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Microtubule dynamics in compatible and incompatible interactions of soybean hypocotyl cells with *Phytophthora sojae*

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The arrangement of microtubules in soybean (*Glycine max*) cells was examined during compatible and incompatible interactions of hypocotyls of soybean cv. Harosoy (susceptible) and cv. Haro 1272 (resistant) with race 1 of the soybean-specific pathogen *Phytophthora sojae*. Both reaction types were similar during the first 3 h after zoospore inoculation in terms of the number of cells penetrated, and depth penetrated into the cortex. By 3 h postinoculation, clear differences had developed between the two interaction types: incompatible interactions were characterized by a hypersensitive response that was confined to single penetrated cells; while compatibly responding cells appeared unchanged. Both types of response were characterized by autofluorescence of cell walls or cytoplasm and, at 6 h after inoculation, complete disorganization of cell cytoplasm. Reorientation and loss of microtubules was seen in the early stages of the incompatible interaction in association with cellular hypersensitivity, but not in compatible responses. In cells adjacent to those that reacted hypersensitively, there was little evidence of change in microtubule orientation. Treatment of hypocotyls with the microtubule depolymerizer oryzalin prior to inoculation did not alter the compatible response, but led to breakdown of the incompatible response. Changes in microtubule orientation and state are thus among the first structural changes that are visible within cells during incompatibility in this system.

Keywords: hypersensitive reaction, incompatibility, microtubules, *Phytophthora sojae*, soybean

Introduction

Compatibility and incompatibility in host–pathogen systems are defined by changes that occur at the molecular, biochemical and physiological levels. In gene-for-gene systems, morphological and ultrastructural changes during the early stages of the infection process appear similar, but as the infection sequence progresses, clear differences arise between these two opposing states. The rapidity with which infection is established, however, varies considerably between host–pathogen combinations. In soybean infected with *Phytophthora sojae*, for example, incompatibility was established in epidermal cells 2 h after inoculation (Ward *et al*., 1989), and was characterized by hypersensitive cell death and membrane and cytoplasmic disorganization. There were also major physiological changes such as a reduction in the level of abscisic acid and systemic stomatal closure that occurred between 2 and 4 h after inoculation (Ward *et al*., 1989; McDonald & Cahill, 1999a; McDonald & Cahill, 1999b). Compatibility was not associated with any of these changes. In contrast, incompatibility was not established during the interaction between flax (*Linum usitatissimum*) and flax rust (*Melampsora lini*) until 24 h after inoculation, even though infection structures such as appressoria and haustoria were formed 12 h after inoculation (Kobayashi *et al*., 1994).

Cellular responses during the initial stages of pathogen growth and penetration of host cell surfaces are beginning to be characterized. Cytoplasmic aggregation, nuclear movement towards the site of penetration, rearrangement of cytoplasmic strands and cytoskeletal elements have all been recorded for both compatible and incompatible interactions (Gross *et al*., 1993; Freytag *et al*., 1994; Heath *et al*., 1996; Hardham & Mitchell, 1998; Skalamera

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& Heath, 1998; Grant & Mansfield, 1999). There is emerging evidence, however, that there are distinct differences at the cellular level between those cells destined for hypersensitivity and those that are, at least in the initial stages of the interaction, unaffected by pathogen ingress.

Microtubules are dynamic components of the cytoskeleton, and various stimuli can promote polymerization and depolymerization, and rapid changes in orientation (Yuan *et al*., 1994; Wymer *et al*., 1996). Microtubules have important roles in many cellular responses and processes, including cell division (Cleary *et al*., 1992; Hyde & Hardham, 1992) and nuclear organization (Baluska *et al*., 1995a; Baluska *et al*., 1995b) and they may also be linked to plasmodesmatal function (Blackman & Overall, 1998). Microtubule arrangement alters in response to external stimuli such as electrical fields, gravity and heat stress (Blackman & Overall, 1995; Smertenko *et al*., 1997; Himmelspach *et al*., 1999) and may regulate stomatal movements (Fukuda *et al*., 1998). Microtubules are also intimately associated with the formation and maintenance of the inter- and intracellular structures of mycorrhizal fungi (Carnero Diaz *et al*., 1996; Genre & Bonfante, 1998; Uetake & Peterson, 1998; Matsubara *et al*., 1999). Alterations to microtubule arrangement within plant cells have also been associated with a number of infection-induced changes that occur during pathogen challenge.

There is clear evidence of changes in the cytoskeleton as an early consequence of recognition from several different host and pathogen combinations. For example, Kobayashi *et al*. (1994); Kobayashi *et al*. (1997a) found that both microtubules and actin filaments were reorganized before penetration of flax cells by *M. lini* and became focused at the site of eventual penetration. Similarly, Grant & Mansfield (1999) and McLusky *et al*. (1999) showed polarization of actin filaments at sites of attempted penetration by *Botrytis allii*. In contrast, Gross *et al*. (1993) found that microtubules were reorganized and depolymerized in suspension cells of parsley at sites of pathogen attack. In addition, chemically induced depolymerization of microtubules has been shown to alter the phenotype of an incompatible response (Kobayashi *et al*., 1997b). Specific and early rearrangements of microtubules during incompatibility, but not compatibility, indicate that microtubules may have a role in determining the outcome of the interaction between host and pathogen. Their role may be as a primary determinant, as suggested by the work of Kobayashi *et al*. (1994); Kobayashi *et al*. (1997a), or the changes in arrangement and state of polymerization may simply be a consequence of the interaction. The extent to which microtubules are involved in incompatibility may depend on the rapidity with which a pathogen can contact and penetrate host cells.

Rps (resistance to *P. sojae*) genes govern resistance against *P. sojae* in soybean (Irwin *et al*., 1995). *Rps* genes encode proteins that recognize specific elicitors produced by *P. sojae* (Umemoto *et al*., 1997). Cultivars can therefore be selected depending on their *Rps* gene combination for either compatibility or incompatibility to particular races or a single race of the pathogen. This study uses race 1 of *P. sojae* that is virulent on soybean cv. Harosoy (*Rps7*) and therefore displays a compatible (susceptible) interaction, and avirulent on the near-isogenic cultivar Haro 1272 (*Rps1a*) that displays an incompatible (resistant) interaction. The study shows that development of incompatibility in this system is a process centred on the rapid induction of a hypersensitive response, and that soybean cells that display these interaction types differ in microtubule arrangement and integrity at very early stages of the infection process. In addition, the microtubule-depolymerizing drug oryzalin changes the incompatible response to one that phenotypically resembles compatibility.

Materials and methods

Pathogen isolates, zoospore production and plant growth conditions

Phytophthora sojae race 1 was grown on 20% V8 juice agar in 9 cm disposable Petri dishes. Production of zoospores followed the method of McDonald & Cahill (1999a). Six-day-old-cultures of *P. sojae* were washed five or six times with sterile distilled water at 25°C, then placed at 18°C overnight. Sporangium production was induced during the washing steps, and zoospore release by sustained and reduced temperature. For inoculation, zoospore densities were adjusted to 1×10^5 mL^{-1}.

Seeds of both cultivars were planted in moistened vermiculite and germinated in the dark in a constant-temperature cabinet (26°C). After 6 days hypocotyls were 10–12 cm in length and of suitable size for inoculation.

Inoculation of hypocotyls with *P. sojae* race 1

Inoculation of soybean hypocotyls was carried out as described previously (Cahill *et al*., 1993). This involved removing seedlings from the vermiculite, laying them horizontally on moist cotton wool in plastic trays, and inoculating at 2·5 cm from the hypocotyl hook with a single 10 μL drop of zoospore suspension. At various time points after inoculation, the region of inoculation was excised from the hypocotyl and immediately placed in fixative. *Phytophthora sojae* race 1 establishes a compatible interaction with soybean cv. Harosoy, and an incompatible interaction with cv. Haro 1272.

Fixation and cryosectioning

Prior to fixation, 3 mm segments of hypocotyl tissue were excised from the site of inoculation at 0, 1·5, 2, 2·5, 3 and 6 h after inoculation. Segments were immediately placed in 20 mL of a fixative solution consisting of 4% paraformaldehyde, 10% dimethylsulphoxide (DMSO) in 50 mM 1,4-piperazine-diethanesulfonic acid (Pipes) buffer (pH 6·9) and infiltrated under vacuum for 1·5 h. Segments remained in fixative for a further 2 h and were then removed and placed in 50 mL of a solution containing 1·0 M sucrose and 0·4% paraformaldehyde. Following

overnight incubation at 4°C, the segments were removed, placed in 50 mL 1·3 M sucrose, and infiltrated under vacuum for a further 60 min.

For sectioning with the cryostat microtome (Cryocut E, Reichert-Jung, Wien, Austria) the sucrose-infiltrated segments were blotted dry and then placed in a foil mould. Embedding compound (Tissue-Tek OCT Compound, Sakura Finetek USA Inc., Torrance, CA, USA) was added to the mould to completely cover the tissue. The mould was then immersed in liquid nitrogen, and the frozen block containing the hypocotyl tissue removed and adhered to a cryostat specimen holder that had been held at –20°C. Serial longitudinal sections, 16 μm thick, were cut at a chamber temperature of –20°C. Sections were placed on gelatine-coated (0·5% w/v in water) microscope slides that were placed in a small, sealed incubation box at room temperature (22°C) for 15 min. Slides were then removed from the box and placed in 50 mL buffer (50 mM Pipes, 5 mM $MgSO_4$, 5 mM EGTA, 5% DMSO, 0·05% Triton X-100) with 1% bovine serum albumin (BSA) and 0·1% gelatine in a Coplin jar. Slides were incubated at room temperature (22°C) for 45 min, then gently washed three times for 3 min each in buffer. Slides were drained onto paper towel and 10 μL of anti-β tubulin monoclonal antibody (Sigma Chemical Co., St Louis, MO, USA) diluted in buffer (1 : 50) were applied to each section. Slides were then placed in a plastic container lined with moist paper towelling, sealed and left in the dark at room temperature (22°C) for 4·5 h. The primary antibody was removed by gently washing sections in buffer three times, then draining. Ten microlitres of sheep anti-mouse antibody conjugated to FITC diluted in buffer (1 : 200) was applied to each section, and the slides were incubated overnight at 4°C. Slides were rinsed in buffer, drained, and sections were mounted in 10–20 μL 1% solution of 1,4-diazabicyclo[2·2·2] octane (DABCO) in glycerol under a glass coverslip. Slides were stored in the dark at 4°C until viewed.

Light and fluorescence microscopy

The arrangement of microtubules in cryosections was examined using an Olympus Provis AX microscope (Olympus Australia Pty Ltd, Oakleigh, Victoria, Australia) with excitation and barrier filters optimized for FITC fluorescence (excitation 450–490 nm, emission 420 nm). Images were collected using a digital camera and processed using image analysis software (OPTIMAS version 5·2, Optimas Corporation, Washington, USA).

Confocal laser scanning microscopy

Labelled hypocotyl sections were viewed with an Optiscan confocal laser scanning system (Optiscan Pty Ltd, Clayton, Victoria, Australia) coupled to an Olympus BX50 compound microscope and a 60× oil-immersion objective lens (numerical aperture 1·4). Images of single or serial optical sections were collected using F900e version 1·5·8 software (Optiscan) and stored in a computer.

Treatment of plants and *P. sojae* cysts with oryzalin

The dinitroaniline herbicide, oryzalin (3,5-dinitro-N_4,N_4-dipropylsulfanilamide) was dissolved in dimethylsulphoxide (DMSO) and made to final concentration with distilled water. The maximum concentration of DMSO in the final solution was <0·01%. Six-day-old dark-grown seedlings of soybean cvs Harosoy and Haro 1272 were gently removed from vermiculite and placed with their root system immersed in oryzalin at a concentration of 1–100 μM in 50 mL plastic centrifuge tubes. Control plants were placed with their root systems immersed in 0·01% v/v DMSO in distilled water. Plants remained with their root systems immersed in oryzalin solution for 14–16 h at 26°C in a growth cabinet. Following treatment, seedlings were removed from the oryzalin solution and washed gently in two changes of distilled water. Seedlings were placed horizontally in trays and inoculated as described previously. Twenty-four hours after inoculation, hypocotyls were examined for lesion development, cut transversely into 2 mm segments, from the centre of the lesion outwards, and plated onto cleared 10% V8 agar to determine the extent of hyphal growth in tissues. The experiment was repeated three times.

To examine the effect of oryzalin on germinating cysts, zoospores of *P. sojae* race 1 were first induced to encyst by briefly vortexing a 1 mL suspension of 10^5 zoospores mL^{-1} within a microfuge tube. Following encystment, 10 μL cyst suspension was added to 90 μL oryzalin, made to the desired concentration, on a standard microscope slide. The cyst suspension was covered with a coverslip; slides were placed in a covered plastic container to maintain high humidity, and kept at 26°C in the dark in an incubator. Germ tube lengths were measured at 4, 6 and 24 h using a microscope fitted with an ocular micrometer.

Results

Time course of the infection process

Following inoculation of hypocotyls of both the resistant and susceptible soybean cultivars with zoospores of *P. sojae* race 1, cysts formed on the epidermal surface within 0·5–1 h. Within 1–1·5 h a single germ tube from each cyst was produced and began to penetrate individual epidermal cells directly or, more frequently, between anticlinal walls (Fig. 1a). Hyphae then moved through the epidermal layer and into the cortex by 2 h after inoculation. By 6 h after inoculation in compatible interactions, hyphae had penetrated to the sixth layer of cortical cells.

In contrast to that of the compatible interaction, there was a rapid response to penetration in the incompatible interaction. Browning of hypocotyl cells was clearly visible between 2 and 3 h after inoculation (Fig. 1b). Single cells of the epidermal layer or groups of penetrated cells (of both epidermis and cortex) displayed a clearly delineated incompatible response. Cells that reacted

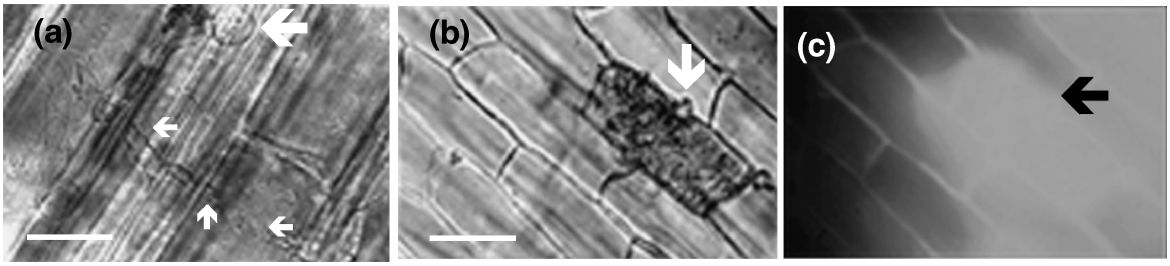

Figure 1 Compatible and incompatible interactions on epidermal peels of soybean cvs Harosoy and Haro 1272 inoculated with *Phytophthora sojae* race 1, 6 h after inoculation. (a) Light micrograph of the compatible interaction with Harosoy. Large arrow shows an empty *P. sojae* cyst on the epidermal surface that has given rise to a germ tube that has penetrated an epidermal cell. The small arrows trace the germ tube through adjoining epidermal cells. ×600; bar = 10 μm. (b) A single hypersensitive epidermal cell surrounded by healthy cells in a hypocotyl epidermal peel from cultivar Haro 1272. The arrow points to a germ tube entry point between cell walls. ×500; bar = 15 μm. (c) Same preparation as in (b), but showing intense blue light-induced fluorescence in the hypersensitive cell (arrow) and in some surrounding cells.

Table 1 Effect of inoculation with *P. sojae* race 1 of hypocotyls of soybean cultivars Harosoy (susceptible) and Haro 1272 (resistant) on cellular autofluorescence and the arrangement of microtubules

Time after inoculation (h)	Soybean cultivar	Number of cells examined	Cells with hyphal contact	Cell wall and cytoplasmic autofluorescence	Microtubule arrangement[a]	
					Normal	Abnormal
0	Harosoy	25 ± 1·6[b]	0	0	25 ± 1·2	0
	Haro 1272	28 ± 0·8	0	0	28 ± 1·2	0
1	Harosoy	30 ± 0·9	11 ± 0·4	4 ± 0·2	28 ± 1·3	1 ± 0·2
	Haro 1272	28 ± 1·2	11 ± 0·8	7 ± 0·5*	21 ± 0·6***	7 ± 0·3***
2	Harosoy	24 ± 1·6	19 ± 0·6	7 ± 0·8	24 ± 0·6	3 ± 0·6
	Haro 1272	24 ± 0·6	20 ± 0·9	11 ± 1·4*	15 ± 0·8***	9 ± 0·6***
3	Harosoy	23 ± 1·8	22 ± 0·4	11 ± 0·5	18 ± 0·5	5 ± 0·4
	Haro 1272	25 ± 0·4	25 ± 0·8	22 ± 0·7**	14 ± 0·6**	11 ± 0·5**
6	Harosoy	28 ± 1·4	27 ± 1·2	15 ± 1·4	15 ± 1·0	13 ± 0·4
	Haro 1272	29 ± 0·7	28 ± 1·0	27 ± 1·2***	12 ± 0·8*	16 ± 0·6

[a]The arrangement of microtubules was 'normal' if similar to that in sections taken from uninoculated control tissues; 'abnormal' if not. Abnormal arrangement of microtubules included microtubules that had showed reorientation, fragmentation and/or were depolymerized.
[b]Mean and standard error of the number of cells examined from each of two fields of view (× 400 magnification) of a single longitudinal section fron each of two lesions on each of two hypocotyls. Mean number of cells in Harosoy and Haro 1272 were compared at each time interval after inoculatio using ANOVA where significance levels are: *, $P < 0{\cdot}05$, **, $P < 0{\cdot}01$, ***, $P < 0{\cdot}001$.

incompatibly, and some cells surrounding them, exhibited strong blue light-induced autofluorescence (Fig. 1c). Autofluorescence was associated with granulation, and aggregation and fragmentation of cytoplasm within single cells that were associated with hyphae. Autofluorescence of cell walls and cellular constituents occurred in both compatible and incompatible interactions, but time of induction and extent of fluorescence differed (Table 1). Significantly higher numbers of cells exhibited some degree of autofluorescence in incompatible interactions compared with compatible interactions. For example, at 2 h after inoculation, 37% of compatible cells and 55% of incompatible cells, respectively, were found to show autofluorescence. By 6 h after inoculation, equal numbers of cells were in contact with hyphae but the majority of cells in incompatible interactions displayed autofluorescence, while only half the contacted cells showed fluorescence in the compatible interaction. In the incompatible interaction, cellular organelles and cytoplasmic strands appeared to be degraded and disorganized (data not shown). Hyphae were restricted to epidermal and cortical cells that reacted hypersensitively. Thus the response was confined to individual penetrated cells with little or no visible impact on adjoining cells.

Organization of microtubules in soybean hypocotyl cells

Arrangement of microtubules in uninoculated hypocotyl cells

In untreated hypocotyl cells, microtubules that were present in epidermal and cortical cells were arranged predominantly in longitudinal or, less frequently, transverse orientation in essentially parallel arrays (Fig. 2a–c). Due to the plane of sectioning passing through the central region of some cells, microtubules were absent and these areas appeared as 'holes' in the image (for example Fig. 2a, middle right).

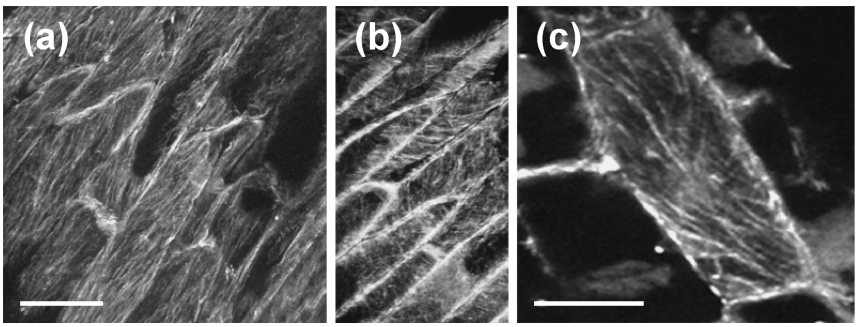

Figure 2 Confocal laser scanning microscopy images of microtubule arrays in longitudinal sections of soybean hypocotyl tissue. (a) Longitudinal and essentially parallel arrays in epidermal cells of cv. Harosoy. Z series of images converted to maximum brightness (20 optical sections, 15 scans per section, total depth 20·4 μm). ×600, bar = 15 μm. (b) Similar to (a), but showing oblique to transverse arrangement of microtubules. Z series of images converted to maximum brightness (20 optical sections, 15 scans per section, total depth 17·8 μm). Magnification and scale same as (a). (c) Single cortical cell of Haro 1272 showing longitudinal arrangement of microtubules. Z series of images converted to maximum brightness image (eight optical sections, 25 scans per section, total depth 5·36 μm). ×1100, bar = 20 μm.

Arrangement of microtubules after infection of hypocotyl cells by P. sojae

In compatible interactions there was little change in distribution or arrangement of microtubules within the first 3 h after inoculation, when hyphae were observed within the epidermis and three to four cortical cell layers (Table 1, Fig. 3a–e). By 3 h after inoculation, 23% of cells had abnormal arrangements of microtubules. Localized wall autofluorescence at sites close to penetrating germ tubes was observed in 48% of cells, but microtubule arrangement was essentially unaffected in those cells and cells adjacent to them (Fig. 3f). Between 3 and 6 h after inoculation there were substantial changes to the microtubule network that included fragmentation and disorganization in cells that were penetrated by hyphae, and there was a complete absence of microtubules within some penetrated cells (Fig. 3f). At 6 h after inoculation, around half the cells observed showed either autofluorescence (54% of cells) or had abnormal arrangements of microtubules (46% of cells).

In incompatible interactions, hyphae penetrated between cell walls and were restricted to the epidermal layer and first three to four layers of the cortex at 6 h after inoculation. Bright spots of FITC-induced fluorescence directly associated with hyphal penetration of cell walls was observed 1–2 h after inoculation (Fig. 4a). Autofluorescence was observed in 25% of cells at 1 h after inoculation, indicating a rapid response to hyphal penetration. By 6 h after inoculation most cells (93%) showed autofluorescence in walls and cytoplasm.

Changes in the arrangement of microtubules were observed in 25% of cells at 1 h after inoculation; by 3 h, 44% of cells had abnormal arrangements. Changes in arrangement were characterized by reorientation, fragmentation and depolymerization, or combinations of the three. In some cells, orientation toward the site of encystment or penetration occurred (Fig. 4d,f,g) or there was reorientation of longitudinal microtubules to short, disarranged microtubules (Fig. 4b). Bright spots of FITC-induced fluorescence were found along longitudinal walls of cells that also showed cytoplasmic autofluorescence and disruption (Fig. 4e). Larger aggregations of both FITC-labelled material and autofluorescent material were also observed in affected cells (Fig. 4c,e–g). Microtubules in cells adjacent to those that were disrupted remained in longitudinal orientation (Fig. 4e,g).

Confocal microscopy was also used to examine changes in microtubules during germ tube penetration through the epidermis and into the first layer of cortical cells during incompatibility (Fig. 5a–d). Composite optical sections showed loss of the cortical microtubule network in areas close to invading hyphae (Fig. 5c,d) and reorientation of microtubules in adjacent cells.

Influence of oryzalin on germ tube growth and hypocotyl interactions with *P. sojae*

A microscope slide assay was used to examine the effect of oryzalin on germ tube growth. For the control treatment, germ tubes were 8·15 ± 0·45 μm long at 4 h, and 11·57 ± 1·61 μm at 6 h after treatment (Fig. 6). At 24 h, germ tubes had grown substantially to 166·15 ± 9·67 μm. Following treatment of cysts with 1 and 10 μM oryzalin, germ tube lengths were not significantly different from controls at 4 and 6 h after treatment, but were less than controls at 24 h. Germination and germ tube growth were significantly reduced following treatment of cysts with 50 μM oryzalin, where no growth was recorded at 4 h, and substantial growth at 6 and 24 h after treatment.

Treatment of seedlings of cv. Harosoy with oryzalin did not alter the response observed in hypocotyls 24 h after inoculation with *P. sojae* (Table 2; Fig. 7a,b). Lesions were phenotypically similar at all concentrations of oryzalin and remained similar to controls, being light in colour with watersoaked margins, typical of compatible interactions. Hyphal spread from the centre of the lesion was approximately 11–12 mm at all concentrations of oryzalin tested (Fig. 8). In interactions with cv. Haro

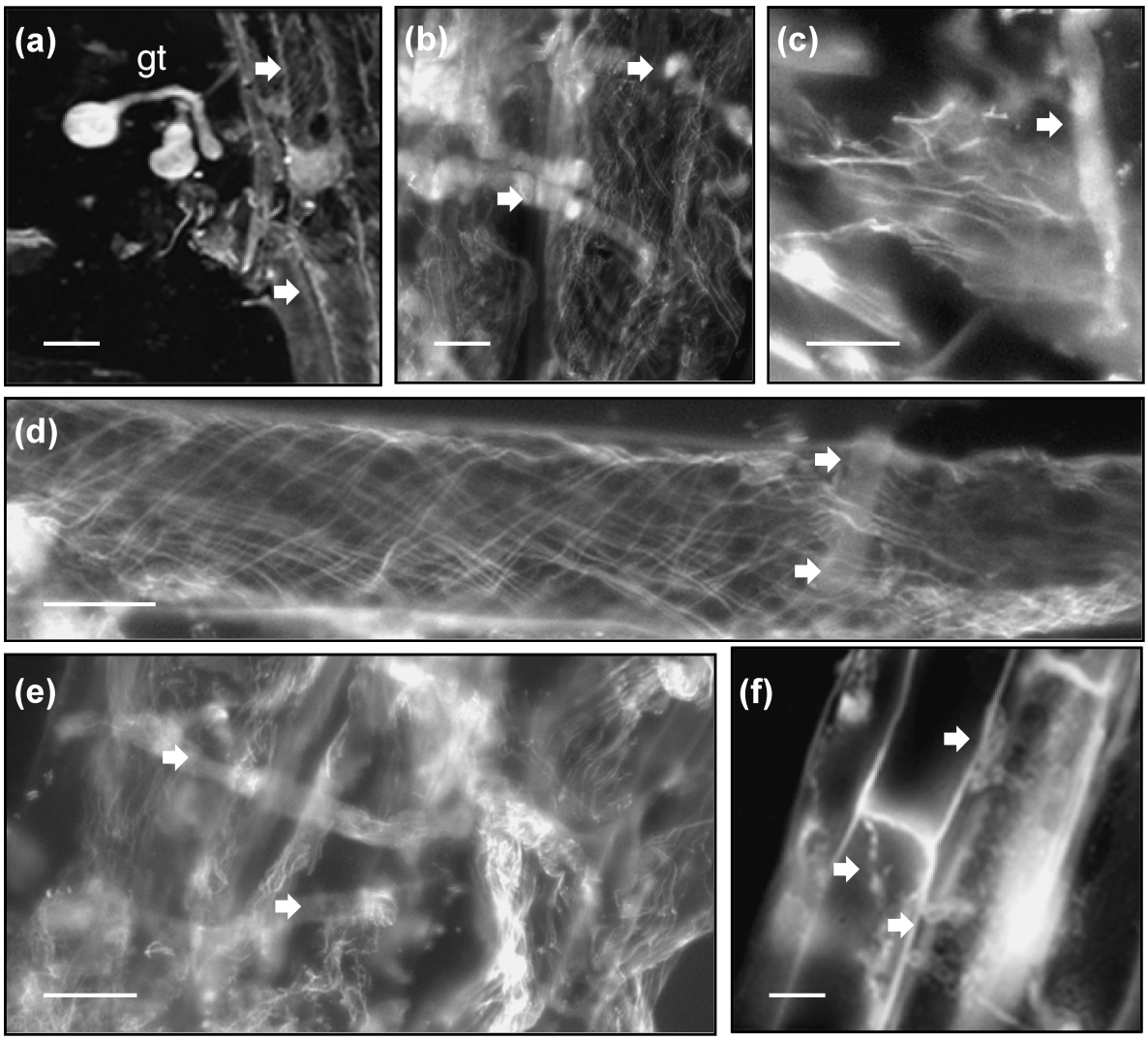

Figure 3 Compatible response of soybean cv. Harosoy to inoculation with *P. sojae* race 1. (a) Confocal image of germinated *P. sojae* race 1 cysts 1·5 h after inoculation of the hypocotyl epidermis, showing intact microtubules in adjacent epidermal cells (gt, germ tube; arrows point to microtubules within epidermal cells). Z series of optical sections, maximum brightness image (26 optical sections, 25 scans per image, total depth 21·3 μm). ×600, bar = 15 μm. (b) Longitudinal section through a region of penetration of cortical cells by hyphae (arrows) 2 h after inoculation, showing associated arrays of microtubules. ×800, bar = 5 μm. (c) Longitudinal section showing epidermal and cortical cells that have been penetrated by hyphae (arrows) 3 h after inoculation. Note fragmented microtubules within the epidermal cell. ×900, bar = 5 μm. (d) Longitudinal section through a single epidermal cell showing a single hypha passing through the cell (arrows) 3 h after inoculation. ×900, bar = 15 μm. (e) Microtubule arrays in epidermal and cortical cells that have been penetrated by several hyphae (arrows) 3 h after inoculation. ×900, bar = 15 μm. (f) Longitudinal section showing epidermal and cortical cells that have been penetrated by hyphae (arrows) 6 h after inoculation. Note fragmentary and disorganized microtubules within epidermal cells and cortical cells, and autofluorescence of cell walls. ×720, bar = 20 μm.

1272, lesions in the untreated controls were typical of the hypersensitive lesions formed during incompatible responses and were dark brown in colour and restricted to the site of inoculation (Fig. 7c). Hyphal spread occurred within hypocotyls after treatment with 1 and 10 μM oryzalin and approached that found for the compatible response (Fig. 7d). Lesions were not as tight as the controls, and lesion spread and 'breakout' occurred. At 50 μM concentration, hyphal spread was reduced to that of the controls and the lesions formed resembled those of the control treatment.

Discussion

The β-tubulin antibody used in the current investigation was specific for microtubules and labelled them in several soybean tissue and cell types. In untreated control tissues the arrangement of microtubules conformed to that described for other plant cells using similar antibodies (Cyr & Palevitz, 1995). In both compatible and incompatible interactions there were major changes to microtubule organization, but there were significant differences both in the timing of changes and in cellular response. The

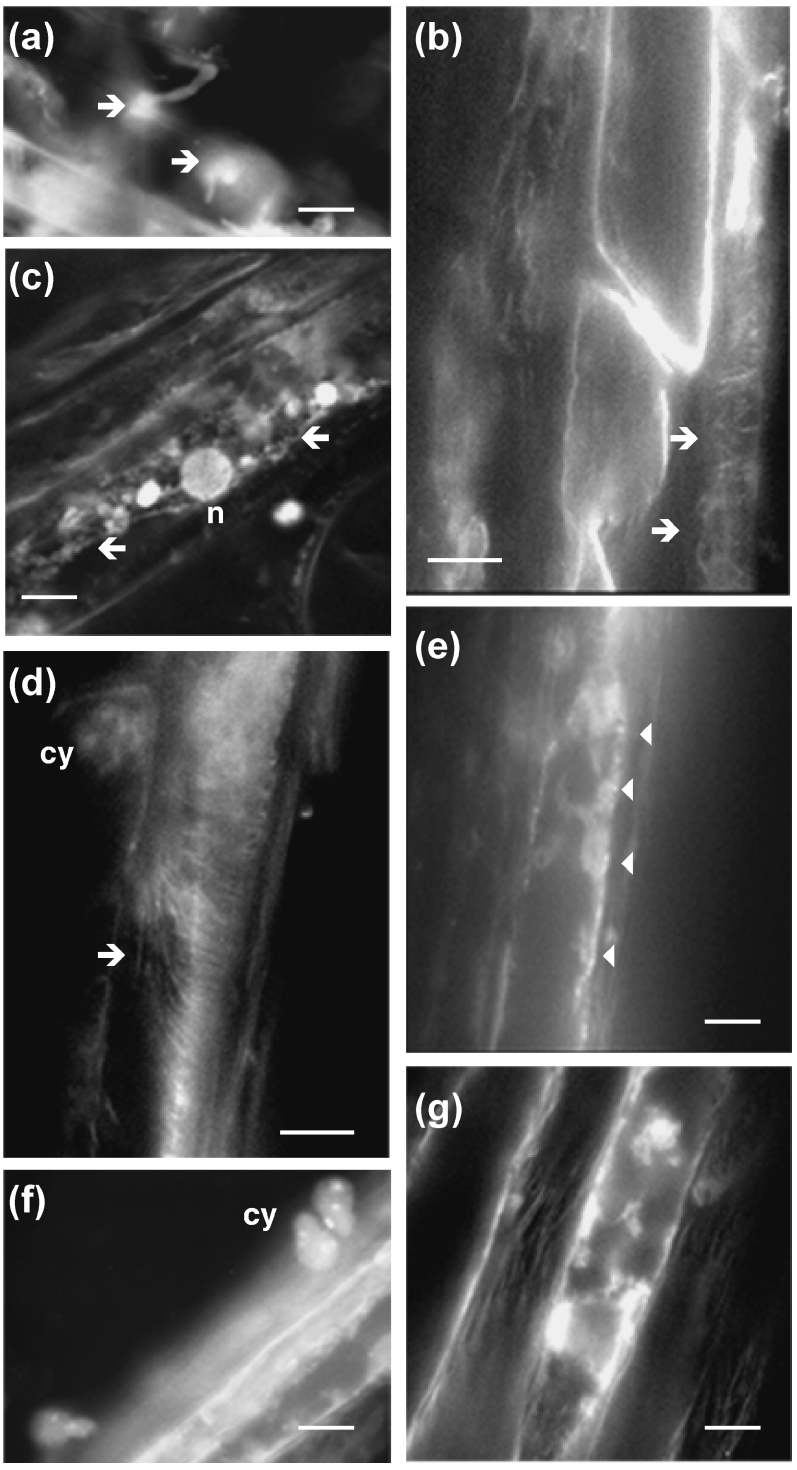

Figure 4 Incompatible interactions between soybean cv. Haro 1272 and *P. sojae* race 1. (a) Bright spots of FITC-induced fluorescence (arrows) at penetration sites in the epidermis 1·5 h after inoculation. ×600, bar = 15 μm. (b) Transverse orientation of microtubules (arrows) in an epidermal cell close to the site of penetration of a hypha between two cortical cells 2 h after inoculation. ×800, bar = 10 μm. (c) Single cell reaction 1·5 h after inoculation showing disruption of the microtubular network within affected cell (arrows), disorganization of the cytoplasm and swelling of the nucleus, n. Note intact microtubule arrays in closely associated cells. ×600, bar = 15 μm. (d) Microtubules (arrow) that appear to show orientation towards a cyst, cy, on the surface of an epidermal cell 1·5 h after inoculation. ×800, bar = 10 μm. (e) Disruption of cellular constituents in a hypersensitive cell 2 h after inoculation. Arrowheads point to bright spots of FITC fluorescence along a longitudinal wall. ×720, bar = 20 μm. (f) Localized disruption of the microtubule network and aggregation of autofluorescent material in the cytoplasm at sites close to penetration sites near several cysts (cy) on the epidermal surface 3 h after inoculation. ×600, bar = 15 μm. (g) Similar cell to (e,f) but showing normal arrays of microtubules in cells adjacent to the reacting cell. ×720, bar = 20 μm.

present results confirm previous reports showing that compatible and incompatible states are established within the first few hours of the interaction of *P. sojae* with its soybean host (Ward *et al*., 1989; Enkerli *et al*., 1997). The rapidity of these interactions contrasts markedly with those of the biotrophic fungi where the establishment phase and the time course of infection may take many hours to complete (Kobayashi *et al*., 1992; Kobayashi *et al*., 1994; Stark-Urnau & Mengden, 1995; Skalamera & Heath, 1998).

There appears to be a number of differences in the way in which the cytoskeleton is involved in different host–pathogen systems. In the present study, reorientation of microtubules around and within incompatible cells were observed very soon after inoculation. Hypersensitivity

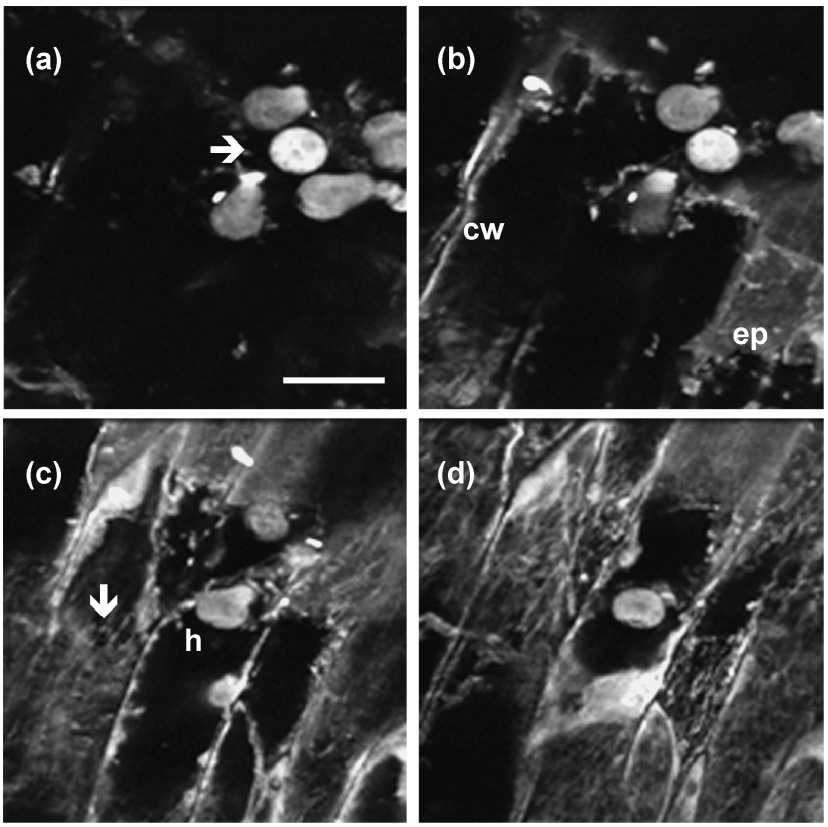

Figure 5 Series of confocal images of microtubules within hypocotyl cells during an incompatible interaction between soybean cv. Haro 1272 and *P. sojae* race 1, 2 h after inoculation. (a) A group of *P. sojae* cysts on the epidermal surface (arrow). (b) Epidermal surface (ep) and cell walls (cw) visible at the penetration site. (c) A hypha (h) within a single epidermal cell. Note microtubules in adjacent cell (arrow). (d) Cortical cell below the cell in (c) showing lack of microtubules around the hypha and reorientation of microtubules in adjacent cells. Z series of confocal images, selected maximum brightness images from 41 optical sections (optical section separation 0·85 μm, total depth 34 μm). ×800, bar = 20 μm.

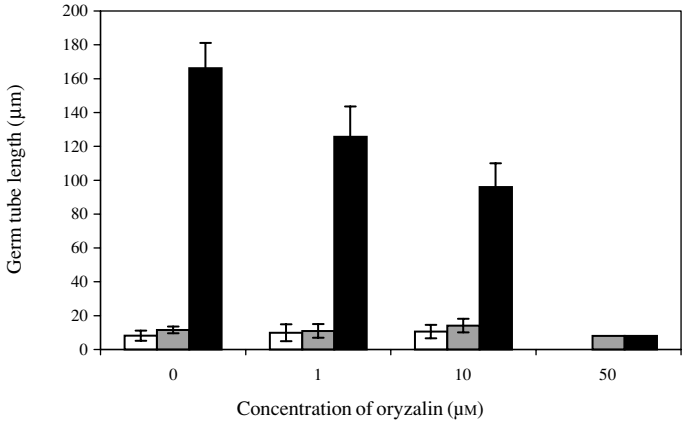

Figure 6 Length of germ tubes of *P. sojae* race 1 after treatment of cysts with oryzalin in a microscope slide assay. Lengths were determined 4 (white), 6 (grey) and 24 h (black) after treatment with oryzalin. Bars represent standard errors of the mean.

and the associated rapid breakdown of cellular constituents and deposition of phenolic materials within the cell and cell walls meant that the window for observation of microtubule dynamics was small and restricted to only one or two hours postinoculation. Reorientation of microtubules before penetration by the germ tube was rarely observed – an obvious and apparently important feature of the flax–flax rust system (Kobayashi *et al.*, 1994). Penetration of cells by *P. sojae* is a rapid process that does not require the production of an appressorium or infection peg, although limited swelling of the germ tube tip often precedes penetration (Ward *et al.*, 1989; Enkerli *et al.*, 1997). Penetration occurs within minutes of germ tube emergence and usually occurs between the walls of adjacent cells – direct penetration of the radial cell wall is extremely rare. Even though there were no obvious

Table 2 Reaction to inoculation with *P. sojae* of soybean cultivars Harosoy and Haro 1272, 24 h after treatment with oryzalin

Oryzalin concn (mM)[a]	Harosoy		Haro 1272	
	Reaction[b]	Lesion type	Reaction	Lesion type
0	S	Light, water soaked	R	Tight necrosis, dark brown, hypersensitive
1	S	Light, water soaked	R/S	Dark brown, slight longitudinal breakout
10	S	Light, water soaked	R/S	Dark brown with longitudinal breakout
50	S	Light, water soaked	R	Tight lesion, hypersensitive, no breakout

[a]Oryzalin was dissolved in DMSO and plants treated for 12–14 h in the dark prior to inoculation (see Materials and methods).
[b]Reaction of hypocotyls to inoculation where S = susceptible, R = resistant.

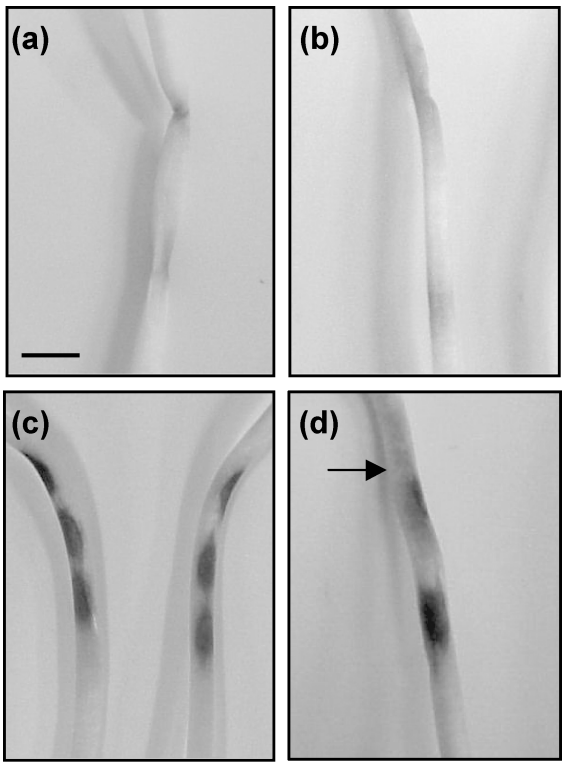

Figure 7 Reactions of hypocotyls to inoculation with *P. sojae* race 1 following treatment with oryzalin. Reactions of (a,b) cv. Harosoy and (c,d) cv. Haro 1272 to inoculation following treatment with distilled water (a,c, control) or 10 μM oryzalin (b,d). Arrow in (d) points to a region where the lesion is breaking out; bar in (a) represents 10 mm.

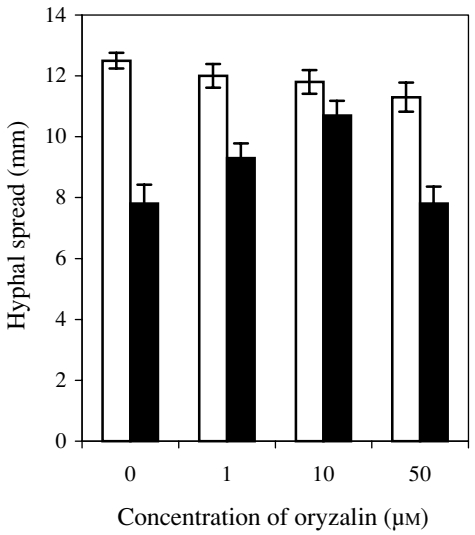

Figure 8 Spread of hyphae of *P. sojae* race 1 within hypocotyls of cv. Harosoy (white) and cv. Haro 1272 (black) 24 h after treatment of seedlings with oryzalin. Bars represent standard errors of the mean.

differences between extent of tissue colonization during the early stages of compatible and incompatible interactions, cellular responses were markedly different. In incompatible combinations the response to invasion was almost immediate and typically hypersensitive. It was shown in a previous study (Ward *et al*., 1989) that within 2 h of inoculation in an incompatible response, 95% of hypocotyl epidermal cells associated with hyphae had undergone a hypersensitive response and were dead. Though they did not study time intervals between 1 and 4 h postinoculation of soybean roots, Enkerli *et al*. (1997) had observed 'extensive host cell necrosis' 4 h after inoculation with *P. sojae*, and the disintegration of cytoplasm and organelles.

Compatibility in the *P. sojae*–soybean interaction appears to result from an initial biotrophic phase with the production of haustoria, that extends for about 3–4 h, followed by a necrotrophic phase in which cells of the cortex are directly penetrated by hyphae and cytoplasmic collapse occurs. As was shown by the previous study (Ward *et al*., 1989) in hypocotyl tissue, compatibility was also associated with the rapid death of cells especially in the epidermis (26% of cells in contact with hyphae dead after 2 h). In contrast, by 7 h after inoculation in incompatible interactions, 100% of epidermal cells and 66% of cortical cells were dead and in compatible interactions 75% of epidermal and 33% of cortical cells were dead. Wall deposits or 'appositions' were associated with hyphal contact in about equal numbers of cells in compatible and incompatible interactions and were formed between 3 and 5 h after inoculation, but did not seem to be related to the resistance response.

Results from this study show that there was no focusing of microtubules at penetration sites in incompatible interactions, as has been described for the biotrophic systems studied. Rather there was rapid disruption, disorganization and depolymerization of microtubules

during incompatibility. Localization of microtubules often occurs beneath developing appressoria but before production of the infection peg (Kobayashi *et al*., 1997a; Grant & Mansfield, 1999). The process of infection, timing and subsequent cellular responses described for the *P. sojae*–soybean interaction contrasts greatly with that of flax–flux rust (Kobayashi *et al*., 1994), and the barley–powdery mildew interaction (Kobayashi *et al*., 1992). In the latter, there is some disagreement as to the timing of the first alteration in microtubules in response to initiation of resistance, but development of the resistant state may occur over several hours. Both depolymerization of microtubules and reorientation and focusing of microtubules have been observed in different systems (Kobayashi *et al*., 1994; Baluska *et al*., 1995a; Skalamera & Heath, 1998). Evidence for more rapid changes in microtubule distribution and state of polymerization are also found in some powdery mildew interactions with barley (Baluska *et al*., 1995a). Between 15 min and 2 h after inoculation in a resistant reaction between *Erisyphe graminis* f.sp. *hordei* and barley, microtubules were reoriented, less densely spaced and became partially depolymerized. In susceptible reactions there were also changes in microtubules, but not to the extent observed for the resistant interaction.

Because of the very nature of the hypersensitive response in soybean cells, in terms of both the rapidity of the response (almost immediately on hyphal penetration) and the resultant cytoplasmic degradation and disorganization that occurs, it is unlikely that microtubules play a significant role in determining the outcome of an interaction in this system. Movement of the nucleus towards or away from the site of hyphal penetration, a feature described in several other systems and probably driven by microtubule changes (Freytag *et al*., 1994; Skalamera & Heath, 1998), was not consistently observed in incompatible interactions in this system. It is thought that pathogen-derived signals (mechanical or chemical, or a combination of both) are necessary for cytoplasmic and microtubule aggregation at the site of penetration (Kobayashi *et al*., 1994; McLusky *et al*., 1999) and are possibly stimuli of nuclear movement. Such signals may also be responsible for the autofluorescence of cell walls in both compatible and incompatible interactions that began about 2 h after inoculation, and may be indicative of major changes or additions to the structure of wall components. Autofluorescence in affected cells is a relatively common feature of the interaction of plant cells with various pathogens (Hardham & Mitchell, 1998; Grant & Mansfield, 1999), but there did not appear to be a relationship between autofluorescence and altered arrangement of microtubules in the present study. It has recently been found that autofluorescence of soybean walls is due to rapid lignification that occurs within 3 h after inoculation, and that it occurs to a greater degree in the incompatible response (Mohr & Cahill, 2001).

One avenue of approach that was used to further examine the role of microtubules in the *P. sojae*–soybean system was the use of oryzalin to depolymerize microtubules. One confounding factor that must be taken into consideration when using oryzalin is that it may also alter the microtubule network within the pathogen, and hence its ability to infect and penetrate cells. Fungal and oomycete microtubules are sensitive to oryzalin, and *P. sojae* appears to be more sensitive to oryzalin than some other pathogens, such as *M. lini* (Wilcox, 1996; Kobayashi *et al*., 1997b; Hyde *et al*., 1999). At the low concentrations used here, normally incompatible interactions were altered such that lesion breakout and some spreading of the lesion occurred, but the lesion remained similar in colour to a normally incompatible response. There was not a complete switch to compatibility as might be expected if microtubules were a primary determinant of the interaction. At the higher concentrations of oryzalin (>50 μM) the growth of the pathogen was affected and the lesion became restricted and resembled that produced in control treatments. In contrast, in compatible interactions there was no difference in lesion size or morphology at any of the concentrations of oryzalin tested. These results suggest that an intact microtubule network in the plant cells is required for maintenance of the resistance response. The reorientation of microtubules observed within cells during the first 2 h during incompatibility may thus be an important component of the cellular response, even though complete disruption of the microtubule network soon follows. A more detailed study of the very early time points during the interaction is warranted, and could include, for example, sensitive real-time analysis of the cytoskeleton in living cells using a GFP reporter gene (Marc *et al*., 1998).

The specific role that microtubules play in host–pathogen interactions is still open to conjecture. Clearly, reorientation of microtubules in incompatible interactions, and their disappearance with time in both compatible and incompatible interactions in the soybean–*P. sojae* system, means that cellular functions that are dependent on them will be dramatically altered. If, as has been proposed by Cyr & Palevitz (1995) and Gundersen & Cook (1999), the cytoskeleton, plasmalemma and cell wall operate as a continuum, then the cytoskeleton must have primary roles in cell-to-cell communication and intercellular signalling during host–pathogen interactions.

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