

Actin Microfilaments are Required for the Expression of Nonhost Resistance in Higher Plants¹

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We investigated the role of actin microfilaments in nonhost resistance of higher plants. Here we present several lines of evidence to indicate that microfilaments are indeed involved in blocking fungal penetration of nonhost plants. *Erysiphe pisi*, a pathogen of pea, normally fails to penetrate into nonhost plants such as barley, wheat, cucumber and tobacco. When tissues of these nonhost plants were treated with cytochalasins, specific inhibitors of actin polymerization, this fungus became able to penetrate and formed haustoria in epidermal cells of these plants. Moreover, treatment of these plants with various kinds and concentrations of cytochalasins allowed several other nonpathogenic fungi, *E. graminis hordei*, *E. graminis tritici*, *Sphaerotheca fuliginea*, *Colletotrichum graminicola*, *Mycosphaella pinodes*, *C. lagenarium*, *Alternaria kikuchiana* and *Corynespora melonis*, to also penetrate the cells of these plants. The degree of microfilament depolymerization varied depending on the kinds and concentrations of cytochalasins applied and we show that this is significantly correlated with the penetration efficiency of *C. graminicola*. This indicates that the polymerized, filamentous state of actin is necessary for plants to block fungal penetration. These results strongly suggest that actin microfilaments may play important roles in the expression of nonhost resistance of higher plants.

Key words: Higher plants — Microfilaments — Nonhost-resistance — Plant-microbe interaction.

A wide variety of fungi attempt to penetrate into plants, however, only a limited number of these interactions are successful. Thus in plant-fungal interactions resistance is the rule and susceptibility is the exception. This implies that all plants have a general host resistance and this idea has been termed nonhost resistance (Heath 1980, Jahnen and Hahlbrock 1988). While the mechanism of

Abbreviations: RH, relative humidity; DMSO, dimethylsulfoxide; PE, penetration efficiency; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; PB, Na-K phosphate-buffer; API, actin polymerization index.

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nonhost resistance remains elusive its very nature indicates that it must be widespread and highly conserved in the plant kingdom. The cytoskeleton meets these criteria and has been shown to respond dynamically in variety of situations (Hejnowicz and Sievers 1981, Hush and Overall 1992, Kadota and Wada 1992, Tanaka and Wakabayashi 1992, Traas et al. 1987) including the fungal penetration process (Kobayashi et al. 1992) making it a likely candidate for the general resistance response.

Microtubules and actin microfilaments may play a role in determining the success or failure of a penetration attempt by a nonpathogenic fungus. Recently, it was reported that the rearrangement of microfilaments and microtubules occurred in plant cells during fungal penetration process. Both of these cytoskeletal elements became radially arranged at the encounter site when a nonpathogenic powdery mildew, *Erysiphe pisi*, attempted to penetrate barley coleoptile cells (Kobayashi et al. 1992). While similar reorganizations of microfilaments and microtubules were observed in coleoptile cells of barley inoculated with a pathogenic powdery mildew fungus, *E. graminis* f. sp. *hordei*, the frequency of these rearrangements was much lower than that induced by the attempted penetration of the nonpathogen. Similarly, Gross et al. (1993) reported that attack of cultured parsley cells by a nonpathogen, (*Phytophthora infestans*) resulted in microfilaments becoming radially arrayed at penetration sites. They also showed that nonhost reactions such as rapid translocation of cytoplasm and movement of the nucleus towards the fungal penetration sites were inhibited by treating the parsley cells with an actin polymerization inhibitor. Although these reports imply a role for cytoskeletal components in the expression of plant resistance to nonpathogens, direct evidence is lacking.

Microfilaments are known to play important roles in a number of cellular functions such as sorting of macromolecules and organelles and signal transduction. If microfilaments are involved in nonhost resistance then it follows that nonpathogens would successfully penetrate plant cells when actin microfilament function is impaired. Since successive polymerization and depolymerization are required for cytoskeletal function it is possible to block processes that depend on actin microfilaments with actin polymerization inhibitors (Bretscher 1995).

In this paper we further investigate the role of actin microfilaments in nonhost resistance. If microfilaments are in-

volved in nonhost resistance of higher plants, a number of nonpathogens may penetrate successfully into plant cells when the function of microfilaments is blocked. We block microfilament reorganization with cytochalasins and observe the effects of this on resistance mechanisms in monocots (barley and wheat) and dicots (cucumber and tobacco) to fungal penetration.

Materials and Methods

Plant and fungal materials—Barley, *Hordeum vulgare* L. cv. Kobinkatagi, and wheat, *Triticum aestivum* L. cv. Norin 61, and cucumber, *Cucumis sativus* L. cv. Suoyo, were grown from seed in vermiculite in a growth chamber under fluorescent lights (ca. 11.8 W m⁻²), 70% RH and 12 h photo period at 20°C for barley and wheat and 16 h photo period at 25°C for cucumber. Tobacco, *Nicotiana tabacum* L. cv. Xanthi nc was seeded in soil and 2 weeks later seedlings with 2–3 fully developed leaves were transplanted to potted soil and grown in a growth chamber under the conditions used for cucumber.

Erysiphe graminis D.C. f. sp. *hordei* Em. Marchal race I, *E. graminis* D.C. f. sp. *tritici* Em. Marchal race t1 and *E. pisi* D.C. race 1 were maintained on barley (a susceptible cultivar Ichibanbosi), wheat (cv. Norin 4) and pea (cv. Midoriusui), respectively, under the conditions described for barley and wheat above. *Sphaerotheca fuliginea* (Schlechtendahl) Pollacci was maintained on cucumber under grown as above. *Colletotrichum graminicola* (Cesati) Wilson was maintained on oatmeal agar medium at 21°C under the continuous fluorescent light. *Mycosphaella pinodes* (Berkeley et Bloxam) Vestergren and *C. lagenarium* (Passerini) Ellis et Halsted were maintained on V-8 juice agar medium and potato sucrose agar medium, respectively, at 21°C in the dark. *Alternaria kikuchiana* Tanaka and *Corynespora melonis* (Cooke) Lindau were maintained on potato dextrose agar medium at 23°C in the dark. These fungi produced a great number of conidia during 7–14 day incubation. Suspensions of conidia of *C. graminicola*, *C. lagenarium*, *M. pinodes*, *A. kikuchiana* and *Cor. melonis* were prepared by adding sterilized distilled water to the culture, followed by rubbing the medium surface with a brush. The density of conidia in the suspensions was adjusted to 5 × 10⁵ conidia per ml using sterilized distilled water.

Inoculation and incubation—Coleoptiles of barley and wheat were excised from seedlings 8 days after sowing and single epidermal cell layers of partially dissected coleoptiles were prepared as described previously (Takamatsu et al. 1978). The primary leaf of cucumber and young, completely developed leaf of tobacco were harvested 10 days after seeding and 1–2 weeks after transplanting, respectively. Lower epidermises of leaves were removed carefully with a forceps, and then specimens were cut into 1 × 1 cm² pieces. Inner epidermis of dissected coleoptiles and upper epidermis of leaf pieces were inoculated with 40–50 freshly harvested conidia of *E. pisi*, *E. graminis hordei*, *E. graminis tritici* or *S. fuliginea* using a brush. For inoculation of other fungal conidia, 5 ml of conidial suspension were deposited on plant tissues. Inoculated tissues were floated on distilled water in watch dishes placed in a moist chamber at 20°C. Since calcium ion enhances powdery mildew infection (Takamatsu et al. 1978), tissues inoculated with these fungi were floated on 10 mM CaCl₂ solution.

Cytochalasin treatments—Cytochalasin A, B, C, D and E (Sigma Co.) were used to disrupt microfilaments and inhibit polymerization of G-actin (Thomas 1978, Wieland 1977, Yahara et al. 1982). All cytochalasins were dissolved in dimethylsulfoxide

(DMSO) at 2 mg ml⁻¹ for the stock solutions. These stock solutions were stored at –20°C until use and diluted to 1, 5, 10 and 20 µg ml⁻¹ with distilled water or 10 mM CaCl₂ solution immediately before use. The final concentration of DMSO in test solutions was adjusted to less than 1%. It was confirmed that 1% DMSO did not affect morphogenesis and/or penetration efficiency of test fungi. In control experiments, 1% DMSO was added to either 10 mM CaCl₂ solution or distilled water. Coleoptiles were floated on 100 µl of various concentrations of cytochalasins in watch dishes immediately after inoculation with test fungi. Leaf pieces were floated on 100 µl of various concentrations of cytochalasins in watch dishes and kept in vacuum chamber for 1 h (to enhance permeation of cytochalasin), followed by inoculation with fungal conidia. Subsequently they were incubated on 200 µl of fresh inhibitor solution. To estimate penetration efficiency of fungi, specimens were observed 36 h (*E. pisi*, *E. graminis* and *S. fuliginea*), 48 h (*A. kikuchiana* and *Cor. melonis*) and 72 h (*C. graminicola*, *C. lagenarium* and *M. pinodes*) after inoculation, respectively. Inoculated barley and wheat coleoptiles were directly observed using differential contrast microscopy (lens × 400, Zeiss Axiophot). Leaf tissues were cleared and stained by the method of Bruzzese and Hasan (1983) with a minor modification as follows. Tissues were placed on tissue paper soaked in decolouring solution (300 ml 95% ethanol; 150 ml chloroform; 125 ml 90% lactic acid; 150 g phenol; 450 g chloral hydrate) for 24–48 h, followed by washing on tissue paper containing distilled water for 24 h. Subsequently, specimens were stained with 0.1% aniline blue and observed with the microscope.

Penetration efficiency (PE) was determined by the following equation: PE = number of haustoria or infection hyphae/number of mature appressoria × 100 (%). Each experiment was repeated at least 3 times, and results were analysed by *t*-test.

Effect of short term treatments with cytochalasin A on PE of *E. pisi* on barley and wheat coleoptiles—A time course of short term treatments is shown in Figure 3a. Barley and wheat coleoptiles were used for the experiment. The coleoptiles which were inoculated with *E. pisi* were incubated on 10 mM CaCl₂ until the treatment. Then they were treated with 1 µg ml⁻¹ cytochalasin A for 2, 4, 6, 8, 10, 12 and 14 h after inoculation (Fig. 3a). After 1 h treatment, coleoptiles were moved on 10 mM CaCl₂ again and incubated until 24 h after inoculation. As a negative and a positive control, inoculated coleoptiles were incubated on 10 mM CaCl₂ including 1% DMSO or 1 µg ml⁻¹ cytochalasin A for 24 h, respectively.

Cytochemistry of microfilaments and evaluation of actin polymerization—The coleoptile system is convenient for cytochemical technique, because microfilaments in coleoptile cells can be readily stained without pre-stain treatments such as enzyme treatment and/or sectioning. Therefore, we chose this system to evaluate the effect of cytochalasins on microfilaments in plant cells.

Microfilaments in coleoptile cells were stained with rhodamine-phalloidin as described previously (Kobayashi et al. 1992) with a slight modification. Coleoptiles were treated with 1 mM *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (Sonobe and Shibaoka 1989) and 0.05% Triton X-100 in 20 mM Na-K phosphate-buffer (PB: pH 7.4, 150 mM NaCl) for 30 min. Specimens were transferred to 1% formaldehyde diluted with PB and treated for 10 min at room temperature. Subsequently, they were stained with rhodamine-phalloidin [3.3 mM rhodamine-phalloidin (Molecular Probes Inc., Eugene, Oregon) 2.5 µl in PB 50 µl per coleoptile] at room temperature for 60–90 min in a highly humid chamber. Specimens were rinsed once with PB for 3 min and mounted with 80% glycerol in distilled water containing 0.1% *p*-phenylene

diamine on glass slides, and observed with a Zeiss Axiophot microscope equipped with an epifluorescence attachment (filter set: BP546, FT580 and LP590).

To evaluate the effectiveness of different cytochalasins on microfilaments, actin polymerization index (API) was defined as follows (Fig. 5): 0, all microfilaments within a cell were completely depolymerized and no actin filaments were observed; 1, fragmentary microfilaments were observed in a part of the cell; 2, fragmentary microfilaments were observed throughout the cell; 3, filamentous microfilaments were arranged almost longitudinally or obliquely to the longitudinal axes of the cells (i.e. they were normal). API was determined in uninoculated barley and wheat coleoptile cells with or without cytochalasin treatment. The index was evaluated 20 cells each on 4 different coleoptiles (total 80 cells) 24 h after the onset of treatment, and average APIs were compared among treatments. In the untreated control coleoptiles, API was always 3.0.

Results

Effects of cytochalasins on penetration efficiency of E. pisi inoculated on monocots and dicots—Obligate parasite, powdery mildews, can not form haustoria in dead plant cells at all (Bushnell and Gay 1978). If the metabolism of host plant cells becomes largely abnormal by a certain reason, this group of fungi can not form haustoria in such plant cells, either. Furthermore, the morphogenesis of powdery mildew during early penetration process is highly synchronized. By these reasons, we decide to use *E. pisi* as a main test fungus in this study. In the absence of cytochalasins *E. pisi* failed to penetrate the cells of all test plants although papillae were formed at the attempted penetration site (Fig. 1, 2a). However, in the presence of cytochalasin fungal penetration of plant cells and formation of haustoria did occur (Fig. 1, 2b). The frequency of penetration was dependent on the type of plant, the kind of cytochalasin and the concentration of cytochalasin used (Fig. 1). Cytochalasin A was most effective on monocot coleoptiles. The highest PE, 63%, was obtained in barley cells by treatment with $1 \mu\text{g ml}^{-1}$ cytochalasin A, but PE was decreased to less than 10% by treatment with more than $5 \mu\text{g ml}^{-1}$ of cytochalasin A (Fig. 1a). Although cytochalasin E treatment allowed *E. pisi* to penetrate into barley cells, higher concentrations of this cytochalasin were required compared to cytochalasin A (Fig. 1a). Cytochalasin B, C and D also enhanced PE but had weak effects. Cytochalasin A, B, C and E has no effects on morphogenesis including in a haustoria formation of *E. pisi* in its host plant, pea, except for high concentrations ($10 \mu\text{g ml}^{-1}$ and higher). Only cytochalasin D severely affected the growth of *E. pisi* even at lower than $1 \mu\text{g ml}^{-1}$. The reason why PE of *E. pisi* at high concentration treatments and cytochalasin D treatments is lower than other treatments is explained by the direct effects of cytochalasins on the fungal growth. Effects of cytochalasins on PE of in wheat coleoptiles were similar to those in barley coleoptiles. One exception was cytochalasin E where PE increased with increased concen-

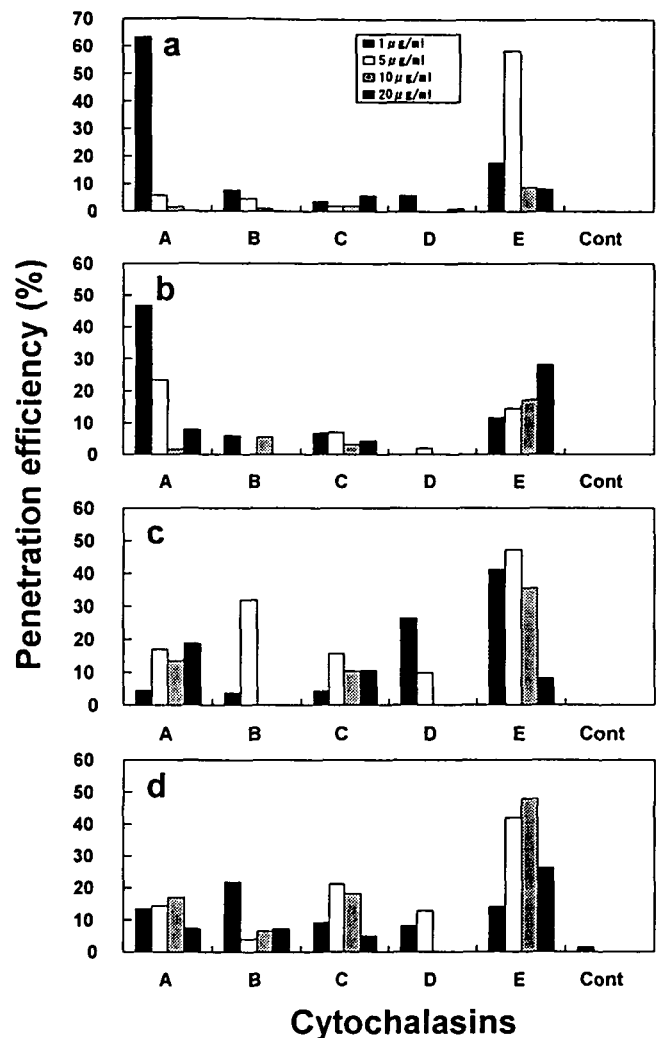


Fig. 1 Effects of cytochalasins on penetration efficiency (PE) of *E. pisi* in barley (a), wheat (b), cucumber (c) and tobacco (d). Cont: untreated control. Haustorial formation of *E. pisi* was observed in coleoptile cells of barley (a) and wheat (b) and in epidermal cells of leaves of cucumber (c) and tobacco (d) 36 h after inoculation, when these plants were treated with cytochalasins.

tration of cytochalasin (Fig. 1b).

In contrast to the monocot coleoptiles, cytochalasin E was most effective on the dicot leaves. In cucumber, PE's of *E. pisi* were 41 and 47% at 1 and $5 \mu\text{g ml}^{-1}$ cytochalasin E, respectively. However, PE was decreased with $10 \mu\text{g ml}^{-1}$ treatment (Fig. 1c). Other kinds of cytochalasins were not as effective at increasing PE. Results with tobacco cells were similar to those of cucumber (Fig. 1d).

Effects of short term treatments with cytochalasin A on PE in both barley and wheat coleoptile cells—To determine a critical timing of cytochalasin treatment to allow nonpathogens to penetrate into nonhost plants successfully and to exclude the possibility that the increase in PE might

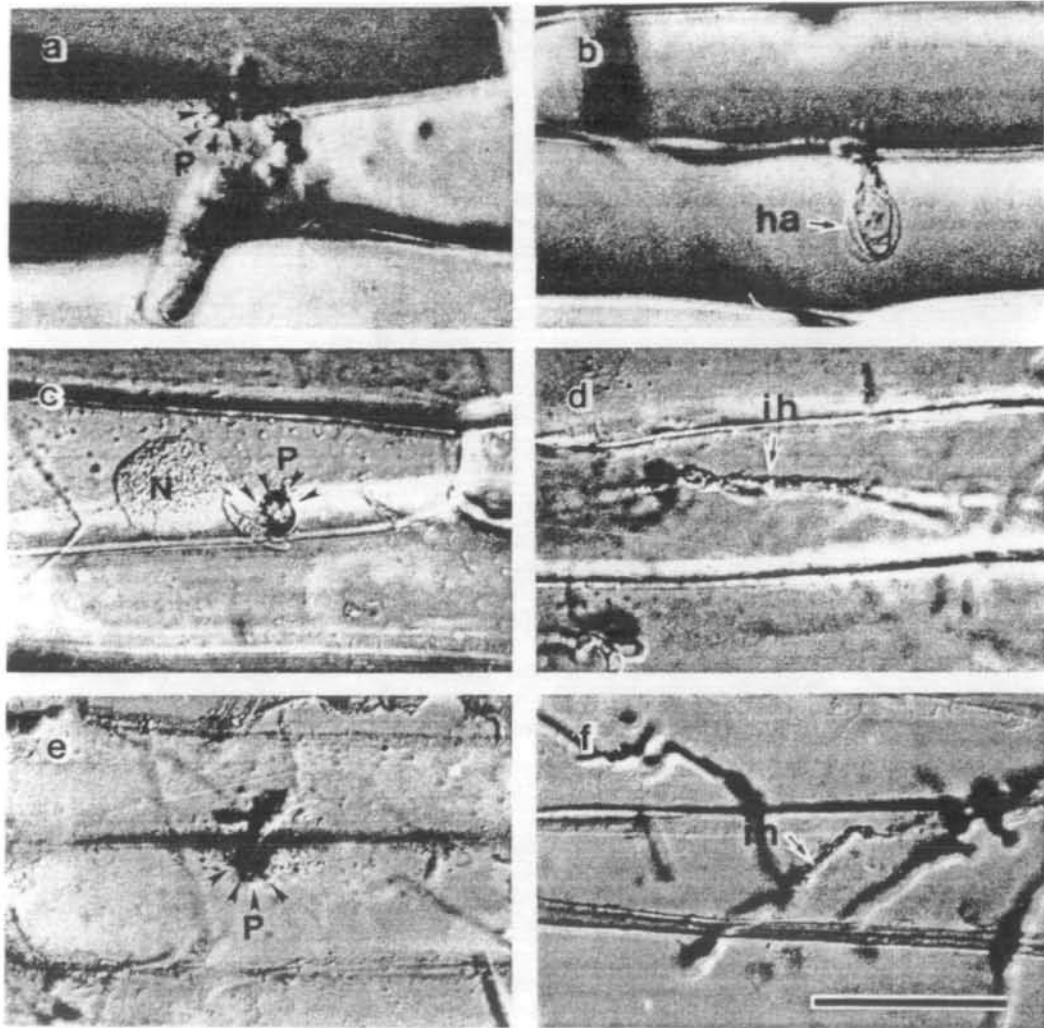


Fig. 2 Light micrographs showing of penetration attempts by *E. pisi* (a, b), *C. graminicola* (c, d) and *M. pinodes* (e, f) in wheat coleoptile treated with $1 \mu\text{g ml}^{-1}$ cytochalasin A. All fungi always failed to penetrate into cells and papillae (P) were observed at penetration sites (a, c, e). When coleoptile cells were treated with cytochalasins, these fungi successfully penetrated into cells and formed haustoria (ha) or elongating infection hyphae (ih). The scale bar represents $50 \mu\text{m}$.

be a result of these secondary effects of cytochalasins, barley and wheat coleoptiles were treated with $1 \mu\text{g ml}^{-1}$ cytochalasin A for 1 h at various times after inoculation (Fig. 3).

The cytoplasmic streaming of coleoptile cells was mostly ceased and most of microfilaments were depolymerized ($\text{API} = 0.2$ in both plants) within 1 h by the treatment with $1 \mu\text{g ml}^{-1}$ cytochalasin A. In the positive control, PE of the nonpathogen reached to 60% and 39% on barley and wheat, respectively, whereas no successful penetration was observed in the negative control of both plants. When coleoptiles were treated with cytochalasin A for only a short term, the effect of the inhibitor was essentially identical to that in the 24 h successive treatment, regardless of the initiation times of the short time treatment except 12 h and 14 h.

On barley coleoptiles, the 1 h treatment was initiated from 0 h to 10 h after inoculation, PEs of the nonpathogen (47–63%) were not significantly different from that of the positive control (Fig. 3b1). On the other hand, when coleoptiles were initiated to be treated with cytochalasin A from 12 h and 14 h, PEs were significantly lower than the positive control ($P < 0.01$) and not significantly different from the negative control ($P > 0.05$). The same tendency was obtained on wheat coleoptiles (Fig. 3b2). As shown in Figure 3a, *E. pisi* initiate to penetrate into coleoptile cells 8–10 h after inoculation. Therefore, only when coleoptiles were treated with cytochalasin A before the initiation of the fungal penetration, the treatment allowed the nonpathogen to penetrate into these nonhost plant cells successfully. Also the results show effects of the short term treatment of cyto-

chalasin A were maintained, at least for 10 h, because the fungus was able to penetrate successfully, even if the treatment was initiated from the time of inoculation (0 h). To determine when the resistance of cells was recovered after removal of the inhibitor, inoculation time of the fungus was delayed gradually. The ability to express resistance recovered mostly by 30 h after removal of the inhibitor (API=2.0 and 2.3 in barley and wheat cells, respectively),

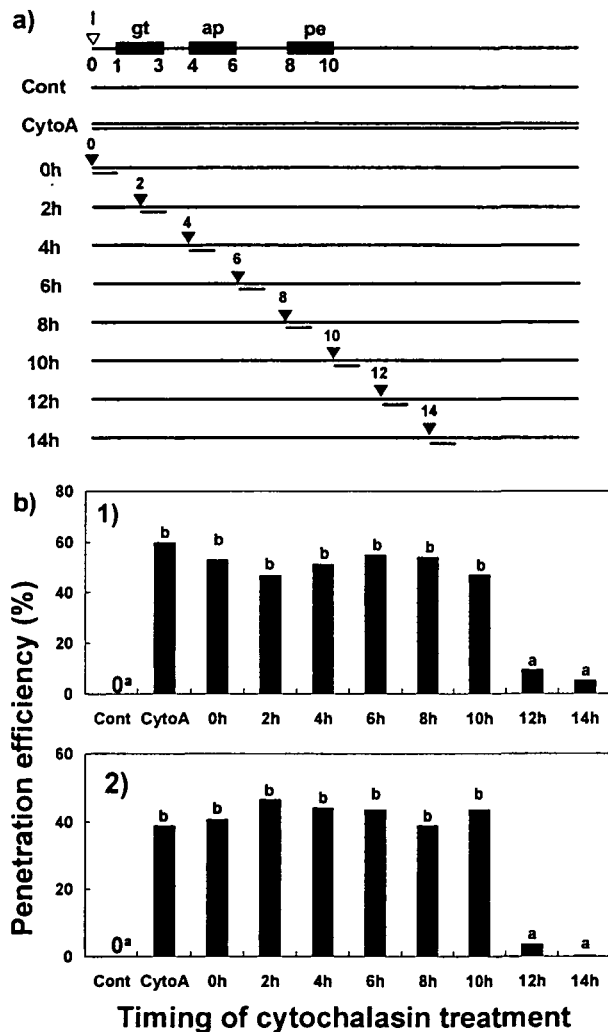


Fig. 3 Effect of short term treatment of $1 \mu\text{g ml}^{-1}$ cytochalasin A on PE of *E. pisi* deposited on barley (b-1) and wheat (b-2) coleoptiles. (a) The time course of treatments with cytochalasin A for 1 h at 0, 2, 4, 6, 8, 10, 12 and 14 h after inoculation. Double line: cytochalasin A treatment. Single line: duration of the incubation on 10 mM CaCl_2 . Cont: untreated control. CytoA: successive treatment with $1 \mu\text{g ml}^{-1}$ cytochalasin A from 0 h to 24 h after inoculation. I: inoculation, gt: germ tube formation, ap: maturation of appressoria, pe: beginning penetration. (b) PE of *E. pisi* at 24 h after inoculation. Cont: untreated control. CytoA: successive treatment with $1 \mu\text{g ml}^{-1}$ cytochalasin A from 0 h to 24 h after inoculation. Different character means statistically significant to successive treatment at 1% level by t-test.

as indicated by 0–16% of PE (data not shown). Cytoplasmic streaming in barley and wheat coleoptile cells recovered at the same time. In these cells, papilla formation was also observed beneath appressoria which failed in penetrating.

Effects of cytochalasin on penetration efficiency of nonpathogens on monocots and dicots—Cytochalasin enhanced the ability of nonpathogens to penetrate plant cells. When *C. graminicola* and *M. pinodes* were inoculated on their nonhost plant, wheat, in the absence of cytochalasins they failed to penetrate. The penetration attempt was aborted following the formation of papillae (Fig. 2c, e). However, when the coleoptiles were treated with cytochalasins, both fungi penetrated into the plant cells and elongated intracellular hyphae were observed (Fig. 2d, f). Similar effects of cytochalasins on penetration attempts of nonpathogenic fungi, were observed for barley coleoptiles and tobacco leaf pieces (Fig. 4). Although some nonpathogens such as *E. graminis* f. sp. *hordei* and *tritici*, *S. fuliginea* and *C. lagenarium* were able to penetrate into tobacco cells

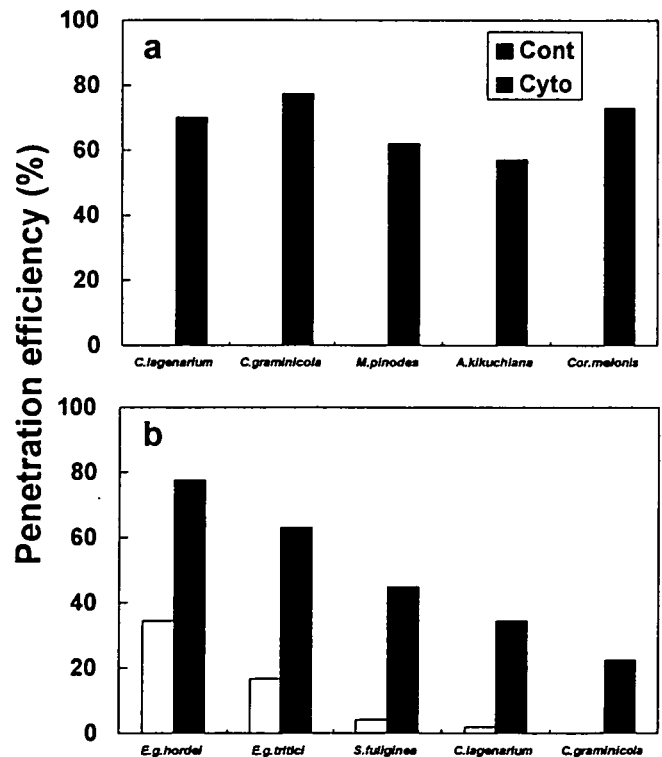


Fig. 4 Effects of cytochalasins on PE of various fungi in their nonhost plants, barley (a) and tobacco (b). Cont: untreated control. Cyto: $1 \mu\text{g ml}^{-1}$ cytochalasin A for barley and $5 \mu\text{g ml}^{-1}$ cytochalasin E for tobacco. Haustorial formation of *E. graminis* f. sp. *hordei*, *E. graminis* f. sp. *tritici* and *S. fuliginea* were observed 36 h after inoculation, and elongating infection hyphae of *C. lagenarium*, *C. graminicola* and *M. pinodes* were observed 72 h and *A. kikuchiana* and *Cor. melonis* were observed 48 h after inoculation.

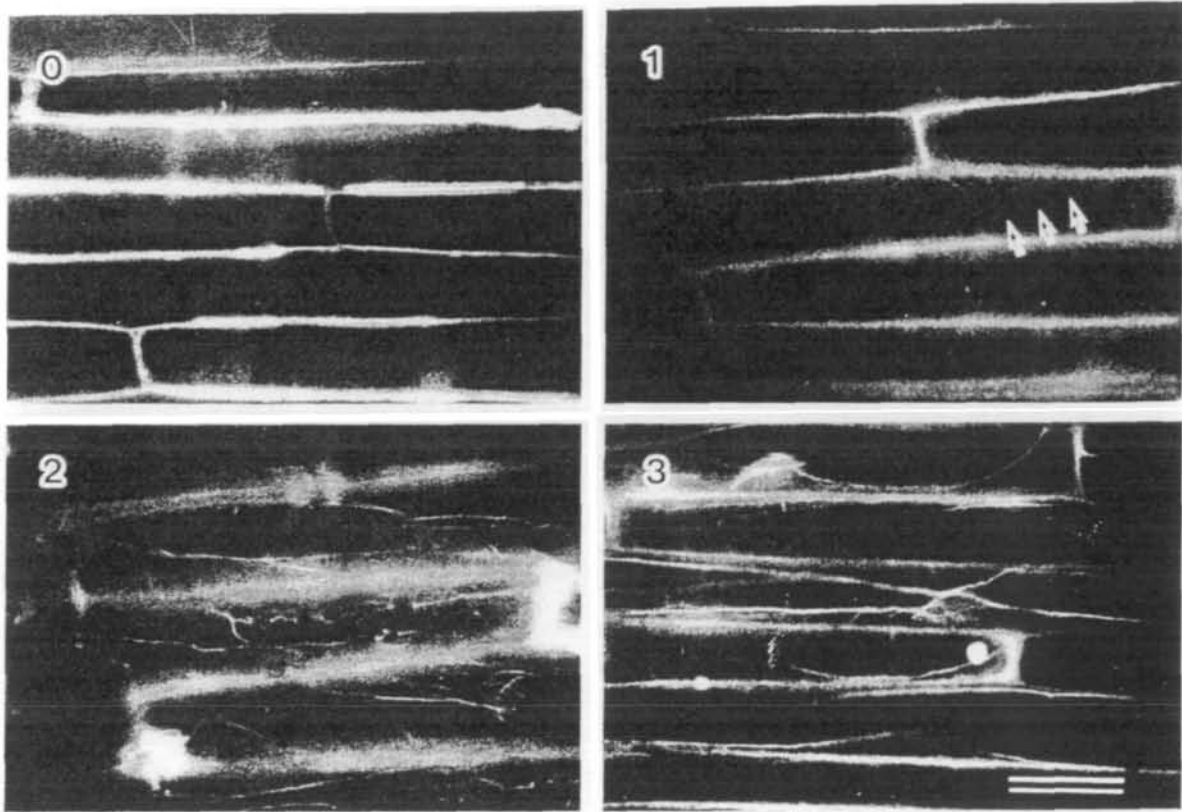


Fig. 5 Micrographs to indicate actin polymerization index (API). Degrees of actin polymerisation in barley coleoptile treated with cytochalasins for 24 h are illustrated. Numbers (0-3) at the upper left corner in each frame show an index representing each degree of polymerization and detailed explanation of the degree is described in text. Similar criteria were applied to determine API of wheat coleoptile. The scale bar represents 50 μm .

without the cytochalasin treatment (PE=2-34%), PE's of all test fungi were significantly increased by cytochalasin treatment (PE=22-78%, $P < 0.01$) (Fig. 4b). Similarly, when wheat and cucumber were treated with $1 \mu\text{g ml}^{-1}$ cytochalasin A and $5 \mu\text{g ml}^{-1}$ cytochalasin E, respectively, *C. graminicola* penetrated successfully into cells of these plants, giving 36 and 62% PE, respectively. Penetration was infrequent without cytochalasin treatment (PE=3 and 6%, respectively). When *E. graminis* f. sp. *tritici* and *S. fuliginea* which are also nonpathogens of barley were inoculated on barley coleoptiles, these fungi were able to penetrate successfully into the cells at considerably high PEs (83% and 85%, respectively). However, their PEs on barley cells were not affected by cytochalasin treatments (data not shown). Nonpathogens which attempted to penetrate into barley or tobacco cells but never or hardly ever succeeded in penetration, were able to penetrate their nonhost plants whose microfilaments were functionally impaired by the inhibitor, cytochalasin.

PE of C. graminicola and actin polymerization are correlated in both barley and wheat cells—We evaluated the effects of cytochalasins on the microfilaments of wheat and

barley coleoptiles (Fig. 5 and 6). In untreated control cells of both plants, microfilaments were observed as thin filamentous structures or thick bundles which were aligned parallel or obliquely (Fig. 5-3). Treatment with cytochalasin for 24 h resulted in varying degrees of microfilament disruption (Fig. 5-0, -1 and -2). The amount of disruption depended on the kind and concentration of the cytochalasin used (Fig. 5). As low as $1 \mu\text{g ml}^{-1}$ of cytochalasin A forced almost all microfilaments to depolymerize in cells of both plants (API=0, as shown in Fig. 5-0). Cytochalasin E also effectively destroyed microfilaments of both plants, although a few intact microfilaments were observed in cells treated with $1 \mu\text{g ml}^{-1}$. Microfilament depolymerization occurred with higher concentrations of cytochalasins B and D were API ranged from 0.2 to 2.9 (Fig. 6). In contrast, cytochalasin C had little effect on microfilament distribution (Fig. 6).

To examine whether successful penetration of nonpathogens was linked with depolymerization of microfilaments in plant cells we compared PE of a nonpathogen with its API in barley and wheat cells treated with cytochalasins. We also considered the effects of the cytochala-

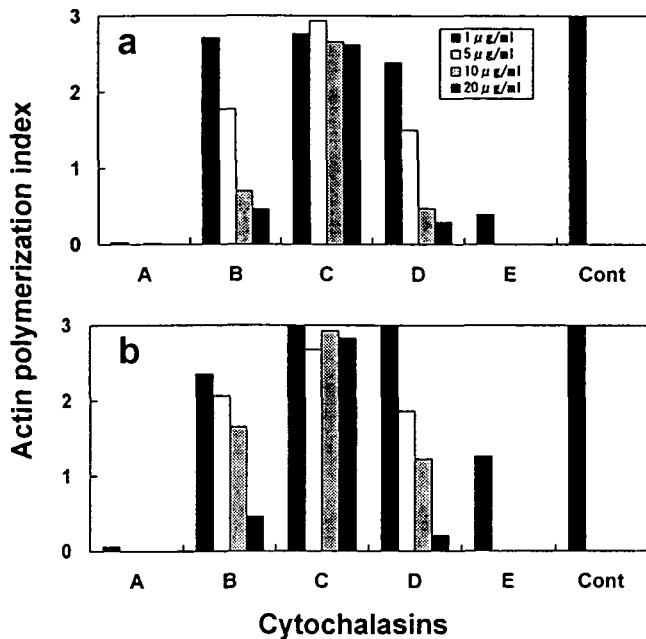


Fig. 6 Effects of cytochalasins on microfilaments in barley (a) and wheat (b) cells. API shows average of 20 cells each of 4 coleoptiles treated with cytochalasins for 24 h (total 80 cells each). Higher indexes show lower effects of cytochalasins to depolymerize actin. Cont: untreated control.

sins on fungi because it is known that their microfilaments are also disrupted by cytochalasins (Thomas 1978). High concentrations of cytochalasins ($10 \mu\text{g ml}^{-1}$ and more) inhibited haustorial formation of *E. pisi* in its host plant, pea (Kobayashi et al. 1997). We found that growth and morphogenesis of all fungi used except *C. graminicola* were affected by cytochalasin concentrations of $10 \mu\text{g ml}^{-1}$ or lower. *C. graminicola* was not inhibited by $10 \mu\text{g ml}^{-1}$ and only rarely perturbed by $20 \mu\text{g ml}^{-1}$ of each of the cytochalasins. Thus, we decided to analyze a correlation between PE and API using *C. graminicola* and inhibitor concentration that did not hinder its growth. As illustrated in Figure 7, PE of this fungus and API in barley and wheat coleoptiles are correlated in both barley and wheat cells ($r_s = -0.8245$ and -0.8756 , respectively, $P < 0.01$, Spearman's ranked correlation coefficient analysis, Zar 1984). A similar significant correlation between PEs and API was also obtained when barley and wheat coleoptiles were inoculated with *E. pisi*, if results of high concentration treatments were omitted (data not shown).

Discussion

We have shown that fungi can successfully penetrate their nonhost plants when the actin microfilaments of the plant are disrupted. Furthermore, we found that *E. pisi*

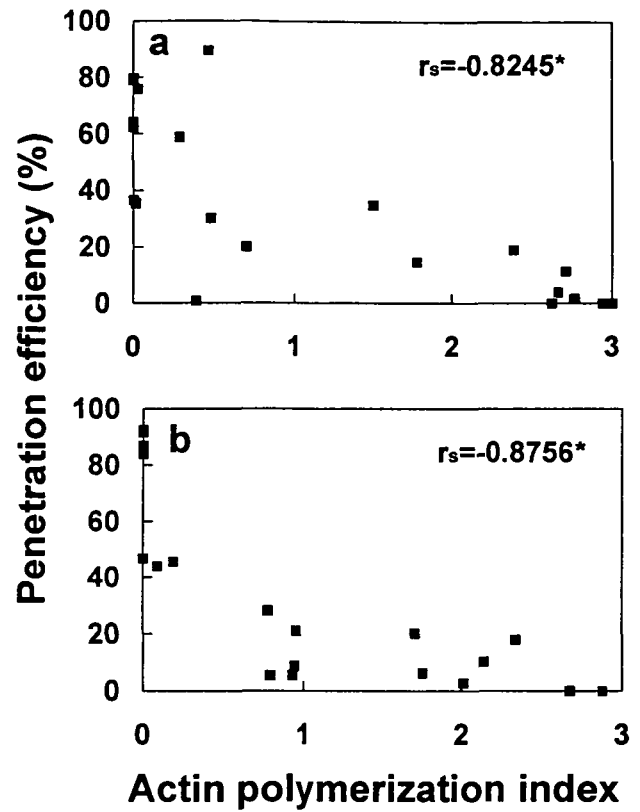


Fig. 7 A correlation between penetration efficiency and actin polymerization index in barley (a) and wheat (b) cells. Each plot shows the penetration efficiency and actin polymerization index at a kind and a concentration of cytochalasin analyzed by Spearman's ranked correlation coefficient analysis. r_s : sample correlation coefficient. *: Statistically significant at 1% level by t-test.

was more successful at penetrating cells when most microfilaments had been depolymerized. We also demonstrate that the ability of *C. graminicola* to penetrate nonhost plant cells is correlated with the degree of microfilament depolymerization. These observations imply that the polymerized, filamentous state of actin is necessary for plants to block fungal penetration. In addition to these results, short term treatments of cytochalasin A allowed the nonpathogen to penetrate into barley and wheat coleoptile cells. Thus, it is hard to consider that the results were caused by secondary effects of the microfilament inhibitor, cytochalasin. This strongly suggests that microfilaments are involved in the expression of plant resistance to nonpathogens making it likely that actin plays an important role in nonhost resistance. Although a distinct difference was shown between genetically defined race-cultivar resistance and nonhost resistance (Heath 1980, Jahnen and Hahlbrock 1988), genetic background and/or molecular bases concerning nonhost resistance have not been available. We have demonstrated the possibility of the involvement of the cytoskeleton in

defense reactions of barley plant (Kobayashi et al. 1992, Kobayashi et al. 1997). The present results strongly support our hypothesis and, furthermore, extend the possibility to nonhost resistance of plants generally. To our knowledge, this is the first report to demonstrate the possibility that a specific cellular component such as actin may be involved in nonhost resistance in plants.

Cytochalasins are secondary metabolites of several fungi and their effects are different depending on test organisms (Thomas 1978, Yahara et al. 1982). Here we show that the ability of cytochalasins to disrupt actin microfilaments was dependent on the type of and concentration of cytochalasin used. At high concentrations of cytochalasins, actin microfilaments of coleoptiles were disrupted but the fungi could not penetrate successfully because the growth of the fungi has been inhibited by cytochalasin. This is not surprising since actin is known to play an important role in hyphal growth (Heath 1990).

Usually, the cytoplasmic streaming and reconstituted actin microfilaments recovered in plant cells after short-term treatment with cytochalasins within a few hours during wash of the specimens (Ishigami and Nagai 1980, Traas et al. 1987). However, the effects of 1 h treatment with cytochalasin A lasted for as long as 30 h in this study. Such a slow recovery of cytoplasmic streaming and resistance to fungal penetration in this system might be attributable to cytochalasin carried over into plant tissues.

Nonhost resistance obviously consists of multiple mechanisms rather than a specific single mechanism. In the present study, we demonstrated that some nonpathogens were able to penetrate into a certain nonhost plant. The results suggest that the penetration stage is not an only critical point to determine success or failure of fungal penetration. In some combinations of plants and fungi, when plant cells were treated with cytochalasin, the nonpathogenic fungus successfully penetrated into nonhost plant cells, but hypersensitive cell death also occurred in the cells, resulting in termination (or cessation) of the fungal growth (unpublished data). Although hypersensitivity is one of the important nonhost reactions at post-penetration stage (Heath 1980, Jahnen and Hahlbrock 1988), formation of a cell wall apposition (papillae) is also a possible significant mechanism of nonhost resistance at fungal penetration stage. When nonpathogens failed to penetrate into plant cells papillae were formed at sites of attempted penetration. Papillae formation is thought to be a general resistance mechanism of plant cells that it is somehow related to nonhost resistance (Ride 1985). As shown in Figure 3, it was necessary to depolymerize microfilaments of plants before initiation of the fungal penetration, in order to allow *E. pisi* to penetrate into nonhost plant cells successfully. This result suggests that microfilaments function in expression of resistant reaction at the stage of fungal penetration. In addition to papilla formation, a number of reports

have demonstrated that defense-related autofluorescent materials and organelles are present at and around fungal penetration sites at the incipient stages of infection process (Aist and Israel 1985, Bayles et al. 1990, Gold et al. 1986, Kunoh et al. 1985a, b, 1982). Interestingly, we found that cytochalasin A suppressed these defense-related reactions induced in barley coleoptile cells by attempted penetration of *E. pisi*, such as cytoplasmic aggregation, papillae formation and accumulation of autofluorescent materials, proteins and carbohydrates (Kobayashi et al. 1997). In this study, we also found that the treatment of cytochalasins strongly suppressed papilla formation in all plants used in the study. Since it is well known that microfilaments play central roles in movement of organelles (Staiger et al. 1994, Williamson 1986) and sorting of molecules (Singer 1992, Sundell et al. 1991) in cells, it is plausible that one of the major functions of microfilaments in expression of nonhost resistance may be polarization of organelles and/or defense-related compounds at fungal penetration sites. Further studies are required to elucidate roles of microfilaments and their mechanism in expression of nonhost resistance in more detail.

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