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Mycoparasitism of *Rhizoctonia solani* by Endophytic *Chaetomium spirale* ND35: Ultrastructure and Cytochemistry of the Interaction

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Abstract

The interaction between endophytic biocontrol agent *Chaetomium spirale* ND35 and the soil-borne plant pathogen *Rhizoctonia solani* was studied by light microscopy and transmission electron microscopy (TEM), as well as further investigated by gold cytochemistry to assess the potential role of cell wall degrading enzymes (CWDEs) during the mycoparasitic process. Macroscopic observations of fungal growth in dual cultures revealed that pathogen growth inhibition occurred soon after contact with the antagonist, followed by the overgrowth of *C. spirale* on the colony of *R. solani*. The coiling of *C. spirale* around *R. solani* and intracellular growth of the antagonist in its host occurred frequently. Moreover, in advanced stage of interaction between the antagonist and the pathogen, The growth and development of *C. spirale* were associated with highly morphological changes of the host fungal cell, characterized by retraction of plasma membrane and cytoplasm disorganization. Further, TEM investigations through localization by gold immunocytochemistry showed that contact between the two fungi was mediated by an amorphous β-1,3-glucan-enriched matrix originating from cell wall of the antagonist *C. spirale* and sticking to its host surface. At the same time, the hemispherical wall appositions which were intensely labeled by the antibodies of β-1, 3-glucan in cell wall of *R. solani* were induced to form at sites of potential antagonist entry. However, the antagonist was capable of penetrating this barrier, indicating that β-1,3-glucanases were produced during the mycoparasitic process. Localization of *N*-acetylglucosamine residues (chitin) with the gold-labelled wheat germ agglutinin (WGA) implicated that chitinases might be involved in the CWD of *R. solani* in this antagonistic process as well. This report is the first evidence about mechanisms of the interactions between *C. spirale* and *R. solani* in ultrastructural and cytochemical aspects.

Introduction

Rhizoctonia species are among the most aggressive soil-borne pathogens, causing losses on almost all vegetables and flowers, several field crops, turf grasses, and even perennial ornamentals, shrubs, and trees throughout the world (Agrios, 1997). Damping-off, mainly caused by *R. solani*, of seedlings in forest nurseries is one of the most serious and widely spread diseases, sometimes causing more than 70% seedling mortality (Kaushik et al., 2000). The wide host range of this pathogen as well as its ability to survive as sclerotia under adverse environmental conditions have markedly reduced the potential of crop rotation as a management strategy. In general, chemical pesticides effectively protect plants from this pathogen. However, public concerns about harmful effects of chemical pesticides on the environmental and human health, as well as the pathogens have developed resistance to the fungicides have promoted a research for safer control approach. Biocontrol is an environmental friendly and efficient alternative to chemical pesticides management of this pathogen.

Chaetomium Kunze ex Fr. is one of the major genera of ascomycetes and more than 300 *Chaetomium* species have been described (Reissinger et al., 2003). In the past several decades, one of the most common, *C. globosum* Kunze ex Fr. has received considerable attention as a potential biocontrol agent for a number of soil- and air-borne plant pathogens, including *R. solani*, *Pythium ultimum*, *Sclerotinia sclerotiorum* and *Venturia inaequalis*, etc. The mechanisms of fungi suppression by *C. globosum* may be mainly based on antibiosis. In addition, mycoparasitism judged by hyphal coiling of the antagonist around *R. solani* and *Alternalia brassicicola* in dual cultures was observed (Andrews et al., 1983; Cullen and Andrews, 1984; Vannaeci *et al*., 1987; Walther and Gindrat, 1988; Di Pietro et al., 1992; Knudsen et al., 1995; Pereira and Dhingra, 1997; Monaco et al., 1998).

Chaetomium spirale ND35 is a strain of dominant endophytic fungus isolated from *Populus tomentosa*

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and displayed antagonistic activities against several common fruit and forest pathogenic fungi, such as *Cytospora chrysosperma* (teleomorph: *Valsa sordida*), *V. mali* and *R. solani*, etc. (Liu et al., 1999). Several modes of action such as antibiosis and production of extracellular lytic enzymes, mainly chitinases and β-1,3-glucanases responsible for mycoparasitism of this antagonist might play very important role in biocontrol of these diseases described above by *C. spirale* ND35. In addition, the biocontrol trials under greenhouse and field conditions have proved the biocontrol potential of strain ND35 against *V. mali*, the causal agent of apple canker (Liu et al., 1999; Gao et al., 2003, 2005). The discovery of this new biocontrol agent *C. spirale* ND35 from poplar, might open up a promising way for its practical applications in agriculture, especially for forest stem diseases management in an environmental safety way. However, one prerequisite for a rational utilization of the biological properties of a potential antagonist is a better understanding of the antagonistic mechanisms. Although hyphal interactions between *Chaetomium* spp. and other pathogens such as *Drechslera sorokiniana* (*Cochliobolus sativus*; Sudhamoy et al., 1999), *Botrytis aclada* in dead onion leaves (Kohl et al., 1997) and *V. mali* (Gao et al., 2005) have been studied by scanning electron microscopy (SEM); little is known about the cytological events during the mycoparasitic process. The main objective of this study was to investigate the cytochemical events of the interaction between *C. spirale* ND35 and *R. solani* through both light microscopy and transmission electron microscopy (TEM) and to delineate the potential role of CWDEs by following the pattern of chitin and β-1,3-glucan labelling in the hyphal cell walls with highly specific gold-complexed probes.

Materials and Methods

Fungal isolates and cultural conditions

The isolate of endophytic *C. spirale* ND35 used in this study was isolated from *P. tomentosa* and was reported as an effective antagonist of several phytopathogenic fungi (Liu et al., 1999). *Rhizoctonia solani* was isolated from the seedling of *Pinus tablaeformis* with damping-off symptom. Both *C. spirale* ND35 and *R. solani* were grown on potato dextrose agar (PDA) at 25°C and stored at 4°C.

Dual culture tests

A dual culture technique (Gao et al., 2002) with partial modification was used to study antagonism. A mycelial disc (5 mm in diameter) of *C. spirale* ND35 was taken from the edge of actively growing colony and placed on the surface of the PDA plate. Two days later, a mycelia disc of *R. solani* (5 mm in diameter) was placed 3 cm apart from *C. spirale* ND35 on the same PDA plate with four replicates. The mycelial discs of pathogen or antagonist on PDA alone served as controls. The plates were incubated at 25°C. The antagonist and its host grew towards each other, and overgrowth of *R. solani* by *C. spirale* ND35 occurred by 3–4 days after inoculation of the pathogen. Mycelial samples from the interaction region were collected at 3, 5 and 7 days after the pathogen inoculation and processed for electron microscopy.

Light microscopy

For the examination of light microscopy, The PDA membranes ($20 \times 20 \times 2$ mm) were cut with sterile scalpel and placed in the centre of slides. *Chaetomium spirale* ND35 and the pathogen were inoculated respectively in the centre of the opposite sides on the same membrane and incubated in big Petri dishes with wet filter paper at 25°C for 5 days. The samples were examined through the direct observation of the slides with the inoculated PDA membranes under the light microscope at 2, 3, 4 and 5 days after inoculation respectively, followed by taking micrographs with a Zeiss Axioscop microscope and MC 100 camera while mycosparasitism occurred.

Transmission electron microscopy

Mycelial samples from the interaction region, as well as samples from single cultures of each fungus on PDA were fixed in 2% (v/v) glutaradehyde in 0.1 M cacodylate buffer (pH 7.4) with 1 mM CaCl and 1% (w/v) sucrose for 3 h at room temperature. Then samples were rinsed six times with the same buffer and postfixed with 1% (w/v) osmium tetroxide in the same buffer for 2 h at room temperature. After rinsed thoroughly with 0.1 M cacodylate buffer (pH 7.4), samples were dehydrated in a graded ethanol series. Fully dehydrated samples were moved from absolute ethanol through a 1 : 1 mixture of ethanol and propylene oxide to pure propylene oxide. Samples were infiltrated through a series of Epon-Araldit-Mixture resin in propylene oxide, followed by embedded in moulds with fresh 100% resin and polymerized at 65°C for 36 h. More than three replicate experiments were performed.

Ultrathin sections cut with a glass knife were collected on formvar-coated slot grids. After drying, the grids were contrasted with uranyl acetate and lead citrate and examined with an EM 10 CR electron microscope (Zeiss, Oberkochen, Germany) at 60 kV or further processed for cytochemical labelling. Three samples per sampling time were examined with an average of 10 grid squares per sample.

Cytochemical labelling

To study the distribution of chitin, a linear polysaccharide of β-1,4-linked *N*-acetylglucosamine residues, WGA, a lectin with *N*-acetylglucosamine-binding specificity (Benhamou, 1989), and 10 nm gold-labelled WGA (purchased from British BioCell Co., Cardiff, UK) was used in this procedure. According to the method of Miosge et al. (1997) with some modification, nickel grids with ultrathin sections were first incubated on a drop of phosphate-buffered saline (PBS; 7.2 mM Na_2HPO_4, 2.8 mM NaH_2PO_4, pH 7.2) for 20 min, then sections were incubated on a drop of gold-labelled WGA (1 : 20 in PBS, pH 7.2) for 2 h at room temperature in a moist

chamber. Sections were washed with PBS, followed by rinse with distilled water, and contrasted with uranyl acetate and lead citrate. The specificity of labelling was tested by preincubating the gold-labelled WGA in a solution of the inhibitory sugar *N*-acetylglucosamine at concentrations of 0.1–0.4 M, according to the method of Winkler and Lünsdorf (2001).

Immunocytochemistry

For studying the distribution of β-1,3-glucan, the antibodies against (1,3)-β-D-glucans were used as the primary antibody. Production, characterization and specificity of the polyclonal antibody against (1,3)-β-D-glucan (no. CH-11-512, ICI, Wilmington, NC, USA) has been described by Northcote et al. (1989). The secondary antibodies [goat antirabbit, immunoglobulin G (IgG)] coupled to 10 nm gold particles were purchased from Aurion company (Wageningen, the Netherlands).

According to the amended method of Kang and Buchenauer (2002, 2003), immunogold labelling was carried out as follows: (i) nickel grids with ultrathin sections were placed on water for 1 min in a high humidity chamber; (ii) incubation of ultrathin sections with blocking solution containing 1% bovine serum albumin (BSA)-C in Tris-buffered saline (TBS; 10 mM Tris-HCl, 150 mM NaCl, pH 7.4) for 20 min; (iii) incubation of the sections with the primary antibody diluted at 1 : 200 in blocking solution for 2 h at room temperature; (iv) washing 10 times with TBS; (v) incubation of the sections with the secondary antibody diluted at 1 : 20 in TBS; (vi) rinsing with TBS, followed by a distilled water rinse. After contrasting with uranyl acetate and lead citrate, the sections were examined with a Zeiss EM 10 CR electron microscope at 60 kV. Three replicate experiments were performed. The specificity of antibody labelling for the immunolabelling of β-1,3-glucan was determined by replacing the primary antibody with buffer, according to the method of Rodriguez-Galvez and Mendgen (1995).

Results

Fungal growth in dual cultures and light microscopy observations

In Petri dish dual cultures, the first apparent contact between *C. spirale* ND35 and its host *R. solani* occurred within 2–3 days after pathogen inoculation, followed by occurring pathogenic growth inhibition soon after contact with the antagonist, and *C. spirale* overgrowth on the colony of *R. solani*, accompanied by retraction of the pathogenic aerial mycelium. By 8 days after inoculation, *C. spirale* began to produce a large number of perithecia.

Chaetomium hyphae, easily recognized, because of their smaller diameter, and its mycelial growth on or along the hyphae of the pathogen, even penetration can be frequently observed under light microscopy. The observations under light microscope showed that *C. spirale* ND35 densely coiled around the hypha of *R. solani* (Fig. 1a). And the intracellular growth in the hyphae of *R. solani* was observed as well (Fig. 1b). At a later stage of the antagonistic process, several hyphae of *R. solani* were strongly degraded and cytoplasm of pathogenic hyphae became to empty, as well as pathogenic hyphae appeared as abnormally shaped, empty pleiomorphic shells (data not shown). To gain a better insight into the pattern of interaction between *C. spirale* ND35 and *R. solani*, more precise information at the ultrastructural level was needed.

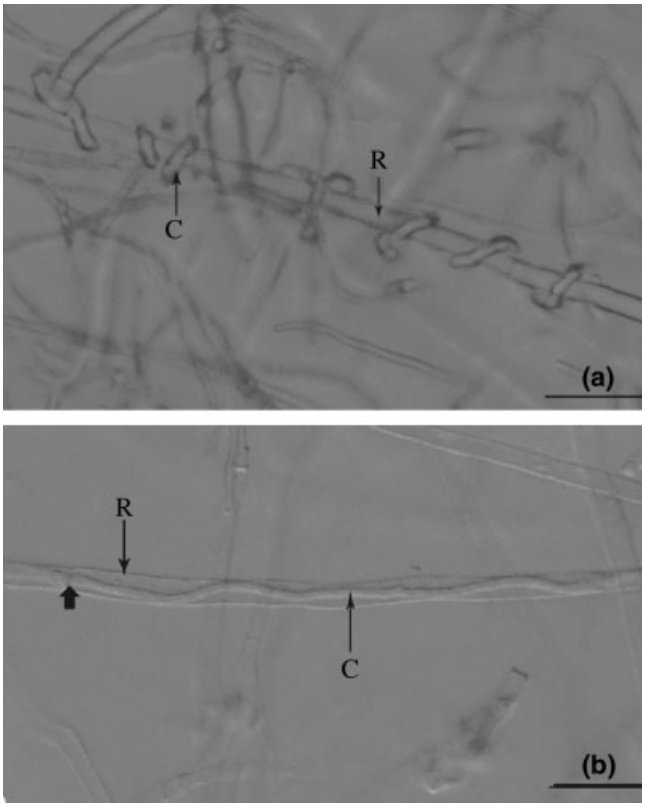

Fig. 1 Light micrographs of the hyphal interacting between *Chaetomium spirale* (C) and *Rhizoctonia solani* (R) in dual cultures. (a) Three days after the pathogen inoculation, the *Chaetomium* hyphae (C) densely coiled around hyphae of *R. solani* (R). (b) Five days after the pathogen inoculation, a *Chaetomium* hypha (C) grew intracellularly in the cell of *R. solani* (R) and penetrated through the septum of host cell (arrowhead). (a and b) Bar = 5 μm

TEM observations

More detailed pictures of the penetration structures in parasitizing process of antagonist was obtained through TEM observations of ultrathin sections. When grown in single culture, *R. solani* showed similar ultrastructure to the results of observations from Benhamou and Chet (1993) and Carisse et al. (2001). Cells were delimited by thick and dark cell walls. They contained a dolipore septum and a dense polyribosome-enriched cytoplasm in which a large number of organelles including mitochondria, vacuole, endoplasmic reticulum, nuclei and lipid bodies (Fig. 2a).

Examination of ultrathin sections from the interaction region of 3-day-old dual cultures demonstrated that hyphae of *C. spirale*, easily recognized by their smaller diameter, frequently encircled hyphae of *R. solani* (Figs 2b, 3d and 4d) and established close contact

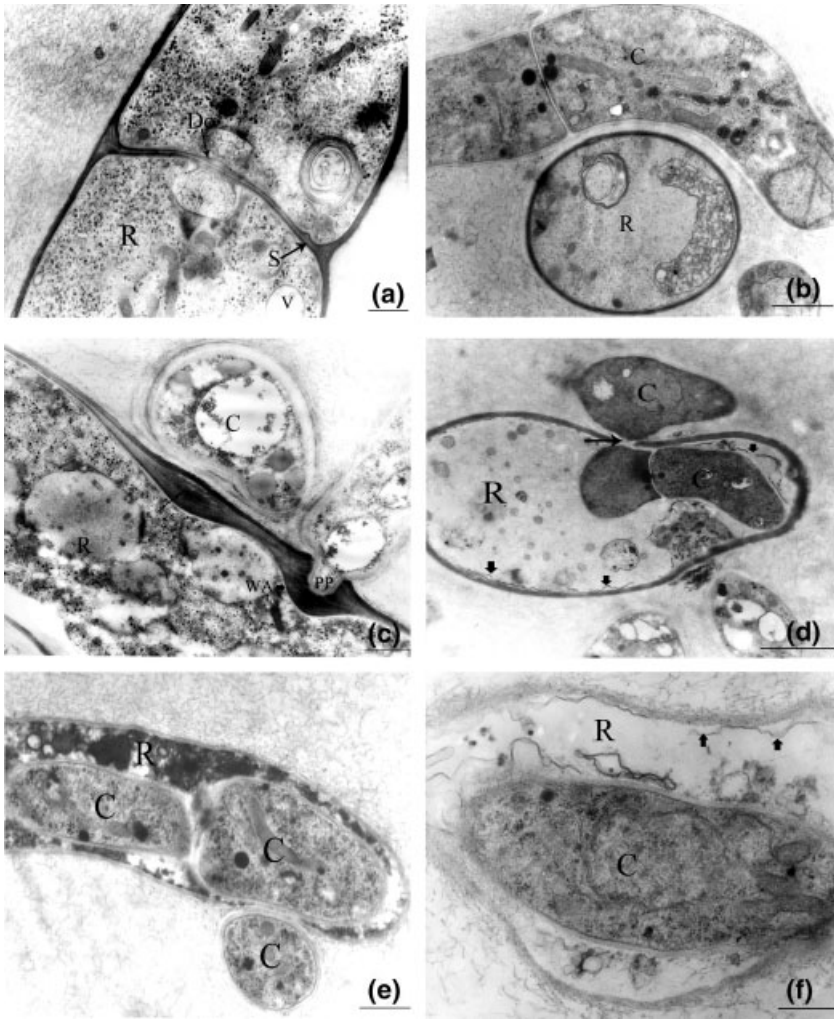

Fig. 2 Transmission electron micrographs of *Chaetomium spirale* (C) hyphae interacting with cells of *Rhizoctonia solani* (R) in dual cultures. (a) Hyphae of *R. solani* (R) in single culture showed a fine ultrastructure with a doipore septum (Do) and dense polyribosome-enriched cytoplasm in which a large number of organelles including mitochondria (M) and vacuole (V; bar = 2 μm). (b and c) Three days after inoculation: (b) a hypha of *Chaetomium* (C) encircled a cell of *R. solani* (R; bar = 2.5 μm); (c) two hyphae of *Chaetomium* (C) was infecting a cell wall of *R. solani* (R) and inducing wall apposition (WA). A *Chaetomium* hypha formed a penetration peg (PP) and another appeared to cause stratification of wall apposition (arrow; bar = 2 μm). (d and e) Five days postinoculation: (d) a hypha of *Chaetomium* (C) penetrated into and grew within a cell of *R. solani* (R; bar = 2.5 μm); (e) a hypha of *Chaetomium* (C) grew in a cell of *R. solani* (R; bar = 2.5 μm). (f) By 7 days after inoculation, because of parasitizing of *C. spirale* (C) a cell of *R. solani* (R) showed signs of pronounced alteration characterized by plasma membrane retraction (arrowheads) and cytoplasm disorganization (bar = 1 μm)

with the host cells. At the same time *Chaetomium* hyphae formed penetration pegs and induced the formation of wall appositions in the hyphal cells of *R. solani* (Fig. 2c). The thickened wall layers appeared splitting signs (Fig. 2c, arrow). Sometimes two to three potential penetration sites were observed in one cell of *R. solani* (Figs 2c and 3d). Investigations of mycelial samples collected from the interaction region 5 days after inoculation showed that the antagonist with intact ultrastructure intracellularly grew (Fig. 2d,e) in pathogenic cells after invading the *Rhizoctonia* hyphae, and went through the septa to extend continuously (Fig. 3h). By 7 days postinoculation, the cytoplasm and protoplast membrane (arrowheads) in some hyphal cells of *R. solani* were markedly disorganized. In most cases, organelles were no longer discernible in the highly altered cytoplasm (Fig. 2f).

Cytochemical localization of *N*-acetylglucosamine residues (chitin)

Ultrathin sections from individual colonies of either *C. spirale* ND35 (Fig. 3a) or *R. solani* (Fig. 3b) were incubated with gold-labelled WGA for localizing *N*-acetylglucosamine residues (chitin). With both fungi, an intense labelling was specifically associated with the cell wall. In contrast, cytoplasm, organelles and vacuoles were nearly devoid of labelling. In control test, no labelling was found over the cell walls of fungi (Fig. 3c). Three days postinoculation with the pathogen, *Chaetomium* encircled the hyphal cell of *R. solani* was frequently observed, moreover three penetration pegs synchronously penetrated into the same cell and induced the formation of wall appositions (Fig. 3d). Observations at a higher magnification showed the early penetration and formation of penetration peg of the *Chaetomium* hypha, as well as the formation of hemispherical wall appositions and a slight retraction of the plasma membrane (PM) from the wall by *R. solani*. The localization situation of some gold particles over them also can be observed (Fig. 3e, arrowheads). Examination of 20 ultrathin sections from the interaction region of 3-day-old dual cultures also revealed the contact of hyphal cell wall between *Chaetomium* and its host (Fig. 3f). At a close observation, the PM of pathogenic cell showed slightly retraction and a decrease in the electron density of the outermost wall layer. Interestingly, cell wall layers of both the antagonist and the pathogen appeared diffuse when in close contact. Incubation of these sections with the gold-labelled WGA resulted in the deposition of gold

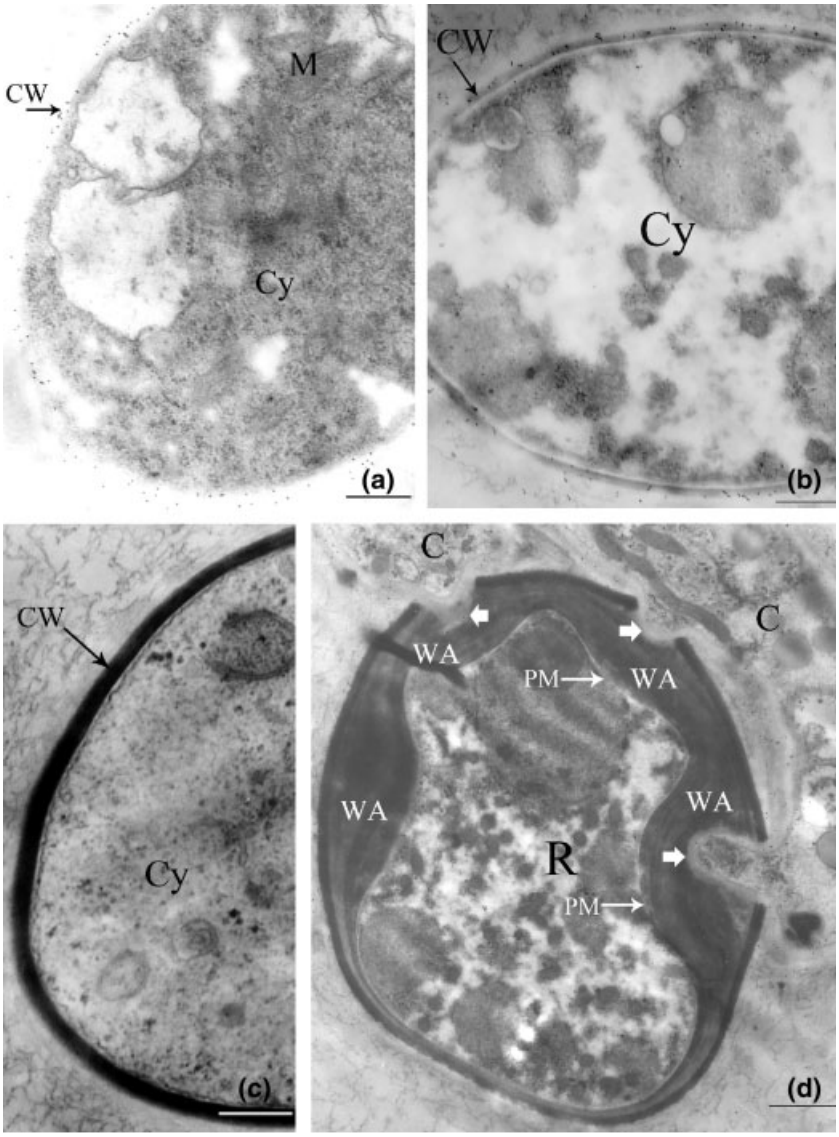

Fig. 3 Transmission electron micrographs of *Chaetomium spirale* and *Rhizoctonia solani* in single cultures or dual cultures. Labelling of chitin with the gold-labelled wheat germ agglutinin (WGA). (a and b) A regular deposition of gold particles (arrows) occurred over the hyphal cell walls (CW) of *C. spirale* (a) and *R. solani* (b) grew in pure culture. Cytoplasm (Cy) and organelles such as mitochondria (M) were unlabeled (bar = 2 μm). (c) Negative control test: no labelling was found over the hyphal cell walls (CW) of *R. solani* (bar = 2 μm). (d–f) Three days after inoculation: (d) a hypha of *Chaetomium* (C) was encircling a cell of *R. solani* (R), producing three penetration pegs (arrowheads), as well as penetrating into the same cell and inducing the formation of wall appositions (WA), accompanied a slight retraction of plasma membrane (PM; bar = 2 μm); (e) portion of (d) at a higher magnification showed an early penetration of the *Chaetomium* hypha, formation of penetration peg (PP) and hemispherical WA, as well as a slight retraction of plasma membrane (PM; bar = 1 μm); (f) the hyphal cell wall of *Chaetomium* (C) contacted with the cell wall of *R. solani* (R) was showing slightly retraction of PM (arrowhead). Gold particles were significantly reduced over the cell wall of *R. solani* (bar = 0.5 μm). (g) Five days postinoculation, the hyphal cell wall of *Chaetomium* (C) did not directly contact with the cell wall of *R. solani* (R). However, the cell wall of *R. solani* showed signs of a decrease in the electron density of the outermost wall layer (arrow) and a few of gold particles over it (bar = 1 μm). (h) Two hyphae of *Chaetomium* (C) grew in a cell of *R. solani* (R) and one of them was passing the septum (arrowhead) of *R. solani*. Some gold particles were observed over the cell wall (arrows) of *Chaetomium* and a few of gold particles were found over the wall and septum (arrowhead) of *R. solani* (bar = 2.5 μm)

particles over cell walls of both fungi. However, gold particles were irregularly distributed over the cell walls of *R. solani* (Fig. 3f). Five days postinoculation, although hyphal cell wall of *Chaetomium* did not directly contact with *R. solani*, the cell wall of *R. solani* also showed signs of a decrease in the electron density of the outermost wall layer and a few of gold particles over it (Fig. 3g, arrow). Two hyphae of *Chaetomium* grew in one cell of *R. solani* and one of them was passing the septum of *R. solani* were also observed (Fig. 3h). Some gold particles were found over the cell wall of *Chaetomium*, and a few of gold particles were observed over the wall and septum of *R. solani*. Hyphae of the antagonist ramified so extensively in host hyphae that it was difficult to delineate some free space in the area that was originally occupied by the host cytoplasm (Fig. 3h). Such a massive colonization frequently resulted in a strong mechanical pressure

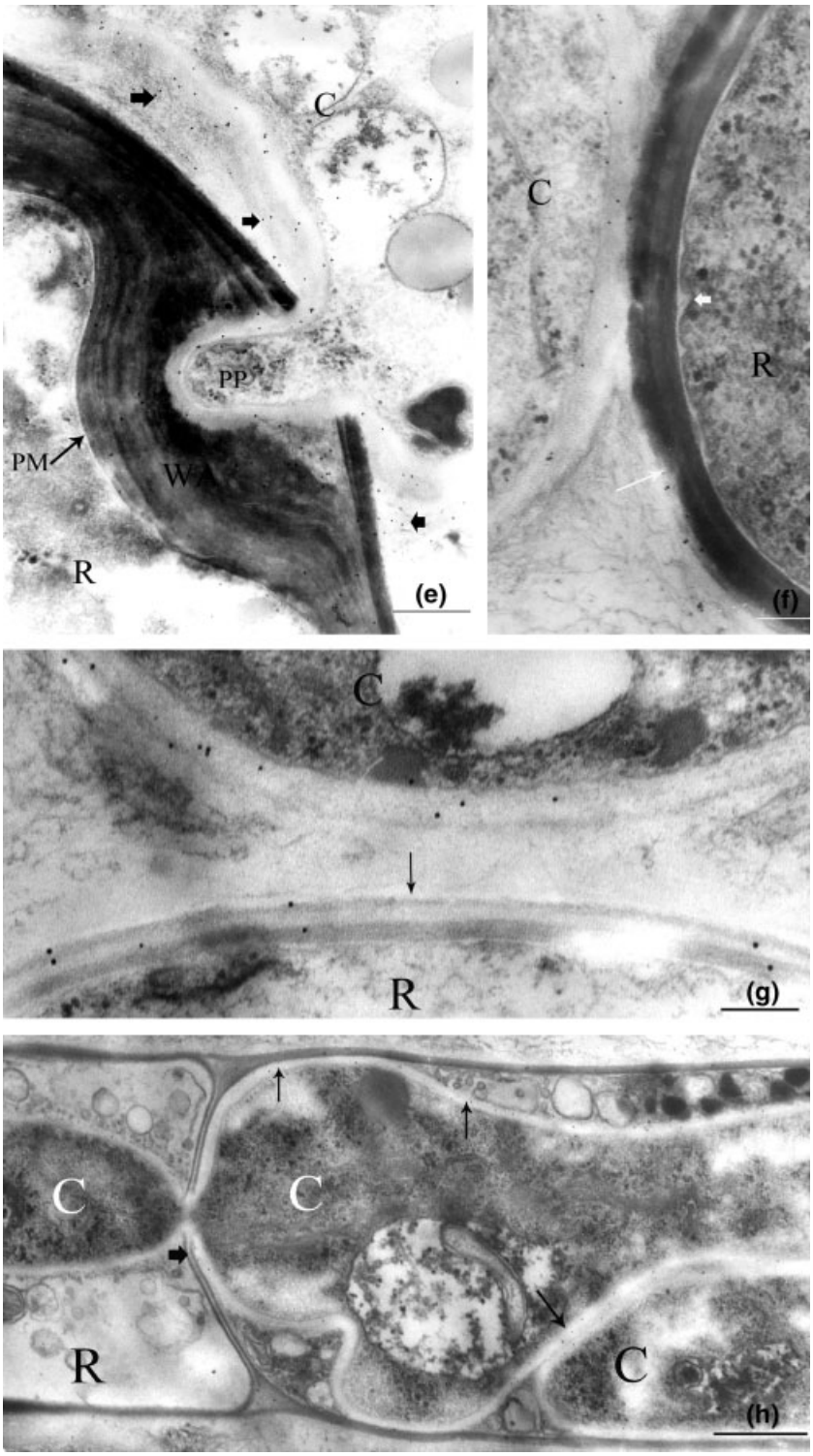

Fig. 3 Continued

against the host hyphal cell walls, ultimately leading to an apparent bursting of the host hyphae.

Immunocytochemical localization of β-1,3-glucan

Gold particles were predominantly associated with the hyphal cell walls of *C. spirale* (Fig. 4a) and *R. solani* (Fig. 4b) grown in single cultures. Three days after inoculation with the pathogen, cells of *R. solani* were encircled by hyphae of *Chaetomium* and produced wall appositions (Fig. 4c,d). A large number of gold particles were distributed evenly over the cell walls of *Chaetomium* and pathogen except at sites of potential penetration by the antagonist and slight alterations occurred over the cell wall of pathogen (Fig. 4d). A

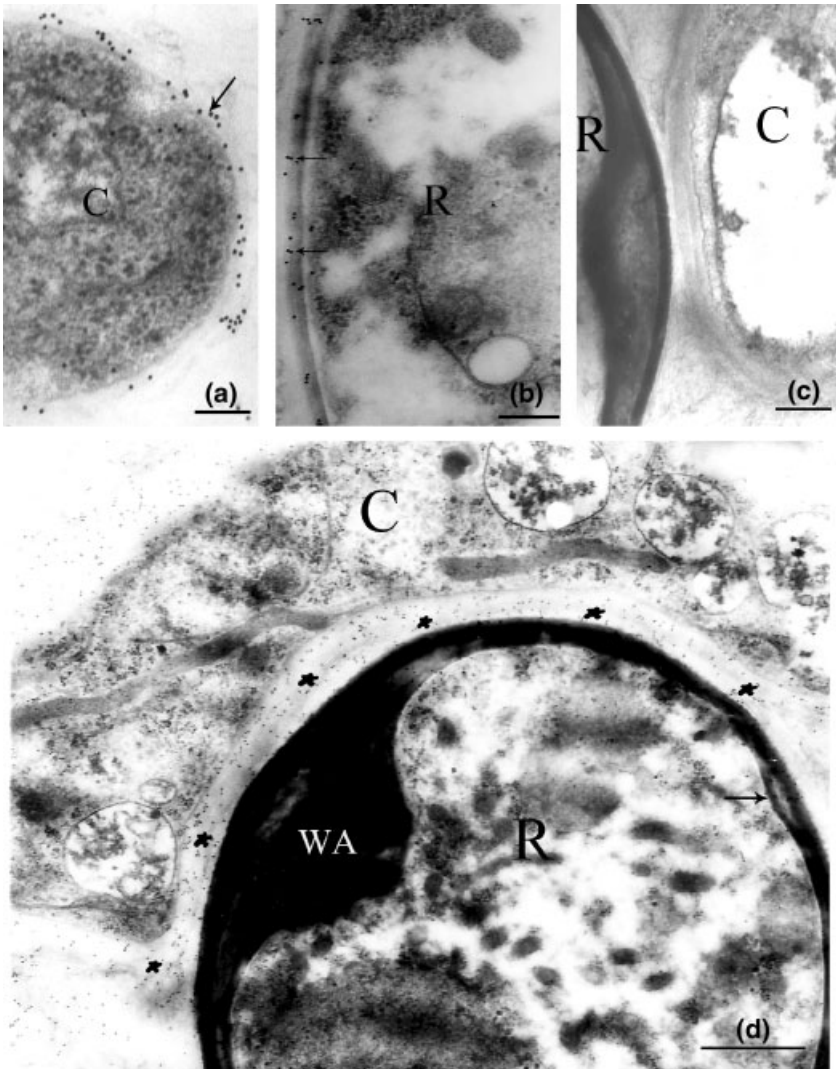

Fig. 4 Transmission electron micrographs of a single culture of *Chaetomium spirale* or*Rhizoctonia solani* and dual cultures between *C. spirale* and *R. solani*. The immunolabelling of β-1,3-glucans with the antibodies of β-1,3-glucan. (a and b) Gold particles (arrows) were mainly observed over the cell wall of a *C. spirale* hypha (a; bar = 0.5 μm) and a *R. solani* hypha (b; bar = 1 μm) grown in single culture. (c) Negative control test: no gold labelling was found over the hyphal cell walls (CW) of *R. solani* and *C. spirale* (bar = 1 μm). (d and e) Three days after inoculation: (d) a cell of *R. solani* (R) were encircled by a hypha of *Chaetomium* (C) and produced a wall apposition (WA). More gold particles were distributed evenly over the cell walls of *Chaetomium* and pathogen (stars) except at sites of potential penetration by the antagonist and slight alteration area (arrow) over the hyphal cell wall of pathogen (bar = 2 μm); (e) at higher magnification, picture showed that contact between antagonist and pathogen was mediated by an amorphous matrix intensely labelled with the antibodies of β-1,3-glucan (stars). A penetration peg (PP) of *Chaetomium* hypha (C) was penetrating into a wall apposition in the hyphal cell of *R. solani* (R; bar = 1 μm). (f) Five days postinoculation, the cell of *R. solani* (R) was parasitized by two *Chaetomium* hyphae (C). The markedly disorganized cytoplasm and some digested wall areas were visible (arrowheads). Gold particles were mainly distributed over the cell walls of antagonist and pathogen (bar = 2 μm). (g) Seven days after inoculation, although hyphae of *Chaetomium* (C) did not directly contact with the cell wall of *R. solani* (R), plasma membrane (arrow) was already retracted, and cytoplasm was highly disorganized (bar = 1.5 μm). (h) At an advanced stage of the interaction, the cell wall of a *R. solani* (R) contacting with hypha of *Chaetomium* was degraded strongly and cytoplasm and all organelles were highly damaged (bar = 1 μm)

close examination revealed that contact between the two fungi was mediated by an amorphous matrix labelled intensely with the antibodies of β-1,3-glucan. This β-1,3-glucan-enriched matrix appeared to originate from the antagonist cell wall and to stick to its host surface (Fig. 4e). Penetration pegs of *Chaetomium* hyphae penetrated into the hemispherical wall appositions in hyphal cells of *R. solani*. More gold particles were observed over the cell walls of penetration pegs and *Chaetomium* hyphae as well as those areas near the invaginated PM on wall appositions in the hyphal cell of *R. solani*. However, a few of gold particles were found along the channel of penetration over the wall appositions in the cell of *R. solani* (Fig. 4e). By 5 days after inoculation, the cells of *R. solani* were parasitized by two *Chaetomium* hyphae (Fig. 4f). Pathogenic cytoplasm was pronouncedly disorganized and some digested wall areas were visible (arrowheads). Gold particles were mainly distributed over the cell walls of antagonist and pathogen. Some gold particles were also scattered over the cytoplasm (Fig. 4f). Seven days postinoculation, although hyphae of *Chaetomium* did

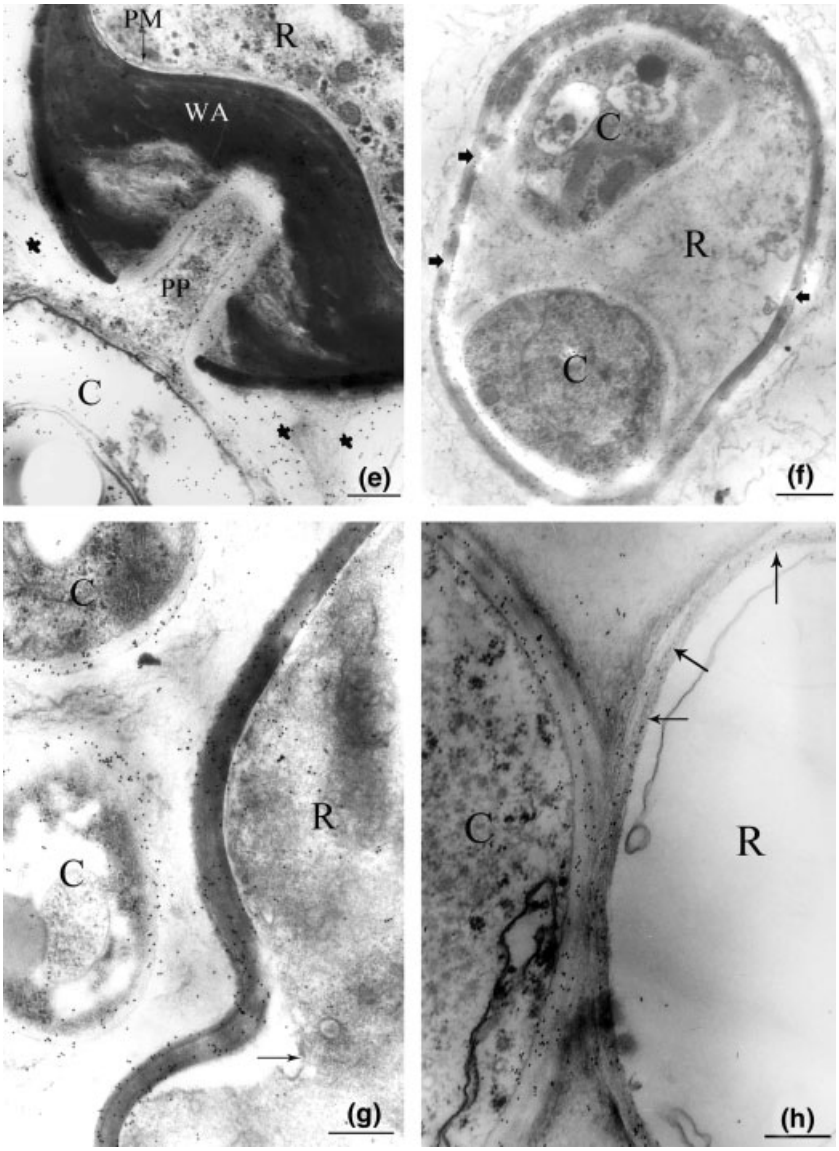

Fig. 4 Continued

not directly contact with the cell wall of *R. solani*, but PM was already retracted, and cytoplasm was highly disorganized (Fig. 4g). At a more advanced stage of the interaction, the cell walls of *R. solani* contacting with hyphae of *Chaetomium* were seriously degraded as well as cytoplasm and all organelles were markedly damaged (Fig. 4h).

All control tests performed on sections of samples collected from the interaction region yielded negative results (Fig. 4c).

Discussion

The ability to produce lytic enzymes has been showed to be a crucial property of the mycoparasitic fungi. Most phytopathogenic fungi have cell wall that contain chitin as a structural backbone arranged in regularly ordered layers and laminarin (β-1,3-glucan) as a filling material arranged in an amorphic manner. The other minor cell wall components are proteins and lipids (Chernin and Chet, 2002). The CWDEs such as chitinases, glucanases and protease, are not only involved in the destruction of the host cell wall; they may also play a role during the initial stages of mycoparasitism, because oligosaccharides generated by CWDEs partially degrading the cell walls of the host may act in turn as elicitors for the general antifungal response of mycoparasitic fungi (Chet and Chernin, 2002).

Our studies demonstrated that *R. solani* is highly vulnerable to an attack by the endophytic antagonist *C. spirale* ND35. The present cytochemical investigations were undertaken to gain a better insight into the cellular and molecular mechanisms in the process of mycoparasitism. Our results provided evidence that early recognition events that mediated by a β-1,3-glucan-enriched amorphous matrix originating from *C. spirale* ND35 triggered the firm binding of the antagonist to the host cell surface, leading to a subsequent programme of specific responses, such as

adhesion and penetration of the antagonist in *R. solani*, precede pathogenic cell alterations, including the formation of wall appositions, cell wall degradation, plasmalemma and cytoplasm disorganization. To our knowledge, the present results provide the first ultrastructural and cytochemical evidence of the interactions between *C. spirale* ND35 and *R. solani* in the mycoparasitic process.

Macroscopic observations of fungal development in dual cultures as well as light microscopy examinations of the interaction region indicated that growth inhibition and structural alterations of the pathogen appeared soon after contact with *C. spirale* ND35. These results are similar to those reports for *Trichoderma harzianum* (Benhamou and Chet, 1993, 1996, 1997) and *P. oligandrum* (Benhamou et al., 1999) as well as *Microsphaeropsis* sp. (Carisse et al., 2001).

TEM observations of the interaction region between the two fungi exhibited that hyphae of *Chaetomium* encircled and established close contact with the host mycelium. These observations provided support to the assumption that the outcome of the interactions was likely determined by early recognition events. Positive correlations between surface-associated components and recognition events in microbial interactions often have been suggested (Chet, 1987; Benhamou and Chet, 1993) and considered to be key determinants in the outcome of a given interaction. Although early studies pointed to a possible role for lectin in the recognition process, Inbar and Chet (1992) proved the role of lectins in *Trichoderma* mycoparasitism. Utilizing lectin-coated nylon fibres through biomimics, *T. harzianum* coiled around the fibres in a way similar to that observed with host hyphae. However, whether such a phenomenon plays a role in the initial recognition between *C. spirale* ND35 and *R. solani* needs further investigation.

With regard to the mechanisms of early recognition and contact between the antagonist and pathogen, there have been some different research results from previous studies on different antagonist and fungal host partners. Benhamou and Chet (1993) observed the interaction between *T. harzianum* and *R. solani* and thought that cells of *Trichoderma* encircled and established close contact with the host mycelium appeared to be mediated by a fine, external matrix originating from *R. solani* hyphae. The polysaccharidic nature of this fine matrix layer was judged with the gold-complexed *Ricinus* lectin. The occurrence of significant amounts of galactose residues in the external matrix of *R. solani* suggested that receptors with galactose-binding affinity were present at the cell surface of *Trichoderma*. Because lectins are sugar-binding proteins, a *Trichoderma* lectin may be responsible for the binding of the galactose-rich matrix of *R. solani*. In addition, observations of the interaction region between *P. oligandrum* and *Fusarium oxysporum* f. sp. *radicis-lycopersici* made by Benhamou et al. (1999) showed that contact apparently was mediated by a fine, external matrix originating from the *Fusarium* hyphae. The occurrence of a significant amount of chitin residues in this matrix suggested that receptors with *N*-acetylglucosamine-binding affinity were present at the cell surface of *P. oligandrum*. A similar interfacial material was not detected in the interactions between the antagonist *P. oligandrum* and *P. ultimum*, *P. aphanidermatum*, or *R. solani*, although intense coiling and adhesion of *P. oligandrum* to the host hyphae could be seen. However, Carisse et al. (2001) made observations of the interaction between *Microsphaeropsis* sp. and *R. solani*. They found that the outcome of the interaction was likely determined by early recognition events often mediated by an amorphous matrix originating from the antagonist, *Microsphaeropsis* sp., but not the pathogen. And a substantial amount of chitin occurred in this matrix suggests that receptors with *N*-acetylglucosamine-binding affinity are present at the cell surface of *R. solani*. Their these findings are almost in accordance with our investigations on acting mode of *C. spirale* ND35 and *R. solani*, except the occurrence of a significant amount of β-1,3-glucan instead of chitin in this matrix suggests that receptors with β-1,3-glucan-binding affinity should be present at the cell surface of *R. solani* hyphae.

One of the most striking features of the interaction between *C. spirale* ND35 and *R. solani* was the abnormal formation of wall appositions at sites of potential antagonist entry. This is similar to the observations on interaction between *Microsphaeropsis* sp. and *R. solani* made by Carisse et al. (2001). Interestingly, the formation of such structural barriers at strategic sites may reflect a defence reaction elaborated by the pathogen in response to antagonist attack. Such a phenomenon, abundantly described in host plant–pathogen interactions (Benhamou et al., 1994; Benhamou and Lafontaine, 1995; Belanger et al., 2003) and induction of defense-related reaction in plant root by non-pathogenic fungi (Benhamou et al., 1997, 2002; Yedidia et al., 1999; Benhamou and Garand, 2001), but little is known in fungus–fungus interactions. The hemispherical wall appositions at sites of potential antagonist entry in hyphal cells of *R. solani* were intensely labelled by the antibodies of β-1,3-glucan. This suggested that the wall appositions were mainly composed of β-1,3-glucans and might function as defence barriers-like callose in plant cells.

Whatever the role played by the wall appositions, the antagonist revealed the ability to penetrate cells of pathogen, and indicated the potential of *C. spirale* ND35 to produce extracellularly hydrolytic enzymes such as chitinases and β-1,3-glucanase in the process of penetrating into cell walls of pathogen through penetration pegs.

Our earlier investigations demonstrated that at least an exo-β-1,3-glucanase and a chitinase from *C. spirale*, induced by cell wall of *R. solani* ND35 were detected both in culture filtrate and on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). *Chaetomium spirale* ND35 penetrating the host mycelia by partially degrading its cell wall and holes in the penetration site on the host mycelia in dual culture with

V. mali were observed by SEM, it revealed that these CWDEs were involved in mycoparasitic attack on the host, as well as in growth and differentiation of itself. Moreover, it is likely that a coordinated action of chitinases and β-1,3-glucanase is a prerequisite to an effective cell wall disruption of the pathogenic fungi (Gao et al., 2005).

On the contrary, antibiosis might be a key determinant of antifungal activity of *C. spirale* ND35. We conducted extraction and purification of antibiotics from *C. spirale* ND35 in a primary experiment and proved growth inhibition of antibiotics against *V. sordida* and *V. mali* respectively *in vitro* (unpublished data). Schirmböck et al. (1994) confirmed that the synergism between CWDEs and antibiotics was also an important feature of the antagonistic process in the *T. harzianum–B. cinerea* interaction. Compared with the previous results of Elad et al. (1980), Di Pietro et al. (1993) and Schirmböck et al. (1994), we could visualize that both hydrolytic enzymes and antibiotics could be involved in the antagonistic action of *C. spirale* ND35 against *R. solani*, as well as their synergistic action might be much more important, according to the hypothetical model (Lorito et al., 1996) that the degrading of the cell wall by the hydrolytic enzymes should facilitate the rate of diffusion of the antibiotic towards the receptor on the cell membrane. In turn, the antibiotic activity on some membrane-associated functions, such as chitin synthesis, should reduce the synthesis and repairing of the cell wall, thus facilitating the action of the CWDEs. The synergistic interaction between fungal CWDEs and antibiotics need to be further investigated to improve the biocontrol efficacy of the strain *C. spirale* ND35.

In conclusion, our results provide evidence that *C. spirale* ND35 has the potential to become a promising biocontrol agent against a wide range of both soilborne and air-borne phytopathogenic fungi.

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