terminal sequence of oligandrin was determined by Picard et al. (21).

Fig. 3. Changes in phenylalanine ammonia-lyase (PAL) activity in root and hypocotyl tissues of sugar beet seedlings treated with distilled water (DW) or cell wall protein fractions from *Pythium oligandrum* isolates MMR2 (D-type) or MMR3 (S-type). Values are the means of three independent experiments and asterisks indicate significant difference ($P < 0.05$) compared with DW treatment according to Dunnett's test.

Fig. 4. Changes in cell wall-bound phenolics content in root and hypocotyl tissues of sugar beet seedlings treated with distilled water (DW) or cell wall protein fractions from *Pythium oligandrum* isolates MMR2 (D-type) or MMR3 (S-type). Values are the means of four independent experiments and an asterisk indicates significant difference ($P < 0.05$) compared with DW treatment according to Dunnett's test.

challenged with *R. solani* 12 h after treatment. Disease severity 14 days after inoculation was significantly reduced by 32.6 and 35.5% on the treatments with D- type and S-type of CWP, respectively, when compared with the control (Table 3). When both types of CWP solution (100 µg in 50 µl) were applied to the paper disk located near the growing margin of fresh cultures of *R. solani* in plates, no reduction of fungal growth was observed (data not shown). These results demonstrate that CWPs do not act as antibiotics but act as elicitors in sugar beet.

Furthermore, wheat bioassay was conducted to determine whether CWPs also induce an effective defense response in wheat by injecting conidial suspension of *F. graminearum* into the same spikelet as was pre-injected with CWP or DW. As shown in Table 3, both types of CWP significantly reduced the number of infected spikelets developed from the injected spikelet compared with DW treatment. CWPs also showed no effect on the growth of *F. graminearum* in plates (data not shown). These results suggested that CWPs also induce defense-related reactions in wheat to reduce the progression of Fusarium head blight in tissues.

DISCUSSION

Evidence is presented in this article that D- and S-types of CWP isolated from *P. oligandrum* display the ability to induce resistance in sugar beet against *R. solani* AG2-2, which is one of the pathogens of root rot and damping-off, and in wheat against *F. graminearum*, which is one of the pathogens of head blight. The finding that both types of CWP had no effect on the growth of *R. solani* and *F. graminearum* in plate tests indicates that CWP has no fungicidal activity against these two pathogens. Three major proteins of ≈28, 27, and 24 kDa were detected from both types of CWP and they are distinct in molecular size from oligandrin reported by Picard et al. (21), which is a 10-kDa protein purified from the culture filtrate of *P. oligandrum* and has the ability to induce resistance in tomato against *Phytophthora parasitica*. Furthermore, although the N-terminal amino acid sequence of these three cell wall proteins were similar up to the 20th residue, they are quite different from that of oligandrin reported by Picard et al. (21). Some *Pythium* spp. have been shown to have one or more species-specific cell wall proteins in the molecular mass range from ≈25 to 40 kDa (29,30). These species-specific cell wall proteins are known to have common epitope or epitopes and common characteristics of amino acid compositions; high content of alanine, and the presence of a significant amount of hydroxyproline. Takenaka and Kawasaki (29) named these cell wall proteins AHWPs. The above three major cell wall proteins of *P. oligandrum* strongly reacted with an antiserum against *P. iwayamai* AHWP (S. Takenaka and Y. Nakamura, unpublished data), suggesting that they belong to the AHWP family. Molecular and biochemical analyses of these three cell wall proteins of *P. oligandrum* are being pursued for their further characterization.

The effect of CWP treatments on the activity of PAL and the amount of cell wall-bound phenolic compounds in sugar beet seedlings were examined. PAL is the initial gateway enzyme in

phenolic compound biosynthesis; therefore, it is critical in determining the production rate of phenolic compounds (13). Previous studies strongly suggested that rapid esterification of phenolic compounds (cinnamic acids, in particular ferulic acid) into plant cell walls is a common and early response in the expression of resistance, resulting in cell strengthening (19). Furthermore, cinnamic acids such as ferulic and coumaric acids are known to be potentially toxic to pathogens (19). A recent study demonstrated the enhancement of PAL activity and accumulation of cell wall-bound phenolic compounds (coumaric and ferulic acids) in wheat plants treated with benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), a novel systemic acquired resistance inducer, in response to powdery mildew infection (27). Similarly, in our study, rapid and transient increase in PAL activities was observed in CWP-treated sugar beet seedlings. Furthermore, sugar beet seedlings treated with D-type of CWP showed significantly higher content of cell wall-bound phenolic compounds than DW-treated seedlings. An ultrastructural and cytochemical study with tomato root tissues inoculated with *P. oligandrum* has shown that phenolic compounds may coat host walls and accumulate inside invaded cells (23). Although both coumaric and ferulic acids were detected in wheat plants as the wall-bound phenolic compounds (27), only ferulic acid was detected in sugar beet. These results suggest that wall-bound esterified ferulic acid may contribute to one of the defense reactions in sugar beet treated with CWP.

The chitinase activity in sugar beet seedlings following CWP treatments also was examined. Chitinase is induced by various factors, including pathogen and pest attack as well as treatment with elicitors, and has been shown to inhibit the growth of many

Fig. 5. Changes in chitinase activity in root and hypocotyl tissues of sugar beet seedlings treated with distilled water (DW) or cell wall protein fractions from *Pythium oligandrum* isolates MMR2 (D-type) or MMR3 (S-type). Values are the means of three independent experiments and asterisks indicate significant difference ($P < 0.05$) compared with DW treatment according to Dunnett's test.

TABLE 3. Disease severities caused by *Rhizoctonia solani* AG2-2 in sugar beet seedlings and *Fusarium graminearum* in spikes of wheat treated with distilled water (DW) or cell wall protein fractions (CWPs) from *Pythium oligandrum* isolates MMR2 (D-type) and MMR3 (S-type)^a

Treatment	Disease severity index of sugar beet ^b	Number of infected spikelet of wheat ^c
DW	2.81	2.36
D-type of CWP	1.90*	1.72*
S-type of CWP	1.81*	1.54*

^a Values are the means of four (in sugar beet) and three (in wheat) independent experiments and asterisks indicate significant difference ($P < 0.05$) compared with DW treatment according to Dunnett's test.

^b Disease severity was estimated 14 days after inoculation.

^c Disease severity was estimated 12 days after inoculation as the number of infected spikelets.

TABLE 2. Composition of cell wall-bound phenolics obtained from root and hypocotyl tissues of sugar beet seedlings 12 h after treatment with distilled water (DW) or cell wall protein fractions (CWPs) from *Pythium oligandrum* isolates MMR2 (D-type) or MMR3 (S-type)^a

Treatment	Ferulic acid (µg g ⁻¹ fresh wt)	Coumaric acid (µg g ⁻¹ fresh wt)
DW	82.9	ND
D-type of CWP	93.6*	ND
S-type of CWP	74.6	ND

^a Values are the means of three independent experiments and an asterisk indicates significant difference ($P < 0.05$) compared with DW treatment according to Dunnett's test. ND = not detected.

fungi in vitro by causing lysis of hyphal tips, which has been considered one of the common plant defense mechanisms (9). In our study, significant but transient increase in chitinase activity was observed in sugar beet seedlings treated with CWP. Similarly, transient increases in chitinase activity also have been observed in cucumber and leek roots during the early stage of colonization by the biocontrol agent *Trichoderma harzianum* (33) and vesicular-arbuscular mycorrhizae (26), respectively, both of whose ability to induce resistance has been suggested. These results suggest that transient increase in chitinase activity may be one of the expressions of defense reactions in plants activated by inducing agents.

Picard et al. (21) observed that oligandrin does not induce hypersensitive reaction-associated necrotic response when infiltrated into tomato leaves, which is a typical reaction in elicitor-treated tobacco leaves. Their ultrastructural investigations showed higher electron density of the cell wall in oligandrin-treated tomato stem tissues followed by challenge with *Phytophthora parasitica* compared with nonoligandrin treated plants. Our CWP also did not induce root necrosis of sugar beet and increased the amount of host cell wall-bound phenolic compounds, indicating the possibility that the CWP described in this study and oligandrin trigger a similar host defense system. Further studies designed to examine the effect of CWP treatments on the accumulation of transcripts of genes associated with host defense are needed.

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