Oligandrin. A Proteinaceous Molecule Produced by the Mycoparasite *Pythium oligandrum* Induces Resistance to *Phytophthora parasitica* Infection in Tomato Plants[1]

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A low-molecular weight protein, termed oligandrin, was purified to homogeneity from the culture filtrate of the mycoparasitic fungus *Pythium oligandrum*. When applied to decapitated tomato (*Lycopersicon esculentum* Mill. var. Prisca) plants, this protein displayed the ability to induce plant defense reactions that contributed to restrict stem cell invasion by the pathogenic fungus *Phytophthora parasitica*. According to its N-terminal sequence, low-molecular weight, acidic isoelectric point, ultraviolet spectrum, and migration profile, the *P. oligandrum*-produced oligandrin was found to share some similarities with several elicitins from other *Phytophthora* spp. and *Pythium* spp. However, oligandrin did not induce hypersensitive reactions. A significant decrease in disease incidence was monitored in oligandrin-treated plants as compared with water-treated plants. Ultrastructural investigations of the infected tomato stem tissues from non-treated plants showed a rapid colonization of all tissues associated with a marked host cell disorganization. In stems from oligandrin-treated plants, restriction of fungal growth to the outermost tissues and decrease in pathogen viability were the main features of the host-pathogen interaction. Invading fungal cells were markedly damaged at a time when the cellulose component of their cell walls was quite well preserved. Host reactions included the plugging of intercellular spaces as well as the occasional formation of wall appositions at sites of potential pathogen entry. In addition, pathogen ingress in the epidermis was associated with the deposition of an electron-opaque material in most invaded intercellular spaces. This material, lining the primary walls, usually extended toward the inside to form deposits that frequently interacted with the wall of invading hyphae. In the absence of fungal challenge, host reactions were not detected.

In the past two decades, various strategies have been considered by plant pathologists toward enhancing resistance of plants to disease. With the development of more and more pesticide-resistant strains, the replacement of chemicals by the controlled use of alternative agents and/or products has become the focus of considerable interest in the context of a sustainable, economically profitable agriculture. As a consequence, a number of biological approaches have been proposed and much attention has been focused recently on the introduction of alternatives that could be efficient, reliable, and safe for the environment (Chet, 1993; Lyon and Newton, 1997). Among the microbial agents that have shown satisfactory degrees of control against root rot pathogens, *Trichoderma* spp. (Chet, 1993) and fluorescent pseudomonads (Kloepper, 1993) have been reported to reduce disease incidence by inhibiting pathogen growth and development in the rhizosphere and by inducing plant defense reactions (Tuzun and Kloepper, 1995; Benhamou et al., 1996; Yedidia et al., 1999). Another mycoparasite that is receiving increasing attention as a promising biocontrol agent of a number of soilborne plant pathogens is *Pythium oligandrum* Dreschsler (Martin and Loper, 1998). Recent investigations have provided the first conclusive demonstration that, in addition to exerting a strong antagonistic activity against a wide range of fungal pathogens (Benhamou et al., 1999), *P. oligandrum* displayed the ability to penetrate the tomato (*Lycopersicon esculentum* Mill. var. Prisca) root system without inducing extensive cell damage (Rey et al., 1998) and to trigger an array of structural defense-related reactions upon challenge with *Fusarium oxysporum* f.sp. *radicis-lycopersici* (Benhamou et al., 1997). Beside the mycoparasitic activity exerted in the rhizosphere and in planta, the formation of structural and biochemical barriers, which adversely affected pathogen growth and development in the host plant, was found to be a major component of the observed induced resistance.

In spite of extensive research on *P. oligandrum*-mediated induced resistance in tomato plants, the

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exact mechanisms underlying the process of elicitation are not yet understood, although it appears realistic to believe that perception of pathogen signals by the host plant might account for the activation of defense responses. Among the fungal signals that have long been reported to elicit plant defense reactions, oligosaccharides including (1-3,1-6)-β-glucans, chitin, and chitosan oligomers (Côté and Hahn, 1994; Benhamou, 1996), Man-rich glycopeptides (DeWit and Spikman, 1982), phospholipids (Creamer and Bostock, 1986), and/or fatty acids such as the arachidonic acid (Ebel and Scheel, 1992) have all been identified as potential elicitors capable of initiating the cascade of events leading to the activation of plant defense genes (Lyon et al., 1995). In recent years, another class of fungal proteinaceous molecules with signaling properties, the so-called elicitins (Ricci et al., 1989), has attracted much attention because of its ability to induce hypersensitive reactions (HR) as well as systemic acquired resistance against fungal and bacterial pathogens in some plant species including tobacco and radish (Kamoun et al., 1993; Bonnet et al., 1996). Unlike other resistance elicitors, elicitins have been reported to induce systemic resistance via their ability to be readily translocated through the vascular system (Devergne et al., 1992; Zanetti et al., 1992).

The identification and characterization of several elicitin-like proteins in some *Pythium* spp. (Huet et al., 1995; Panabières et al., 1997) recently have led to the concept that production of such molecules was a common feature shared by the fungal genera *Pythium* and *Phytophthora* in the Pythiaceae family. Although the structure of these *Pythium* spp.-produced metabolites has been deeply studied (Panabières et al., 1997), their biological functions in terms of potential induction of plant disease resistance have not been well defined. In an attempt to bring new insights into the mechanisms underlying *P. oligandrum*-mediated induced resistance in tomato (Benhamou et al., 1997), we became interested in finding out whether *P. oligandrum* produced a metabolite similar to those identified in other *Pythium* spp. and whether this metabolite could trigger a resistance response. An experimental model, consisting of decapitated plants infected by *Phytophthora parasitica* (Ricci et al., 1989), was chosen to investigate the potential effect of the *P. oligandrum* metabolite on the rate and extent of tomato plant colonization.

Our results provide the first conclusive evidence that *P. oligandrum* secretes a low-molecular mass protein, termed oligandrin, which induces resistance against *P. parasitica* in tomato. Data are presented demonstrating that treatment with the oligandrin triggers ultrastructural and biochemical modifications in tomato stem cells, and that such changes correlate with marked alterations of the invading fungal cells. To our knowledge, this is the first detailed report on the cytologically visible consequences induced by a protein of fungal origin in tomato.

RESULTS

Oligandrin Purification

The purification procedure (including the HPLC steps) used in this study allowed the recovery of large amounts of oligandrin in a pure form from the culture filtrates of *P. oligandrum*. The HPLC profile of a crude filtrate of *P. oligandrum* revealed the presence of one major peak at retention time of 10.2 min (Fig. 1a). After ion-exchange chromatography and reverse-phase liquid chromatography, a purified protein termed oligandrin was visualized by HPLC (Fig. 1b). Crude culture filtrate of *P. oligandrum* was found to contain 53 mg/L of oligandrin. The protein was purified to homogeneity as demonstrated by SDS-PAGE (Fig. 2). Analysis of the electrophoretic pattern revealed that the oligandrin exhibited a molecular mass of approximately 10 kD. Oligandrin behaves as an

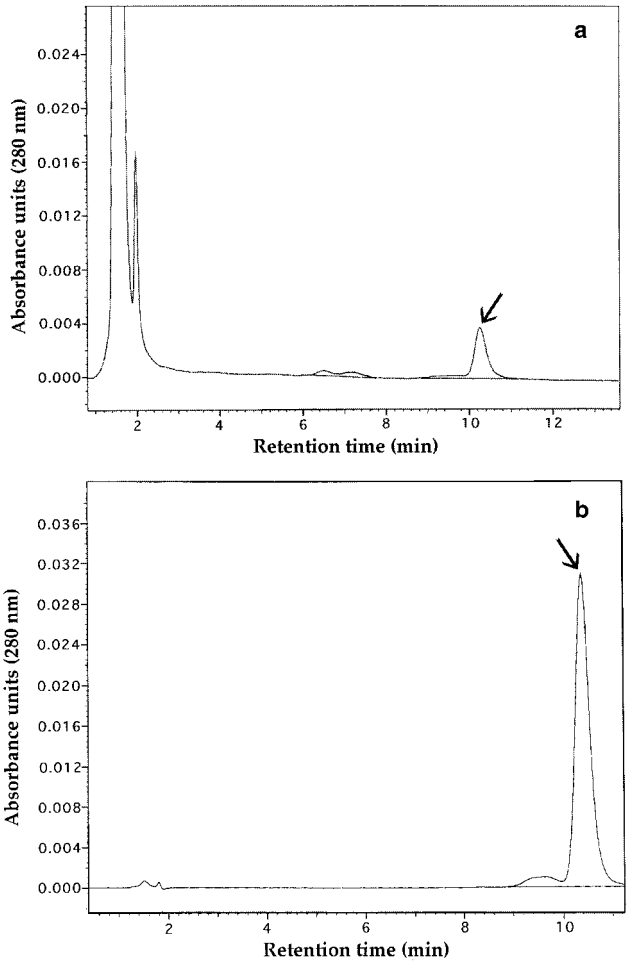

Figure 1. Analytical HPLC profiles of *P. oligandrum* crude filtrates and of the purified oligandrin. a, Crude culture filtrate of *P. oligandrum* (arrow). b, Purified oligandrin eluting at 40% CH_3CN (arrow).

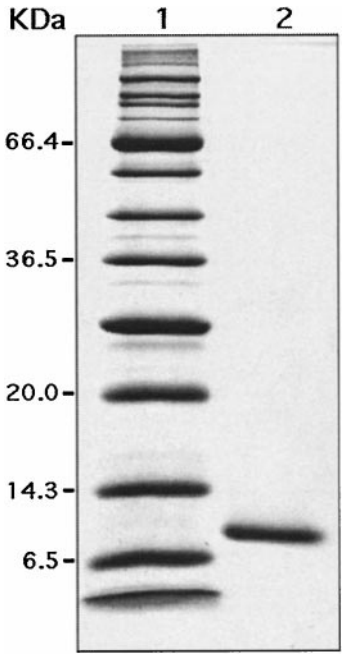

Figure 2. Electrophoretic profile of the oligandrin after SDS-PAGE. One single-stained band (lane 2) with a molecular mass of about 10 kD, as compared with molecular mass markers (lane 1), is detected.

acidic protein as evidenced by ion-exchange chromatography (pI of about 4.5) and analysis of its UV spectrum allowed to exclude Trp and to identify Tyr and Phe (data not shown).

Amino Acid Sequencing of Oligandrin

The N-terminal sequence of the 10-kD protein was determined up to Leu-39; its alignment with sequences from 13 elicitins secreted by some *Phytophthora* sp. and *Pythium* sp. is illustrated in Figure 3. Analysis of the oligandrin sequence revealed that Thr and Ser accounted for about 30% of the amino acids whereas Trp, His, and Arg were missing (Fig. 3a). WU-BLASTp under standard settings gave significant homology with the elicitins from *Phytophthora* and *Pythium* species. The percent match between these sequences reached nearly 50% including identical and strongly similar residues (Fig. 3b). However, in spite of such similarities, significant differences were observed between oligandrin and the known elicitins as illustrated by the gaps introduced in the ClustalW multiple alignment (version 2.0), thus indicating that oligandrin might be considered as an elicitin-like protein harboring original features.

Antifungal Potential of Oligandrin

Observation of mycelial samples exposed to sterile distilled water showed the presence of typical hy-

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                5                  10                  15                  20
Ala Thr Cys Thr Asp Glu Gln Phe Ser Asp Ser Ile Ile Lys Leu Thr Pro Ala Ile Gly

                25                  30                  35                  40
Ser Val Ser Gly Cys Thr Ala Asp Ser Gly Phe Thr Met Ile Pro Pro Thr Gly Leu
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a

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                    10         20         30         40
                     |          |          |          |
Cry       TACTATQQTAAYKTLVSILSDASFNQCSTDSGYSMLTAKAL
Cin-b     TACTATQQTAAYKTLVSILSESSFSQCSKDSGYSMLTATAL
Dre-b     TACTSTQQTAAYTTLVSILSDSSFNKCASDSGYSMLTAKAL
Meg-b     TACTTTQQTAAYKTLVSILSESSFNQCSKDSGYSMLTATAL
Cap       ATCTTTQQTAAYVALVSILSDSSFNQCATDSGYSMLTATAL
Par       TTCTTTQQTAAYVALVSILSDTSFNQCSTDSGYSMLTATSL
Cac       ATCTSSQQTAAYVALVSILSDTSFNQCSTDSGYSMLTATSL
Cin-a     TTCTSTQQTAAYVALVSILSDSSFSQCATDSGYSMLTATSL
Dre-a     TTCTSTQQTAAYVTLVSILSDSSFNQCATDSGYSMLTATAL
Meg-a     TTCTSTQQTAAYVTLVSILSDSSFNQCATDSGYSMLTATAL
Inf       TTCTTSQQTVAYVALVSILSDTSFNQCSTDSGYSMLTATSL
Vex1      TACTTTQQTAAYVALVSILSDDNFSQCSTDSGYSMLTATAL
Vex2      TACTTTQQTAAFVALVSVLSTDNFNQCSTDSGYSMLTATAL
Olig      ATCTDEQFSDSIIKLTPAIG--SVSGCTADSGFTMIPPTGL

Align     ::**  * : :   *.. :.  ... *: ***::*:....*
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b

Figure 3. Comparison of the N-terminal sequence of oligandrin with typical elicitins from *Phytophthora* spp. and *Pythium* spp. a, N-terminal sequence of oligandrin determined both from native and reduced-alkylated protein. b, Clustal W multiple alignment of oligandrin with 13 typical elicitins from *Phytophthora* spp. and *Pythium* spp. Align: asterisk (*), identity (10 residues, 24.4%); colon (:), strongly similar (nine residues, 21.9%); period (.), weakly similar (10 residues, 24.4%). *Cry*, Basic cryptogein from *Phytophthora cryptogea* (Ricci et al., 1989). *Cin-b*, Basic cinnamomin from *Phytophthora cinnamomi* (Huet and Pernollet, 1989). *Dre-b*, Basic drechslerin from *Phytophthora drechsleri* (Huet et al., 1992). *Meg-b*, Basic megaspermin from *Phytophthora megasperma* (Huet and Pernollet, 1993). *Cap*, Acidic capsicein from *Phytophthora capsici* (Ricci et al., 1989). *Par*, Acidic parasiticein from *P. parasitica* (Mouton-Perronnet et al., 1995). *Cac*, Acidic cactorein from *Phytophthora cactorum* (Huet et al., 1993). *Cin-a*, Acidic cinnamomin from *P. cinnamomi* (Perez et al., 1999). *Dre-a*, Acidic drechslerin from *P. drechsleri* (Huet et al., 1992). *Meg-a*, Acidic megaspermin from *P. megasperma* (Huet and Pernollet, 1993). *Inf*, Acidic infestin from *Phytophthora infestans* (Huet et al., 1994). *Vex1* and *Vex2*, Acidic vexins from *Pythium vexans* (Huet et al., 1995). *Olig*, Oligandrin from *P. oligandrum* (present work).

phae mainly characterized by a dense cytoplasm containing numerous organelles and a large number of vacuoles (Fig. 4a). Exposure of samples to purified oligandrin at 5, 15, and 30 μg/mL for 1 to 4 h did not result in any significant morphological and ultrastructural alteration. Prolonged incubation (12 h) with the oligandrin at the highest concentration did not affect cell integrity as evidenced by the occurrence of regularly shaped hyphae in which the plasma membrane was not retracted from the cell wall and the cytoplasm appeared metabolically active (Fig. 4b).

Symptomatology

Decapitated tomato plants, treated with the purified oligandrin or non-treated, were inoculated with *P. parasitica*, strain 149, to determine their susceptibility to fungal attack (Fig. 5, arrowheads). Typical disease symptoms, mainly characterized by the formation of enlarged brownish lesions at the sites of fungal contact, were visible by 4 d after inoculation in control plants. Between 6 and 7 d post-inoculation, these plants showed severe symptoms of wilting (Fig. 5a) and about 30% of the plants were dead by 7 to 8 d after fungal inoculation. Noninfected, oligandrin-treated tomato plants showed no symptoms of phytotoxicity during the course of the experiment.

Treatment with the oligandrin at a concentration of 3 nM resulted in much less disease development than occurred in non-treated tomato plants (Fig. 5b). By 7 d after fungal inoculation, treated plants were free of apparent symptoms such as wilting or senescence and exhibited a reduced number of stem lesions (Fig. 5b). Although some lesions could be seen in areas where the fungus was applied (Fig. 5d, arrow), the extent of these lesions never reached levels similar to those observed in control plants (Fig. 5c). Oligandrin-treated, decapitated tomato plants did not exhibit necrotic features typically associated with HR. Necrotic spots similarly were not seen when the oligandrin, at all concentrations tested, was introduced by direct infiltration into the leaves through the abaxial epidermal layer. Oligandrin treatment significantly reduced disease severity provoked by *P. parasitica* in tomato and striking differences in plant mortality were observed. At the end of the experimental period, treatment with the oligandrin reduced plant mortality by more than 60% (Table I).

Oligandrin Migration

To determine whether the oligandrin could be translocated throughout the plant in a way similar to the *Phytophthora* sp. elicitins (Devergne et al., 1992; Zanetti et al., 1992), radioiodinated oligandrin was applied to tomato plants on either the decapitated apex (Fig. 6a, arrow) or the wounded petiole (Fig. 6c, arrow). Three hours after treatment, examination by autoradiography revealed that radioactivity could be detected not only in the area of elicitin application but also at a distance from it (Fig. 6, b and d). Although a great amount of the radioactivity remained at the application site (Fig. 6, b and d, arrows), a signal was observed at some distance in both the stem and the leaves. In the leaves, radioactivity was

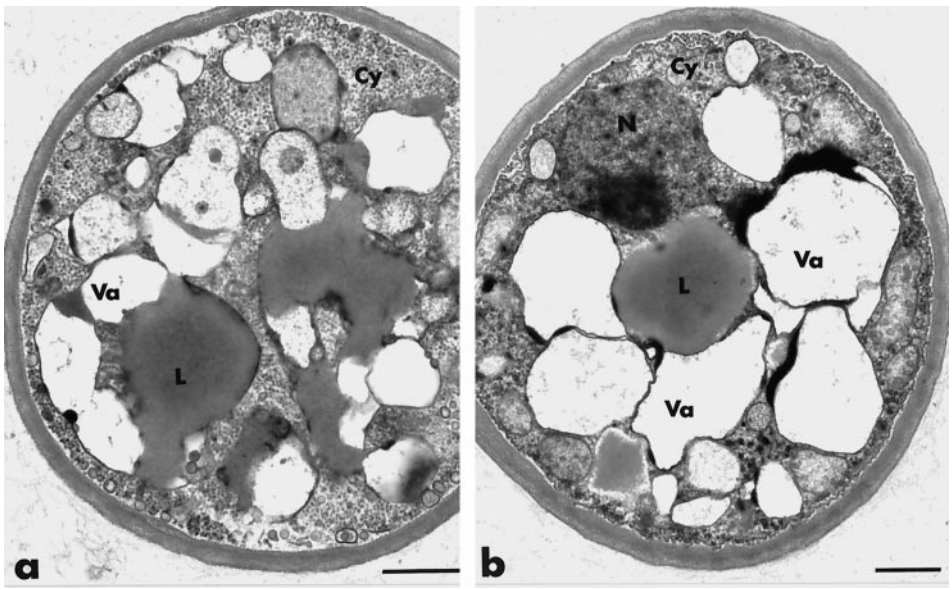

Figure 4. Antifungal potential of the oligandrin. a, *P. parasitica* hyphae exposed to sterile distilled water. A typical hyphal cell mainly characterized by a dense cytoplasm (Cy) containing numerous organelles such as lipid bodies (L) and a large number of vacuoles (Va). Bar = 0.5 μm. b, *P. parasitica* hyphae exposed to oligandrin at 30 μg/mL. No morphological or structural alterations are visible. The hyphal cell is similar to that shown in a with cytoplasm in which organelles including the nucleus (N), lipid bodies, and vacuoles are noticed. Bar = 0.5 μm.

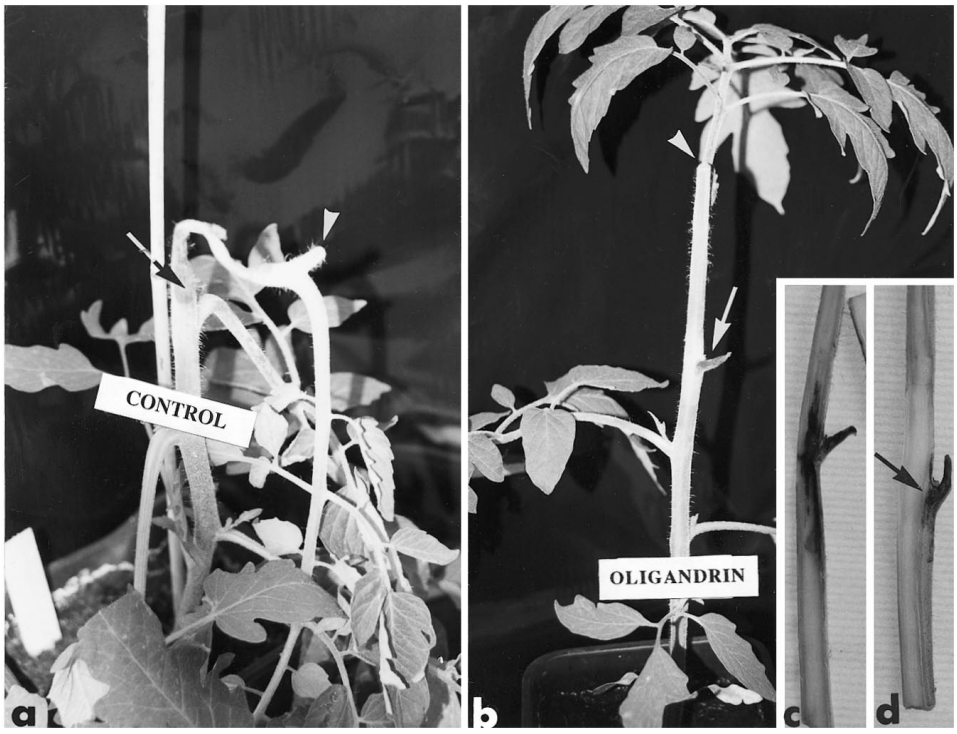

Figure 5. Effect of oligandrin treatment on the expression of symptoms in tomato plants infected by *P. parasitica*. Oligandrin was applied onto the decapitated apex (arrows) and fungal inoculation was performed on the section of the leaf petiole in position 2 (arrowheads). a, In the absence of oligandrin treatment, tomato plants exhibit severe symptoms of wilting by 7 d after inoculation. b, Upon oligandrin treatment, tomato plants appear free of apparent symptoms, except in the area of fungal inoculation (arrow). c and d, Effect of oligandrin treatment on the stem of tomato plants upon slicing off the outer stem layers. The extent of the lesion induced by *P. parasitica* is much reduced in the oligandrin-treated tomato plant (d, arrow) than in the control, non-treated plant (c).

intense in the veins (Fig. 6b, arrowhead) and apparently diffused in the lamina (Fig. 6b, double arrowheads). This profile of migration suggests that oligandrin is translocated in tomato through the vascular system as previously reported for other elicitins in tobacco (Devergne et al., 1992; Zanetti et al., 1992).

Histological Observations of *P. parasitica*-Infected Tomato Stem Tissues

Observations of transversally sectioned stem samples from non-treated plants that were inoculated with *P. parasitica* showed that all tissues were massively invaded by hyphae of the pathogen (Fig. 7a). By 7 d after fungal inoculation, stem tissues were intensely colonized as evidenced by the occurrence of pathogen hyphae through much of the epidermis, the cortex, the endodermis, the paratracheal parenchyma cells, and occasionally the vascular stele. Fungal growth was mainly intercellular (Fig. 7a, arrow) but it could also occur intracellularly. Pathogen ingress toward the vascular stele coincided with extensive cell disorganization as judged by marked alterations

Table I. *Effect of oligandrin treatment on symptom expression in tomato plants infected by Phytophthora parasitica*

Decapitated tomato plants were treated with 30 μL of oligandrin solution (3 nmol/plant) and inoculated simultaneously with *P. parasitica* on the section of the leaf petiole in position 2 beneath.

Oligandrin Treatment	Disease Severity[a]			Stem Invasion[b]	Protection[c]
	I	II	III		
nmol	*no. of plants*[d]			cm^3	%
0 (control)	6	26	13	0.75 ± 0.72	–
3000	19	23	3	0.30 ± 0.35	60

[a] Disease index was as follows: I, Near absence of stem lesions; II, small lesions restricted to specific stem tissues including the epidermis and the cortex; III, enlarged stem lesions. [b] Volume of stem tissues showing symptoms (lesions) of fungal invasion. Values are standard errors of the mean. [c] Relative restriction of stem invasion as compared to controls. [d] Disease severity as estimated on 15 tomato plants per treatment in triplicate.

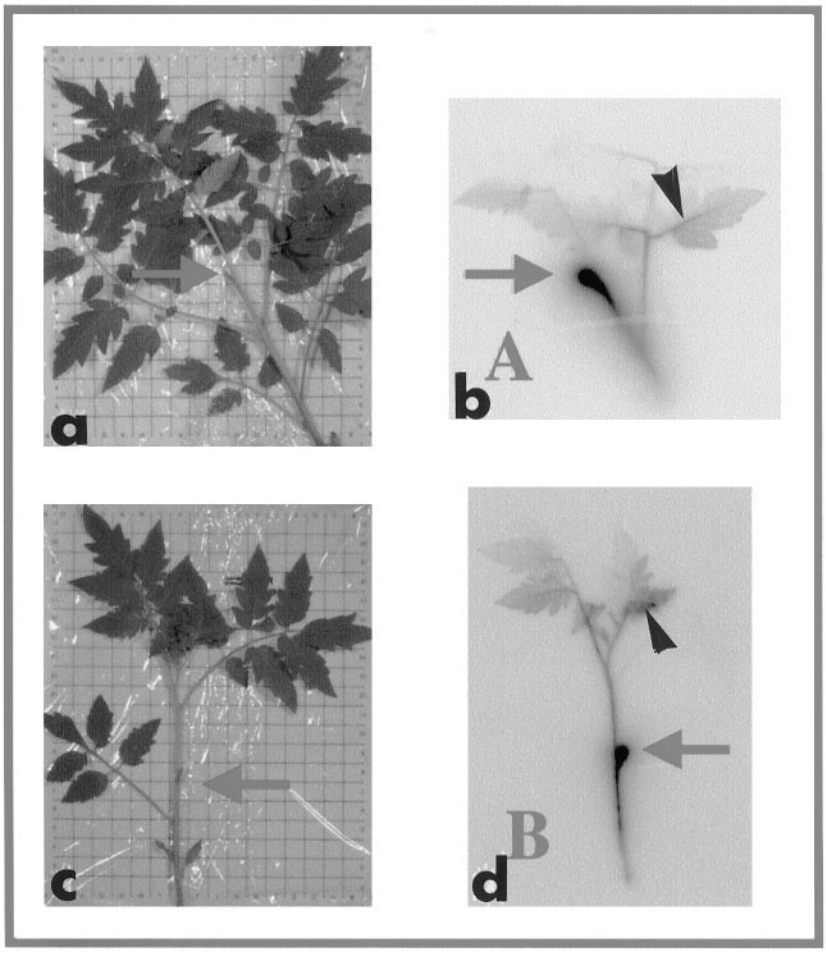

Figure 6. Migration of radioiodinated oligandrin in tomato plants. Three nanomoles of [^{125}I]oligandrin (specific activity = 1.5 Ci/mmol) was applied to tomato plants either onto the decapitated apex (a and b, arrows) or on the wounded petiole (c and d, arrows). Three hours after [^{125}I]oligandrin treatment, migration of radioiodinated oligandrin was detected by autoradiography (b and d).

of the cytoplasm, which was frequently reduced to aggregated remnants (Fig. 7a, double arrows). Host reactions such as wall appositions, intercellular plugging, and xylem vessel coating were not detected. In all cases, the observed pattern of fungal colonization and host cell disorganization coincided with the occurrence of macroscopically visible symptoms along the stems, leading to severe plant wilting and eventually plant death.

Examination of stem sections from oligandrin-treated plants that were challenged with *P. parasitica*, either on the same day or 24 h later, showed that fungal colonization occurred, but to a much lesser extent than in non-treated, inoculated plants (Fig. 7b). Fungal growth was mainly restricted to the outermost host cell layers and occurred only in some intercellular spaces. Invading hyphae appeared to be severely damaged as evidenced by morphological changes (Fig. 7, b and d, arrows) and increased staining density with toluidine blue. Pathogen penetration in the outer host cell layers always coincided with host cell changes mainly characterized by the elaboration of structural barriers in the regions proximal to potential fungal penetration (Fig. 7, c–f). A typical host reaction was the deposition of an amorphous material in some infected cells. Hyphae, trapped in this material, were apparently immobilized (Fig. 7c). Other typical features of the host response included the formation of small wall appositions at the sites of potential pathogen penetration (Fig. 7e) and the plugging of most intercellular spaces with a material that stained densely with toluidine blue (Fig. 7f). Such host reactions were never seen in the uncolonized tissues beneath the invaded cell layers.

Ultrastructural Features of Noninfected Tomato Stem Tissues

Examination of stem samples from oligandrin-treated tomato plants that were not challenged with *P. parasitica* showed a high preservation of the host cell integrity in all tissues. Host cells in the epidermis and the cortex were characterized by the occurrence of enlarged vacuoles and by a layer of dense cytoplasm appressed against the cell wall (not shown). Such host cells resembled those observed in non-treated tissues and typical host responses such as formation of wall appositions, intercellular space plugging, and vessel coating were never detected.

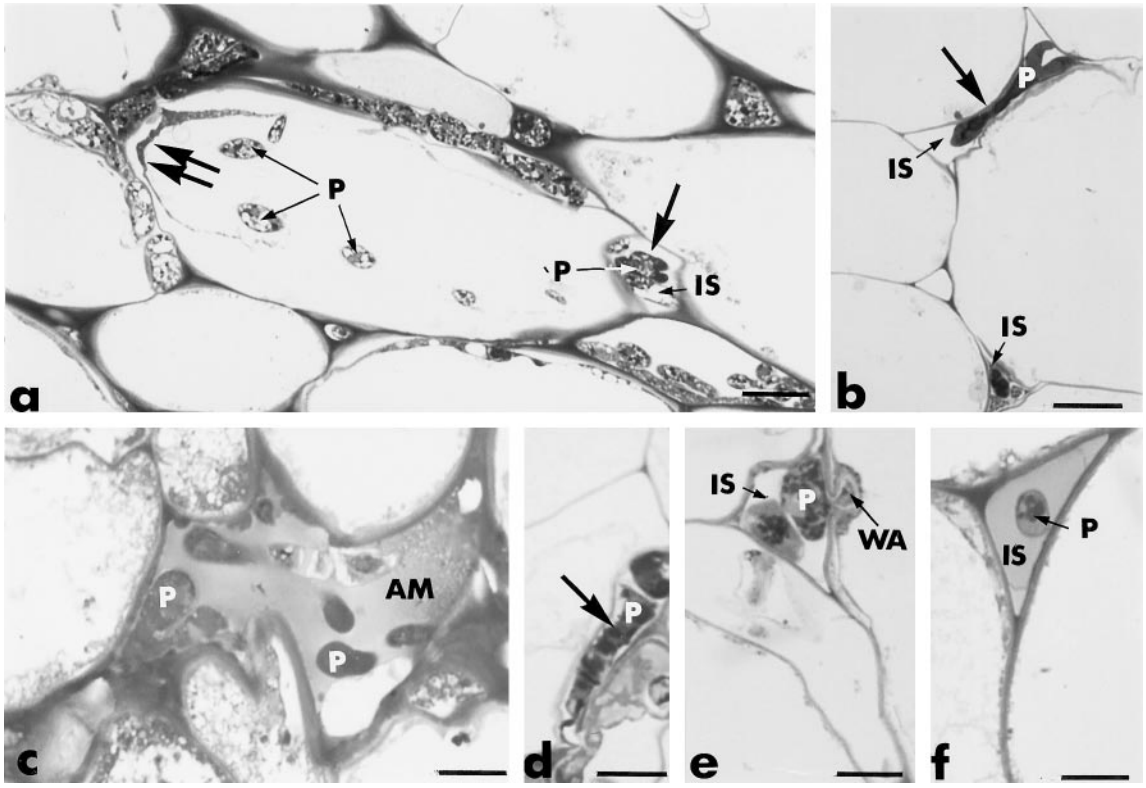

Figure 7. Light micrographs of stem samples from control (a) and oligandrin-treated tomato plants (b–f). a, Samples from oligandrin-free (control) tomato stems, collected 7 d after inoculation with *P. parasitica.* Hyphae of the pathogen (P) multiply abundantly in all tissues. Fungal growth occurs intra- and intercellularly (arrow). Pathogen invasion coincides with marked cytoplasm alterations (double arrows). IS, Intercellular space. Bar = 10 μm. b through f, Samples from oligandrin-treated tomato stems, collected 7 d after inoculation with *P. parasitica.* Fungal growth is mainly restricted to the outermost host cell layers and occurs only in some intercellular spaces. Invading hyphae appear severely damaged (b and d, arrows). Wall appositions (WA) are seen in the regions proximal to potential fungal penetration (e). An amorphous material (AM) accumulates in some infected cells. Hyphae of the pathogen (P), trapped in this material, are apparently immobilized (c). An intercellular space is plugged with a material that stains densely with toluidine blue (f).

Ultrastructural Features of *P. parasitica*-Infected Tomato Stem Tissues

Non-Treated Tomato Plants

In the absence of oligandrin treatment, inoculation with *P. parasitica* resulted in an intense fungal development in nearly all tissues except for the xylem vessels, which were seldom colonized (Fig. 8). Pathogen growth was mainly intercellular (Fig. 8b) but it could also occur intracellularly through direct host wall penetration, which was achieved by means of constricted hyphae (Fig. 8c). It is surprising that such a massive fungal invasion did not result in marked host wall alterations as estimated by the pattern of gold labeling following incubation of sections with the gold-complexed exoglucanase (Fig. 8, b and c). Gold particles were regularly distributed over the host cell walls, even in the areas closely adjacent to the channels of fungal penetration (Fig. 8c, arrows). In contrast, the host cytoplasm was markedly altered and usually reduced to fibrillar and vesicular remnants (Fig. 8c, arrowheads). Fungal cell walls were also evenly labeled by the gold-complexed exoglucanase (Fig. 8c). In these control tomato plants, pathogen invasion failed to stimulate host reactions such as wall appositions, intracellular deposits, intercellular plugging, and xylem vessel occlusions (Fig. 8d).

Oligandrin-Treated Tomato Plants

In oligandrin-treated tomato plants, the pattern of stem colonization by *P. parasitica* differed markedly from that observed in control plants no matter what the timing of fungal inoculation (Fig. 9). Although extensive fungal multiplication was seen at the stem surface (Fig. 9a), fungal growth in planta was mainly restricted to the outermost cell layers, including the epidermis and the outer cortex. Hyphae of the pathogen were seldom seen in the inner tissues and they were never detected in the endodermis or the vascular stele. One of the most striking changes observed in oligandrin-treated plants as compared with controls was the obvious alteration of most fungal cells, the cytoplasm of which appeared either highly disorganized (Fig. 9b) or aggregated and filled with

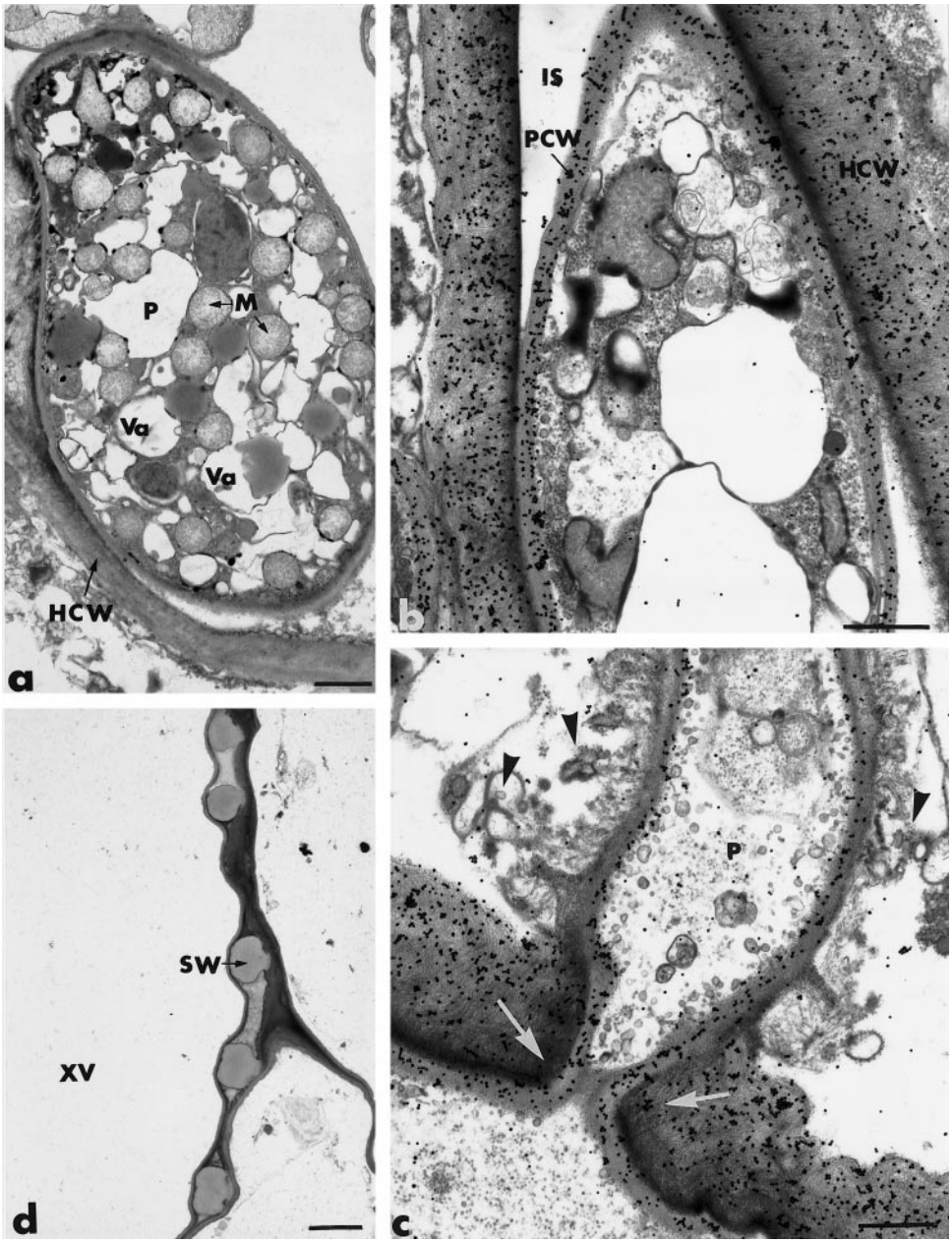

Figure 8. Transmission electron micrographs of *P. parasitica*-infected tomato stem tissues (control), collected 7 d after inoculation. In a through c, Pathogen (P) growth occurs intra- (a) and intercellularly (b). *P. parasitica* hyphae appear metabolically active as judged by the presence of numerous mitochondria (M) and vacuoles (Va). Direct host wall penetration is achieved by means of constricted hyphae (c). Labeling of cellulose with the gold-complexed exoglucanase shows that gold particles are regularly distributed over the host cell walls (HCW), even in the areas closely adjacent to the channels of fungal penetration (c, arrows). The *P. parasitica* cell wall (PCW) is also evenly labeled (b). The host cytoplasm is reduced to fibrillar and vesicular remnants (c, arrowheads). a, bar = 1 μm; b and c, bar = 0.5 μm. d, In uninvaded xylem vessels (XV), defense reactions such as secondary wall (SW) coating are not seen. Bar = 3 μm.

electron-dense, polymorphic inclusions (Fig. 9c). A number of hyphal cells (about 20%) showed various degrees of alteration including distortion, retraction, and even breakdown of the plasma membrane (Fig. 9b, arrowheads) as well as pronounced degenerescence of the cytoplasm in which typical organelles such as the nucleus and mitochondria were no longer discernible (Fig. 9b). Most of the other invading hyphae were characterized by an apparent densification of the cytoplasm associated with the formation of enlarged, osmiophilic inclusions, which extended in most of the space initially occupied by the cytoplasm (Fig. 9c). Attempts of host cell wall penetration by morphologically and structurally altered hyphae of

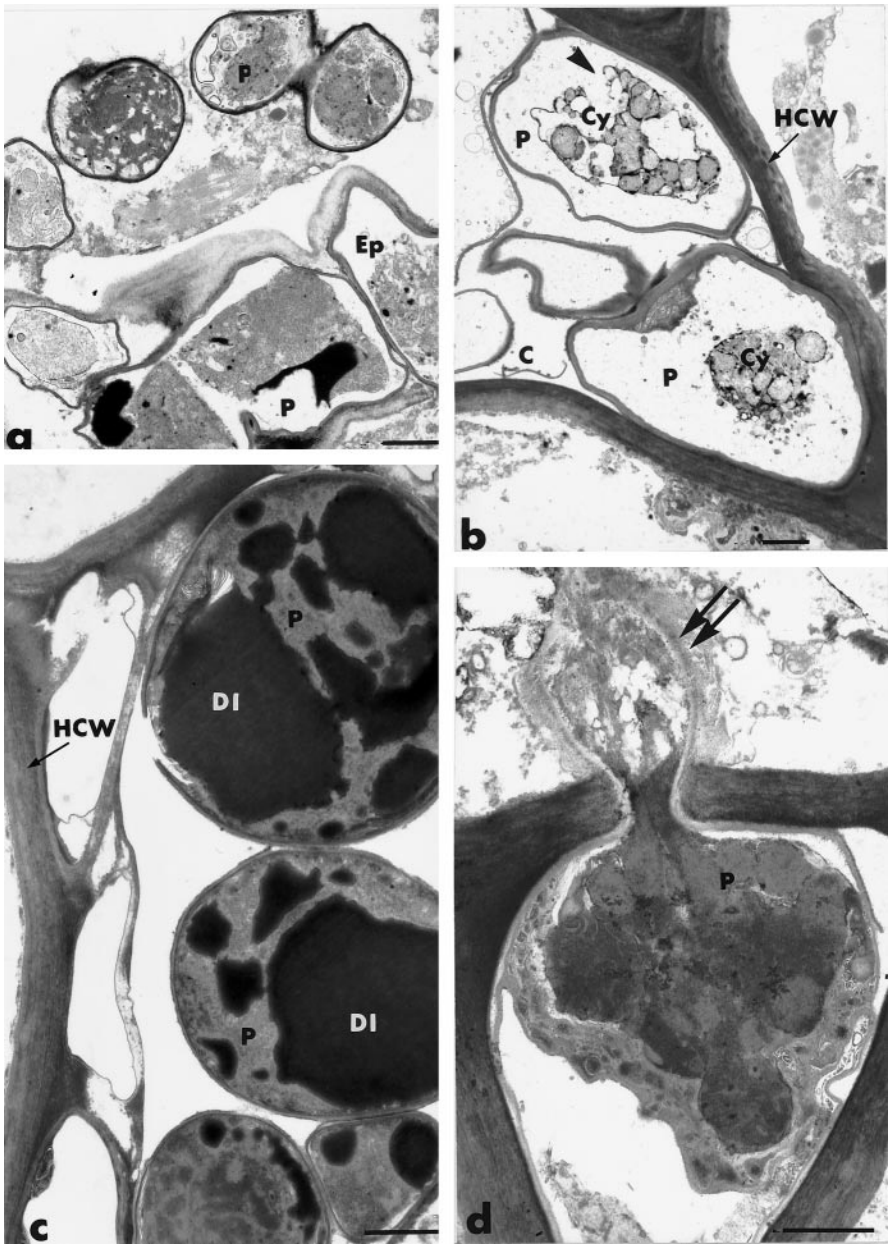

Figure 9. Transmission electron micrographs of stem tissue from decapitated tomato plants treated with the oligandrin and collected 7 d after inoculation with *P. parasitica*. In a through c, although extensive fungal multiplication is seen at the stem surface (a), fungal growth in planta is mainly restricted to the outermost cell layers including the epidermis (EP) and the outer cortex (C). Hyphae of the pathogen (P) are highly disorganized as evidenced by their cytoplasm (Cy), which is either reduced to vesicular and fibrillar fragments (b, arrowhead) or filled with dense inclusions (DI) (c). The host cell wall (HCW) is apparently well preserved. Bar = 1 μm. d, Attempts of host cell wall penetration by morphologically and structurally altered hyphae of the pathogen (P) are occasionally seen. An amorphous material is deposited around the penetrated hyphal portion (double arrows). Bar = 1 μm.

the pathogen were occasionally seen (Fig. 9d). An amorphous material was found to accumulate along the penetration peg (Fig. 9d, double arrows). Although apparently intact, fungal cell walls were, in most cases, distorted and circumvoluted (Fig. 10a). The dense cytoplasmic matrix in which organelles were no longer visible was found to undergo gradual dissolution to be reduced finally to fragments in which vesicular structures and membrane remnants could be seen (Fig. 10b, arrowheads). Upon incubation with the gold-complexed exoglucanase, a regular deposition was detected over the walls of damaged hyphae of *P. parasitica* (Fig. 10b).

Beside fungal cell alterations, treatment with the oligandrin triggered the elaboration of several host reactions (Figs. 10, c and d, and 11). Pathogen ingress

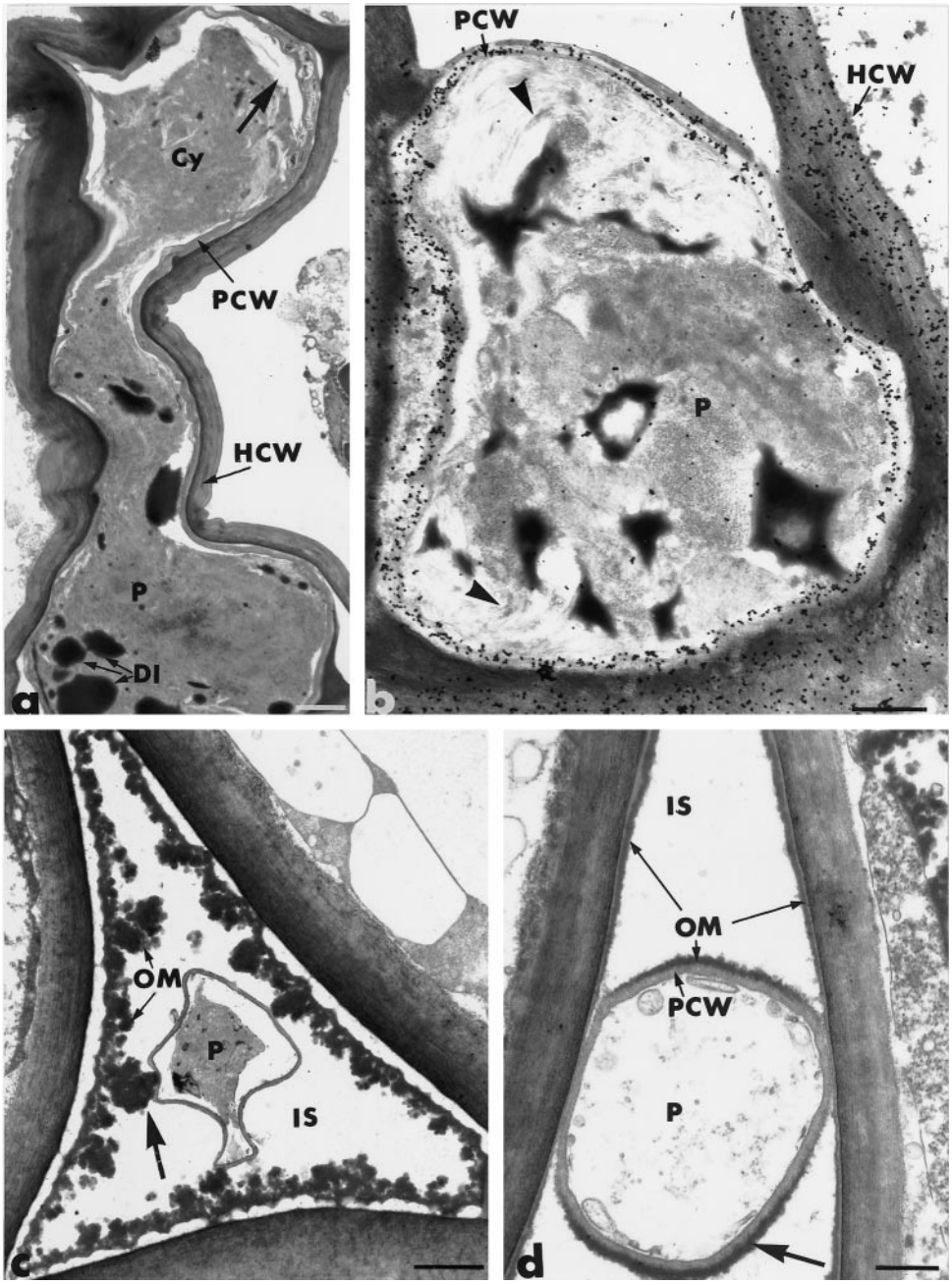

Figure 10. Transmission electron micrographs of stem tissue from decapitated tomato plants treated with the oligandrin and collected 7 d after inoculation with *P. parasitica*. a, The fungal cell wall (PCW) is distorted and circumvoluted. The dense cytoplasmic (Cy) matrix shows various degrees of disorganization to finally be reduced to small fragments in which vesicular structures and membrane remnants are seen (c, arrowhead). The dense inclusions (DI) are the only fungal structure discernible in these highly altered hyphae. Bar = 1 μm. b, Upon incubation with the gold-complexed exoglucanase, a quite regular deposition of gold particles is seen over the walls (PCW) of damaged hyphae of *P. parasitica*. The host cell wall (HCW) is evenly labeled. The host cytoplasm is highly degraded and reduced to membrane fragments in places. Bar = 0.5 μm. c and d, Host defense reactions elaborated in response to pathogen invasion include the deposition in some intercellular spaces (IS) of an electron-opaque material (OM), which extends toward the inside to form polymorphic deposits that interact with the pathogen (P) cell wall (c) and form a coating band at the fungal cell surface (d, arrow). c, Bar = 1 μm; d, bar = 0.5 μm.

in the epidermis was associated with the deposition of either an electron-opaque material (Fig. 10c) or a fibrillar network (not shown) in most invaded intercellular spaces. The osmiophilic material lining the primary walls usually extended toward the inside to form polymorphic deposits that frequently interacted

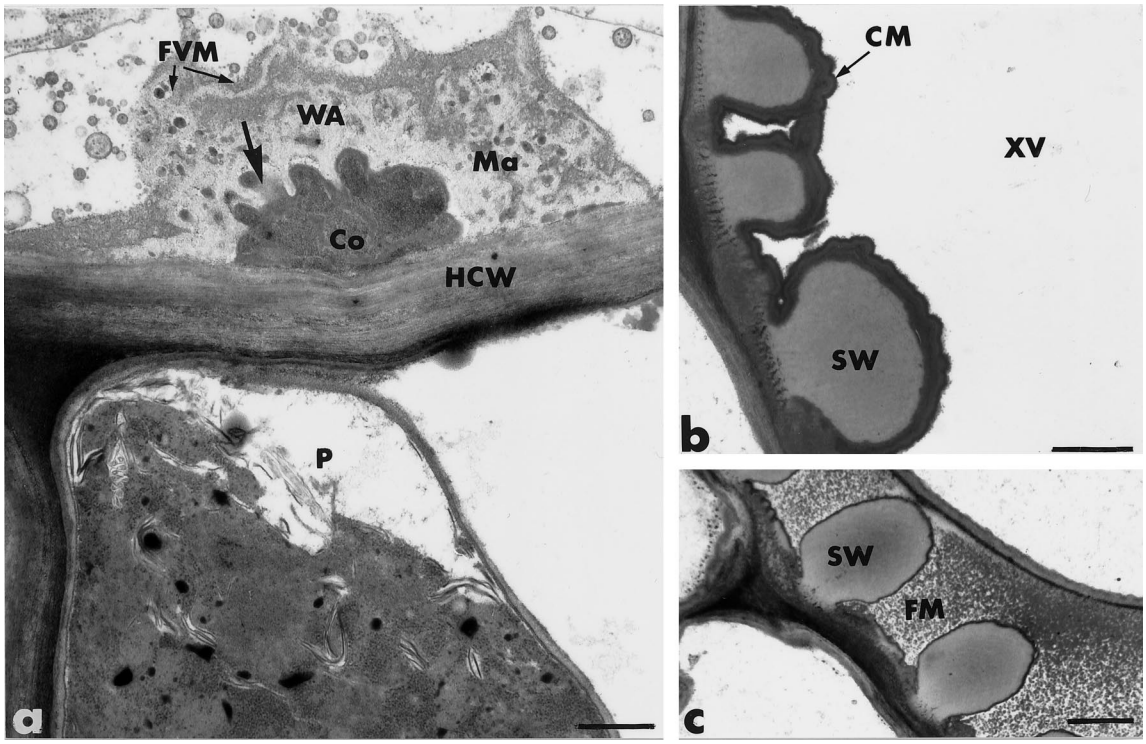

Figure 11. Transmission electron micrographs of stem tissue from decapitated tomato plants treated with the oligandrin and collected 7 d after inoculation with *P. parasitica*. a, A heterogeneous wall apposition (WA), made of an amorphous matrix (Ma) and containing a central multilobed core (Co) is formed at a site of potential pathogen (P) penetration. It is delimited by a fine fibrillo-vesicular material (FVM). Bar = 0.5 μm. b and c, Host reactions including the coating of secondary walls (SW) with a band of osmiophilic material (CM) (b) or deposition of an electron-opaque, fibrillar material (FM) (c) are seen in noninvaded xylem vessels (XV). b, Bar = 1 μm; c, bar = 2 μm.

with the wall of invading hyphae (Fig. 10c, arrow) and occasionally formed a coating band at the fungal cell surface (Fig. 10d, arrow). Another feature, occasionally seen in reacting host cells, was the formation of multitextured wall appositions at sites of potential fungal penetration (Fig. 11a). These appositions, which could vary greatly in size, shape, and texture, were usually found to be made of an amorphous matrix that was impregnated by osmiophilic substances and was delimited by a loosely arranged layer of fine fibrillo-vesicular material (Fig. 11a). The core of wall appositions was frequently made of osmiophilic aggregates that formed short finger-like projections in the amorphous matrix (Fig. 11a, arrow). The host cell wall itself displayed a higher electron density than normal, thus indicating the probable infiltration of structural molecules. Both the impregnated host cell wall and the wall appositions were efficient in preventing fungal ingress since successful hyphal penetration of these structures was not observed. It is interesting that host reactions were detected in noninvaded xylem vessels. The host reactions were mainly characterized by the coating of secondary walls with a band of osmiophilic material (Fig. 11b) or the deposition of an electron-opaque, fibrillar material in the vessel lumen (Fig. 11c). Control tests, including pre-incubation of the exoglucanase-gold complex with β-1,4-glucans prior to section labeling, resulted in the absence of labeling over both the cell walls and the wall appositions (not shown).

DISCUSSION

We recently reported that tomato plants, when preinoculated with the aggressive mycoparasite *P. oligandrum*, gained increased protection against crown and root rot caused by *F. oxysporum* f.sp. *radicis-lycopersici* (Benhamou et al., 1997). In direct line with these earlier observations, attempts were made in the present study to delineate the mechanisms by which *P. oligandrum* could exert its beneficial effects on tomato plants. Evidence is presented in this paper that *P. oligandrum* secretes a proteinaceous metabolite that displays the ability to operate as an elicitor of resistance. Although the three-dimensional structure and the biophysical properties of this small, water-soluble molecule need to be further characterized, the present results support the view that this metabolite, termed oligandrin, shares some similarities with the elicitins identified in some *Phytophthora* spp. and *Pythium* spp. (Huet et al., 1994, 1995; Panabières et al., 1997; Ponchet et al., 1999). Among the criteria so far identified for the assignment of a given protein to

the elicitin family is the amino acid composition of the N-terminal end. In that context, the absence of Trp, His, and Arg residues and the relative abundance of Thr and Ser residues in the oligandrin terminal end are key characteristics that define an "elicitin signature" (Ponchet et al., 1999). The lack of Trp, which was confirmed by the UV absorption spectrum (not shown), the low molecular mass (10 kD, about 100 amino acids), and the migration profile within the plant also provide a helpful signature for including the oligandrin into the elicitin family. In spite of those similarities, significant differences characterize the oligandrin with respect to its biological activity. The major difference probably relies on the finding that oligandrin infiltration into tomato leaves failed to provoke the HR-associated necrotic response, a reaction consistently found to occur in tobacco plants treated with true elicitins (Ricci, 1997; Ponchet et al., 1999). By contrast, a high level of protection against the oomycete fungus, *P. parasitica*, was noticed, thus substantiating the concept that the oligandrin can be considered as a resistance elicitor. Although the reasons why the oligandrin failed to mediate HR in tomato plants are still unknown, one possibility could be the trapping of some oligandrin molecules by components of the tomato cell wall matrix. In line with this concept, recent investigations have disclosed that effective binding of the elicitins from *Phytophthora* species to membrane receptors required higher concentrations of proteins in tomato than in tobacco, mainly because a large number of molecules were trapped in the tomato cell wall, thus preventing sufficient access of the elicitins to their target receptors (Ponchet et al., 1999). In that context, one might consider that the tomato cell wall acts as a filter that controls oligandrin diffusion in such a way that membrane-bound receptors are not fully saturated. Although it is clear that the early events involved in the recognition of oligandrin molecules by tomato cells must be more fully investigated, the finding that resistance was expressed upon oligandrin treatment suggests that the receptors involved in the specific oligandrin signaling pathway are functional.

Evidence is provided from the present ultrastructural study that oligandrin-treated tomato plants afford increased protection against *P. parasitica* and that this protection is at least partly associated with a reduction in pathogen biomass and an increase in hyphal structural alterations. To our knowledge, this is the first report about the effect of a protein of fungal origin on the cytology of pathogen colonization in a host plant. A decrease in the amount of fungal cell colonization and pathogen viability, as illustrated by the frequent occurrence of modified and/or highly damaged fungal cells, were typical features of reactions observed in oligandrin-treated tomato plants only. Whether such alterations are attributable to the creation of a fungitoxic environment associated with the synthesis and accumulation of antimicrobial compounds by the reacting host cells or simply relate to a direct antifungal effect of the oligandrin in planta deserves to be biochemically investigated.

If one considers that the oligandrin is mobile within the plant, as evidenced by the migration profile, then it seems realistic to believe that a direct fungitoxic effect might account for the observed fungal damage. However, the observation that exposure of *P. parasitica* hyphae to pure oligandrin did not lead, under our conditions, to structural alterations similar to those detected in planta (see Fig. 4) tends to suggest that the oligandrin has no fungicidal activity against *P. parasitica*. Such a conclusion should, however, be viewed with caution since the possibility that the molecule might have undergone structural alterations once in the plant needs to be considered. Extraction and structural characterization of [I^{125}]oligandrin from tomato leaves as well as bioassays would answer the question as to what extent the oligandrin can be converted into a fungitoxic compound that might operate against the invading hyphae in the tomato plant tissues.

The observation that invading fungal cells were markedly damaged at a time when the cellulose component of their cell walls was quite well preserved (Fig. 10b) favors the hypothesis of a specific plant defense reaction. Synthesis of phytoalexins as a site-specific response to fungal ingress is a well-documented response to elicitor treatment (Hammerschmidt, 1999). Several lines of evidence have shown that the fungitoxic effect of both phytoalexins and preformed phenolics was related to their interaction with membrane-bound lipids or phospholipids, resulting in an increase in fungal membrane permeability, pore formation, and leakage of cell contents (Weete, 1980). Phenolic-induced perturbations in the permeability of the plasma membrane in *P. parasitica* cells might have promoted internal osmotic imbalances, leading to the observed disturbances such as plasmalemma retraction, cytoplasm aggregation, condensation, and in some cases, complete loss of the protoplasm. In oligandrin-treated tomato plants, phenolic compounds might be involved in at least two key biological functions. First, the accumulation of phenolic compounds at sites of pathogen penetration might cause inhibition of fungal growth as illustrated by the distorted and degenerative aspect of all fungal hyphae. Second, the impregnation of phenolic compounds in the host cell walls (as indicated by the higher electron density of cell walls than normal) and their accumulation in noninvaded xylem vessels as a coating along secondary walls might contribute to enhancing the mechanical strength of these defensive barriers.

Restriction of fungal growth to the outermost stem tissues was also found to correlate with the formation of heterogeneous wall appositions beyond the infection sites. Reinforcing the host cell walls by either the

impregnation of hydrophobic substances or by the deposition of new wall-like polymers (Ride, 1983; Hahlbrock and Scheel, 1989) is an essential prerequisite for preventing enzymatic degradation of the host cell walls, a phenomenon that is considered to be one of the most harmful events associated with the infection process by pathogenic fungi (Collmer and Keen, 1986). Support for the close association between oligandrin treatment and induced resistance also came from the observation that intercellular spaces, known to be strategic sites for pathogen spread, were often filled with an electron-dense material in which invading hyphae were trapped. Such host reactions, obviously designed to halt pathogen ingress, were never seen in control plants where the pattern of fungal colonization was similar in many respects to colonization known to occur with necrotrophic fungi. Thus the present observations are of particular relevance since they bring further insight to the concept that oligandrin is capable of evoking biochemical events usually associated with the natural plant disease resistance process.

Although wall appositions could be seen in the outer stem tissues, their extent never reached that observed in tomato plants treated with elicitors such as chitosan (Benhamou et al., 1994; Benhamou and Lafontaine, 1995) or with *P. oligandrum* itself (Benhamou et al., 1997). Although a clear explanation for such a difference in the rate and extent of this structural response is still difficult to give, the possibility that the reduced number of wall appositions correlates with a reduced level of callose synthesis can be raised. Several lines of evidence have shown that callose formation is modulated directly or indirectly by the intracellular concentration of free Ca^{2+}, which is known to control the activity of one of the key structural enzymes, β-1,3-glucan synthase (Köhle et al., 1985). Recent investigations of the earliest events leading to elicitin-mediated HR in tobacco have shown that huge Ca^{2+} uptake occurred within minutes following elicitin application (Tavernier et al., 1995). Because the elicitin receptor is thought to be a ligand-dependent calcium channel (Ponchet et al., 1999), it was suggested that Ca^{2+} influx was of ligand-dependent type, thus indicating that saturation of the receptor by sufficient elicitin molecules was a prerequisite for optimal Ca^{2+} uptake (Tavernier et al., 1995; Keiser et al., 1998). In light of these results, one might speculate that the trapping of a large number of oligandrin molecules in the tomato plant cell wall might have hampered receptor saturation, leading to moderate Ca^{2+} influx and consequently to a reduced activity of the callose synthesis-involved β-1,3-glucan synthase.

Even though the exact mechanisms by which oligandrin operates to trigger resistance in tomato are not fully elucidated, the present results demonstrate that the beneficial effect exerted by this fungal proteinaceous molecule results from an integrated action of biochemical and anatomical factors that develop at the onset of pathogen penetration. The observation that defense reactions were expressed in oligandrin-treated plants only upon challenge with *P. parasitica* supports the hypothesis that a signal produced by the pathogen is essential for triggering synthesis and accumulation of defense gene products. A similar conclusion was reached in the case of chitosan-treated tomato plants (Benhamou, 1996). It was shown that defense reactions accumulating in chitosan-coated tomato roots infected with *Fusarium oxysporum* f.sp. *radicis-lycopersici* were seldom seen in noninfected, chitosan-treated tomato roots. Benhamou et al. (1996), similarly studying the protective effect of endophytic bacteria against fungal plant pathogens, found that extensive defense reactions occurred in bacterized plants only following pathogenic attack. These observations together with the present results suggest that biotic or abiotic agents sensitize the plant to respond more rapidly to microbial attack without causing accumulation of defense gene products that would require extensive loss of energy.

In summary, evidence is provided in this study that oligandrin, a small proteinaceous molecule produced by the mycoparasite *P. oligandrum*, is an elicitin-like protein that displays the ability to trigger a resistance response in tomato without inducing symptoms of phytotoxicity such as those observed during elicitin-mediated HR (Ricci et al., 1989). Among the resistance elicitors identified so far, fungal-derived proteinaceous molecules with high elicitor activity (Yu, 1995) are attracting a lot of attention not only because of their specific mechanisms of action on gene expression in plants (Ponchet et al., 1999) but also because their simple nature offers the best prospects for the production of synthetic analogs that can be introduced as a new biocontrol strategy in plant disease management.

MATERIALS AND METHODS

Plant Material

Tomato (*Lycopersicon esculentum* Mill. var. Prisca) seeds were sterilized by immersion in 7% (v/v) calcium hypochloride for 7 min and thoroughly rinsed in sterile distilled water. Seeds were sown in sterilized vermiculite in a 37- × 23-cm plastic tray. One-week-old tomato seedlings were uprooted and transferred into pots (11 × 11 × 11 cm) containing peat (Vapogro, Griendtsveen, Netherlands) at a density of one plantlet per pot. Plants were grown in a greenhouse at 22°C with a 14-h light period and were fertilized twice a week with a commercial plant nutrient solution (Solufeed soluble fertilizer, ICI Agrochemicals, Paris). Experiments were performed with 2-month-old plants harboring five to six fully expanded leaves.

Fungal Cultures

Pythium oligandrum Drechsler, strain 1010, was isolated from pea roots in Denmark (provided by Dr J. Hockenhull,

The Royal Veterinary and Agricultural University, Copenhagen). *Phytophthora parasitica* (isolate 149, highly virulent on tomato) was obtained from the collection maintained at Institut National de la Recherche Agronomique (Antibes, France). Both fungi were cultivated on potato-dextrose agar medium (Difco, Detroit) at 25°C in the dark.

Production of the Culture Filtrates

Liquid cultures of *P. oligandrum* were obtained by growing the fungus in a defined medium containing for 1,000 mL of deionized water: 0.6 g of KH_2PO_4, 0.7 g of KNO_3, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.125 g of $K_2HPO_4 \cdot 3H_2O$, 0.3 g of $Ca(NO_3)_2$, 1 mg of H_3BO_3, 1.5 mg of $MnSO_4 \cdot H_2O$, 4 mg of $ZnSO_4 \cdot 7H_2O$, 0.1 mg of $Na_2MoO_4 \cdot 2H_2O$, 20 μg of KI, 20 μg of $CuSO_4 \cdot 5H_2O$, 20 μg of $CoCl_2 \cdot 6H_2O$, 8 mg of $FeNa_2$ EDTA, 1 mg of nicotinic acid, 1 mg of pyridoxin, 1 mg of calcium panthotenate, 1 mg of thiamine hydrochloride, 1 g of $Asn \cdot H_2O$, and 20 g of Glc. This medium was chosen for its known potential of stimulating elicitin production (Bonnet et al., 1996). The flasks were incubated in the dark for 8 d at 24°C. Culture filtrates of *P. oligandrum* were recovered after mycelium removal on a GF/C filter (Whatman, Clifton, NJ) under vacuum.

Protein Purification from the Culture Filtrates

Culture filtrates of *P. oligandrum* (5 L) were concentrated 10-fold by evaporation under vacuum at 35°C and dialyzed against deionized water for 24 h at 4°C. Fifteen milliliters of 0.34 M sodium-acetate was added to the concentrated filtrate (495 mL) and the pH of the resulting solution was adjusted to 3.5 with 10% (v/v) aqueous trifluoroacetic acid. The concentrated filtrate was loaded on a 20-mL cationic exchange Macroprep sulfopropyl High S column (Bio-Rad, Ivry sur Seine, France) previously equilibrated with 10 mM sodium-acetate (pH 3.5). The retained fraction was eluted with 10 mM sodium-acetate containing 0.25 M NaCl (pH 3.5) and adjusted to a pH of 7.0 before being subjected to reverse-phase liquid chromatography using a Synchroprep C4 column (30 μm, 300 Å, Synchrom Inc., distributed by Eichrom Technologies, Paris) that was pre-equilibrated with 10 mM sodium-acetate containing 0.25 M NaCl (pH 7.0). Elution was carried out at room temperature using a gradient of acetonitrile (CH_3CN) (20%, 30%, 40%; v/v) in 50 mM aqueous sodium-formate (HCOONa). A purified protein, termed oligandrin, was recovered from the 40% CH_3CN fraction.

Each chromatographic step was qualitatively assayed by HPLC. HPLC (625 LC system solvent delivery, Waters, Milford, MA) was performed by loading the active fractions on a Hema RP C18 column (10 μm, 150 × 4.6 mm i.d., Interchim, Montlucon, France). Elution was carried out with the following solvents: A [20% CH_3CN, 10 mM $(NH_4)_2SO_4$, and 20 mM HCOONa] and B (40% CH_3CN and 100 mM HCOONa) using a linear gradient: 100% A → 100% B (10 min), and 100% B hold for 2 min. The flow rate was 1 mL/min. Elution was monitored with a Waters 996 diode array detector (200–400 nm, resolution: 1.2 nm). Integration at 280 nm, spectra, peak purity, and all calculations were achieved with Millenium software (version 3.2, 1999, Waters). For the last step, a peak was visualized from the 40% CH_3CN fraction. After removal of CH_3CN under vacuum, the pure protein was extensively dialyzed against ultrapure water (Millipore, Bedford, MA) and freeze-dried. Purity of the protein was further assessed by SDS-PAGE on 15.4% (v/v) polyacrylamide-SDS gels (20 mA/gel in 0.25 mM Tris [tris(hydroxymethyl)aminomethane], 1.92 M Gly, and 0.1% SDS) (Le Berre et al., 1994).

Protein Sequencing

The purified protein (1 mg) was reduced with dithiothreitol in 8 M urea and alkylated with iodoacetamide. The reduced and alkylated protein was extensively dialyzed against ultrapure water and freeze-dried. N-terminal sequencing of oligandrin was performed on both the native and the alkylated proteins. The two sequences were found to be identical, with the exception of one Cys residue missing in the native protein.

For sequence determination, the freeze-dried protein was resuspended in 1% (w/v) trifluoroacetic acid-20% acetonitrile solution at a final concentration of 2 mg/mL. An aliquot (0.5–1.5 nmol protein) was loaded on polybrene-treated glass fiber and N-terminal sequence determination was performed by automated Edman degradation using an Applied Biosystems (Foster City, CA) 470 A sequencer. Phenylthiohydantoin (PTH) amino acids were identified on-line with a 120 A Applied Biosystems PTH-Analyser by reverse-phase HPLC using a PTH-C18 cartridge (2.1 × 220 mm, Brownlee, Applied Biosystems, Roissy, France). All products, reagents, and programs used for sequencing were from Applied Biosystems.

Multiple sequence alignment was performed using the method of Thompson et al. (1994) with Clustal W (version 2.0). Similarities between proteins were revealed using the WU-BLASTp in the Swall database at the European Bioinformatics Institute (Cambridge, UK, http://www.ebi.ac.uk/).

Antifungal Potential of Oligandrin

Mycelial samples (1 mm^3), collected from an actively growing colony of *P. parasitica*, were subjected to oligandrin at a concentration ranging from 5 to 30 μg/mL in distilled water for 1, 2, 4, and 12 h at room temperature. Samples were rinsed thoroughly thereafter with distilled water and processed for electron microscope investigations. Controls included mycelial samples immersed in sterile distilled water.

Radioiodination and Migration of Oligandrin

The protein was iodinated according to a previously described procedure (Wendehenne et al., 1995). The protein (100 μg) was incubated in 100 μL of 50 mM phosphate buffer (pH 7.4) with 1 mCi of Na-^{125}I (Amersham, Buckinghamshire, UK) and iodogen as the catalyst for 20 min at

20°C. The [^{125}I]oligandrin was purified by gel filtration on a G-25 Sephadex column (5 mL, Pharmacia, Uppsala) equilibrated with 50 mM Tris-HCl buffer (pH 7.4). Eluted fractions were collected and the radioactivity counted with a 6000TA liquid scintillation analyzer (Beckman Instruments, Fullerton, CA). Fractions containing the radioactive protein were pooled and stored at −20°C.

Migration of the radiolabeled oligandrin in tomato plants was determined by applying 3 nmol of [^{125}I]oligandrin (specific radioactivity was about 1.5 Ci/mmol) onto the decapitated apex or onto the wounded petiole. Three hours after [^{125}I]oligandrin treatment, migration of the protein was monitored by a 10-min exposure of the tomato plants with a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA).

Oligandrin Application and Challenge Inoculation

Tomato plants at the five- to six-leaf stage were decapitated above the fifth fully expanded leaf (Ricci et al., 1989) just prior to applying 30 μL of oligandrin (3 nmol/plant) onto the fresh wound. Control plants were decapitated and treated with sterile water. At the same time or 24 h later, decapitated tomato plants were inoculated by placing a plug of actively growing mycelium of *P. parasitica* on the section of the leaf petiole in position 2 (beneath). Control plants were treated similarly but with fungus-free agar plugs. One week after fungal inoculation, the stem was longitudinally sectioned, allowing visualization of pathogen spread. Each plant was categorized according to the following scale: I, near absence of stem lesions; II, small lesions restricted to specific stem tissues including the epidermis and the cortex; and III, enlarged stem lesions. Stem invasion was estimated by the volume of discolored tissues (in cm^3) and percent of protection was computed as the relative reduction of invasion compared with water-treated, inoculated control plants. The experiment was repeated three times with 15 plants for each treatment.

The oligandrin, at concentrations ranging from 0.1 to 100 nmol/20 μL, was directly infiltrated into tomato leaves through the abaxial epidermal layer to determine whether HR (necrotic lesions) was expressed in tomato leaves.

Tissue Processing for Electron Microscope Studies

Mycelial samples and tomato stem samples (2 mm^3), collected at or near the necrotic lesions 7 d after fungal inoculation, were fixed by immersion in 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h at room temperature and post-fixed with 1% (w/v) osmium tetroxide in the same buffer for 1 h at 4°C. Samples were dehydrated in a graded ethanol series and embedded in Epon 812 (JBEM Chemicals Pointe-Claire, Quebec, Canada). Thin sections (0.7 μm), cut from the Epon-embedded material using glass knives, were mounted on glass slides and stained with 1% (v/v) aqueous toluidine blue prior to examination with an Axioscope microscope (Zeiss, Jena, Germany). Ultrathin sections (0.1 μm), collected on nickel grids, were either contrasted with uranyl acetate and lead citrate for immediate examination with a transmission electron microscope (model 1200 EX, JEOL, Tokyo) operating at 80 kV or further processed for cytochemical labeling. For each treatment, an average of five samples from three different stems were investigated. For each sample, 10 to 15 ultrathin sections were examined under the electron microscope.

Cytochemical Labeling

Colloidal gold, with particles averaging 12 nm in diameter, was prepared according to Frens (1973) using sodium citrate as a reducing agent. The β-1,4-exoglucanase-gold complex used for localization of cellulosic β-1,4-glucans was prepared according to Benhamou et al. (1987) using a β-1,4-D-glucan cellobiohydrolase (EC 3.2.1.21) complexed to gold at pH 9.0.

Labeling with the gold-complexed exoglucanase was performed by first incubating the ultrathin tomato stem sections for 5 to 10 min on a drop of phosphate-buffered saline containing 0.02% (w/v) polyethylene glycol 20,000 at pH 6.0, and then transferring them to a drop of the enzyme-gold complex for 30 min at room temperature in a moist chamber. After careful washing with phosphate-buffered saline (pH 7.2) and rinsing with distilled water, sections were contrasted with uranyl acetate and lead citrate and observed with a JEOL 1200 EX transmission electron microscope operating at 80 kV.

Specificity of the labeling was assessed by the following control tests: (a) incubation of the probe on which was previously added β-1,4-glucans from barley, 1 mg/mL^{-1}; (b) incubation with the un-complexed protein followed by incubation with the gold complex; and (c) incubation with colloidal gold alone.

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