Expression of Tolerance to the Host-Specific Toxin of *Alternaria alternata* (AT Toxin) in Cultured Cells and Isolated Protoplasts of Tobacco

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ABSTRACT

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Crude AT toxin extracted from the culture filtrate of *Alternaria alternata* pathotype tobacco was applied to the suspension-cultured cells of tobacco cultivars that differed in susceptibility to the fungus, the causal organism for tobacco brown spot disease. Crude AT toxin at a concentration of 3% (v/v) inhibited cell growth in cell lines of all the tobacco cultivars, irrespective of the resistance of the cultivar to the fungus. However, cell mortality values, as judged by a vital staining method, were much lower for cell lines of resistant plants than for those of susceptible ones. These differences were also observed when leaf mesophyll protoplasts cultured for 4 days were treated with the toxin for 24 hr. Cell division and further colony formation occurred with protoplasts from resistant, but not from susceptible, plants. Thus, application of AT toxin to cultured cells or protoplasts might be an efficient alternative for evaluating the degree of resistance of tobacco lines to tobacco brown spot.

Tobacco brown spot is a major foliar disease throughout tobacco production areas of the world. The causal organism, *Alternaria alternata* (Fr.) Keissl. pathotype tobacco, is a fungal pathogen that parasitizes the tobacco leaves at a late stage of maturity. Breeding of disease-resistant cultivars, which may be considered the least expensive and most practical approach to combating this disease, has not been effective in the control of brown spot due to lack of a reliable artificial inoculation method. Evaluation of disease resistance is the most difficult step in breeding brown spot resistant cultivars. Wide variability in response among individuals within a given genotype, and even among tissues of an individual, indicates that resistance is a dynamic condition dependent upon not only genetic, but also numerous environmental factors.

It has been shown that *A. alternata* pathotype tobacco produces several metabolites that can reproduce a part or all of the symptoms of this disease (7). Recently, a new metabolite, AT toxin, was isolated and characterized by Kohmoto et al (4). The AT toxin can induce typical symptoms that consist of a necrotic lesion surrounded by a chlorotic area. Although this toxin did not induce any symptom to nonhost plant species, differential reactions between susceptible and resistant tobacco cultivars have also been observed (5). Thanutong et al (11) directly used this toxin to select brown spot resistant tobacco out of protoplast-derived colonies.

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The objective of this study was to measure the effect of AT toxin on intact tobacco plants as well as on cultured cells, a system that may provide a simpler physiological state than that found in intact plants. The goal of this work was to develop a new artificial screening method featuring the application of AT toxin instead of the fungus itself.

MATERIALS AND METHODS

Plant materials. Three cultivars of tobacco (*Nicotiana tabacum* L.) susceptible to tobacco brown spot, i.e., Bright Yellow 4, Burley 21, and Tsukuba 1, were used in this study. The cultivar Tsukuba 1 was used only in the protoplast experiments. *N. tabacum* 'Beinhart 1000-1' and *N. suaveolens* Lehm. were used as the resistant controls. Reciprocal F_1 hybrids between Beinhart 1000-1 (resistant) and Burley 21 (susceptible) were also used.

Cell cultures. Leaf segments from aseptically growing seedlings were excised and placed on callus-inducing medium consisting of Linsmaier and Skoog basal salts (6) supplemented with 3 mg/L naphthaleneacetic acid (NAA), 3 mg/L indole-3-acetic acid, and 0.1 mg/L kinetin. Friable calli were transferred to a liquid medium of the same composition as that used to induce calli. Liquid suspension stock cultures were maintained in the dark on a gyratory shaker (125 rpm) by diluting fourfold into fresh medium every week.

Isolation and culture of protoplasts. Mesophyll protoplasts were isolated from young plants at 6–10 wk from seed

Table 1. Reaction of leaves of different cultivars to AT toxin[a]

Cultivar or species	Pathogenic								Nonpathogenic	
	Toxin concentration								Toxin concentration	
	100%		10%		1%		0.1%		100%	
	Necrosis	Chlorosis	Necrosis	Chlorosis	Necrosis	Chlorosis	Necrosis	Chlorosis	Necrosis	Chlorosis
Bright Yellow 4	90[b]	90	70	70	10	10	0	0	0	0
Burley 21	100	100	100	100	60	60	0	10	0	0
Beinhart 1000-1	60	0	70	10	0	0	0	0	0	0
Nicotiana suaveolens	0	0	0	0	0	0	0	0	0	0
F_1 (Beinhart 1000-1 × Burley 21)	60	30	40	20	0	0	0	0	0	0
F_1 (Burley 21 × Beinhart 1000-1)	50	10	10	0	0	0	0	0	0	0

[a] Reaction measured 4 days after toxin application.
[b] Percent of application points giving symptoms.

sowing by immersing 1-mm-wide leaf strips in an enzyme solution consisting of 1.0% cellulase R-10 (Onozuka), 0.25% macerozyme R-10 (Onozuka), 0.5% dextran sulfate, and 0.6 M mannitol for 14 hr. Protoplasts were filtered through one layer of Miracloth (CalBiochem), collected by centrifugation (200 rpm for 5 min), and rinsed with 0.6 M mannitol. This cleaning process was repeated three times. Finally, in order to remove debris, the protoplast suspension was layered onto 0.6 M sucrose and centrifuged at 500 rpm for 10 min. Protoplasts were cultured in a medium consisting of Nagata and Takebe salts (9), 3 mg/L NAA, 1 mg/L 6-benzylaminopurine, 17 g/L sucrose, and 0.45 M mannitol, at 25 C in the dark for the first three days, followed by incubation under dim light conditions (about 500 lx) for the next three days, and then growth under bright light (2,000 lx).

Isolation of AT toxin. AT toxin was isolated by a modification of the methods of Thanutong et al (11). Isolate number 122 of *A. alternata* pathotype tobacco, isolated at Utsunomiya Experiment Station of Japan Tobacco Inc., was used in this study. Cultures of *A. alternata* were grown in stationary liquid culture in the medium of Mikami et al (8) at 28 C under UV lights for 3 wk. The cultures were filtered through filter paper (Whatman No. 2). Activated charcoal was then added to the culture filtrate to give a final concentration of 0.5% (w/v). After 30 min, the charcoal was collected and washed with water. The toxin was eluted by washing the charcoal with 70% acetone. The acetone was removed by evaporation. The water phase was adjusted to a pH of 4.0 with 1 N HCl and extracted with ethyl acetate to remove tenuazonic acid, another major metabolite of the fungus. The water phase was concentrated in vacuo at 40 C. The residue was diluted with distilled water (1/100 volume of the initial culture filtrate), and the pH was adjusted 6.0 with 1 N NaOH. This preparation was referred to as 100% crude AT toxin.

Table 2. Viability[a] of the suspension-cultured cells treated with various doses of crude AT toxin

Cell line	Toxin concentration					
	0%	0.5%	1%	2%	3%	5%
			24 Hr after treatment			
Bright Yellow 4	88.9 (3.0)[b]	···	81.8 (3.1)	96.1	8.6 (4.8)	···
Burley 21	85.9 (3.0)	59.9	2.1 (2.0)	5.3	2.8 (2.0)	0
Beinhart 1000-1	91.0 (1.2)	85.7	89.7 (2.0)	91.8	79.7 (2.5)	77.3 (5.0)
Nicotiana suaveolens	91.1 (1.3)	89.0	79.6	···	84.8 (6.6)	81.5 (5.2)
			48 Hr after treatment			
Bright Yellow 4	87.0 (5.1)	···	87.9 (0.8)	81.9	11.4 (9.2)	···
Burley 21	83.6 (3.5)	56.7	1.9 (1.9)	0.6	1.6 (1.6)	0
Beinhart 1000-1	91.5 (1.6)	77.9	84.5 (5.6)	90.0	80.7 (4.2)	70.4 (6.3)
N. suaveolens	87.9 (2.7)	86.8	73.1	···	81.9 (1.3)	82.5 (1.0)

[a] Cell viability was estimated by staining with phenosafranin. More than 300 cells were counted for each plot.
[b] Each value with parentheses is the average of three separate experiments. Standard deviation is in parentheses, and the value without parentheses shows the average of a single experiment.

Application of AT toxin to intact leaves. Leaves were excised from plants 8 wk after transplanting. Six aliquots of 10 μl of the crude AT toxin at a given concentration were applied to the surface of each leaf. The leaves were then incubated in the dark at 25 C in 100% RH. After 4 days of incubation, the number of both necrotic and chlorotic lesions was counted for each leaf. The degree of resistance was based on the rate of lesion formation of both types, regardless of the severity of the lesion.

Treatment of cultured cells with AT toxin. A filter-sterilized (0.22 μm) solution of AT toxin was added to 3-day-old suspension cell cultures. About 0.1 ml of the suspension was harvested at given time intervals. The cells were stained with 0.01% phenosafranin, and the ratio of the stained (dead) and unstained (alive) cells was determined. Growth of the cultured cells was determined from the volume of cells present in a centrifugation tube after settling for 20 min.

Treatment of protoplasts with AT toxin. AT toxin solution dissolved in 0.6 M mannitol was added at the final concentration of 3.0% (v/v) to a population of protoplasts cultured for 0, 2, and 4 days. Treated protoplasts were maintained at 25 C in the dark for 24 hr. The toxin was then removed from the protoplast suspension by washing with 0.6 M mannitol three times. At various time intervals, small volumes of the protoplast suspension were harvested and stained with 0.01% phenosafranin for the determination of cell mortality. After the untreated protoplasts started to divide, the ratio of dividing cells to nondividing cells was measured for the treated population.

RESULTS

Treatment of intact leaves with AT toxin. *N. suaveolens* and *N. tabacum* 'Beinhart 1000-1', which have been shown to be resistant to tobacco brown spot (10), produced fewer chlorotic lesions even when an undiluted solution (100%) of AT toxin was applied (Table 1). In contrast, when the toxin was applied to leaves of two susceptible tobacco cultivars, Bright Yellow 4 and Burley 21, typical symptoms of this disease were induced at concentrations as

low as 1%. Although Beinhart 1000-1 produced a few necrotic lesions at higher concentrations, they were much smaller and fainter than those observed in the susceptible cultivars. Among the susceptible cultivars, Burley 21 appeared to be more sensitive to the toxin, which is consistent with the observation that white types of tobacco carrying yb_1yb_1,yb_2yb_2 genes tend to form larger lesions in response to *A. alternata* infection than normal green tobacco. The reciprocal F_1 hybrids between Beinhart 1000-1 and Burley 21 exhibited a reaction that was similar to that of the resistant parent.

Within a given cultivar, however, symptoms induced by AT toxin were affected by several factors, such as stalk position and nutrient conditions, as is the case when the plants are inoculated with the fungus (7). For example, the lower leaves tended to form larger lesions than the higher ones in a given individual (*data not shown*).

Reaction of the cultured cells. Various concentrations of AT toxin were applied to well-established suspension cell cultures of four genotypes. Frequencies of viable cells at various toxin concentrations are listed in Table 2. At the concentration of 0.5%, little effects were observed except with Burley 21 cells, which showed slightly lower viability than untreated cells. High sensitivity of cultured cells of Burley 21 was again consistent with the earlier observation on intact leaves. At a concentration of 3%, differential reactions were observed between susceptible and resistant lines. Therefore, the dose of 3% was used throughout the following experiments.

AT toxin was added to suspension culture cells at a final concentration of 3% and the growth of the treated cells was measured. Figure 1 shows the growth of cells of Burley 21 and Beinhart 1000-1 in the presence or the absence of the toxin. Neither of the cell lines showed any sign of growth in the presence of the toxin. This was also true when much lower concentrations (0.5%) were applied to the cultures. Irrespective of the degree of the resistance, cell growth was inhibited by the toxin in all the cell lines used in this study (*data not shown*). Thus, in terms of cell growth, there appeared to be no differential response between resistant and susceptible cultivars.

When cell mortality was measured (by the staining of cells with phenosafranin), cultures of the susceptible cell lines Bright Yellow 4 and Burley 21 had greater than 90% mortality 24 hr after toxin application (Fig. 2, Table 2). Cell lines derived from Beinhart 1000-1 and *N. suaveolens* exhibited tolerance to the toxin with 80.7 and 81.9%, respectively, of the cells surviving 48 hr after treatment.

Differential staining reaction by phenosafranin has been reported to discriminate dead from live cells (12). Biological significance of this staining was confirmed in separate experiments from those shown in Table 2. Frequencies of unstained cells of Burley 21 and Beinhart 1000-1 that were not exposed to AT toxin were 88.2 and 97.1%, respectively. After 24 hr of 3% AT toxin treatment, almost all of the cells of the Burley 21 cell line were stained. After a thorough rinse with fresh liquid medium, these cells were plated onto agar medium that was free of the toxin. Even 60 days after the plating, none of the cells proliferated. In contrast, when the Beinhart 1000-1 cell line was treated with the toxin for 24 hr, 95.7% of the cells remained unstained. Cell proliferation and confluent colony formation were observed as early as about 20 days after the plating of these cells on agar medium

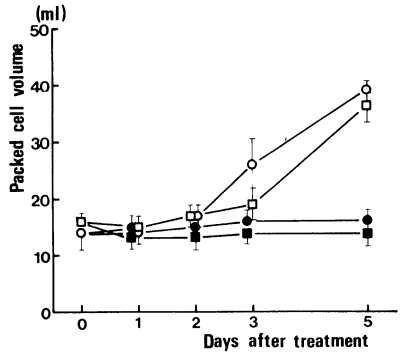

Fig. 1. Effect of AT toxin (3.0%) on the growth of suspension-cultured cells. Closed circle = Beinhart 1000-1 (resistant) with toxin, open circle = Beinhart 1000-1 without toxin, closed square = Burley 21 (susceptible) with toxin, open square = Burley 21 without toxin. Each point represents the average of three separate experiments with standard deviation.

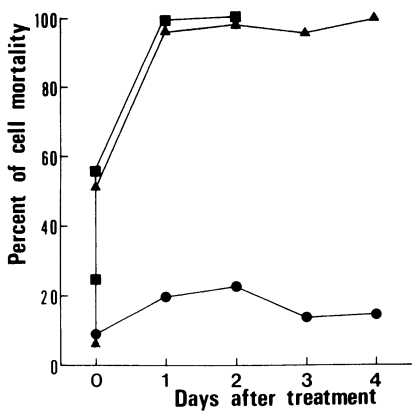

Fig. 2. Mortality of cultured cells in the presence of AT toxin (3.0%). Cell mortality was estimated by staining with 0.01% phenosafranin. Closed circle = Beinhart 1000-1 (resistant), closed square = Burley 21 (susceptible), closed triangle = Bright Yellow 4 (susceptible).

Table 3. Viability[a] of cultured protoplasts treated with 3% AT toxin for 24 hr

Protoplast source	Viability (%)			
		Time of toxin treatment		
	Control	Day 0	Day 2	Day 4
Bright Yellow 4	50.9 (2.9)[b]	0	29.2 (6.5)	34.8 (5.1)
Burley 21	62.8 (7.1)	0	46.0 (1.0)	42.3 (0.4)
Tsukuba 1	24.5 (3.5)	0	39.3 (1.6)	13.0 (0.1)
Beinhart 1000-1	54.1 (7.2)	0	38.0 (2.3)	40.0 (3.2)
F_1 (Beinhart 1000-1 × Burley 21)	50.1 (4.3)	0	35.3 (1.2)	41.9 (0.6)

[a] Cell viability was measured 48 hr after toxin release by staining with phenosafranin.
[b] Each value is the average of three separate experiments with standard deviation in parentheses.

Table 4. Cell division frequency of 4-day-old cultured protoplasts treated with 3% AT toxin for 24 hr

Cultivar	Protoplasts undergoing cell division[a] (%)		Ratio (%) treated/ untreated
	Untreated	Treated	
Bright Yellow 4	30.6 (4.8)[b]	2.2 (2.2)	7.1
Burley 21	33.0 (0.3)	2.0 (0.1)	6.1
Tsukuba 1	18.0 (1.6)	0.8 (0.5)	4.4
Beinhart 1000-1	42.4 (5.0)	20.7 (4.1)	48.8
F_1 Beinhart 1000-1 × Burley 21	50.6 (3.1)	36.1 (5.9)	71.3
F_1 Burley 21 × Beinhart 1000-1	37.9 (4.6)	26.5 (1.2)	69.9

[a] Percent cell division measured 10 days after toxin release.
[b] Each value is the average of three separate experiments with standard deviation in parentheses.

free of the toxin. Although cell viability varied to some extent, colony formation was always observed only in toxin-treated Beinhart 1000-1 cells and not in Burley 21. Based on these observations, it was concluded that the staining with phenosafranin was a reliable method for measuring viability of cultured cells.

Reaction of protoplasts toward AT toxin. When freshly isolated protoplasts were treated with AT toxin, all the protoplasts were dead within 48 hr (Table 3). Although the difference between untreated and treated populations was clear, except for Tsukuba 1, no clear difference between cultivars was detected in terms of the frequency of viable protoplasts.

Usually, the first cell division in a protoplast population occurs 4–6 days after the protoplast release. The cell division frequency was measured for treated protoplasts (Table 4). When 4-day-old protoplasts were treated with the toxin, the cell division frequency at 10 days was around 2% in the susceptible cultivars such as Bright Yellow 4 and Burley 21. The cell division frequency of the resistant cultivar, Beinhart 1000-1, was as high as 20%. Furthermore, both reciprocal F_1 hybrids between Beinhart 1000-1 and Burley 21 showed the same or greater tolerance as compared with the resistant parent. Thus, a clear-cut difference was observed between resistant and susceptible cultivars.

The same experiments were conducted using 2-day-old protoplasts. The results were somewhat variable with susceptible cultivars showing 1–4% cell division, and the resistant cultivar and F_1 hybrids showing 0–11% cell division (*data not shown*). Untreated protoplasts at 2 and 4 days were stained with Calcofluor White and observed under a fluorescence microscope. The 2-day-old protoplast exhibited variable staining intensity of the cell wall, whereas 4-day-old protoplasts were stained strongly and uniformly.

DISCUSSION

AT toxin was first isolated from culture filtrate of *A. alternata* by Kohmoto et al (4). The toxin can induce a typical symptom on leaves upon treatment of susceptible tobacco cultivars, whereas it induced only slight or no symptoms on those of resistant tobacco cultivars or nonhost plants (5). Our results described here confirm the differential reactions to the toxin between susceptible and resistant tobacco cultivars, although the toxin preparation used here was a crude one. Nevertheless, as is the case when the fungus itself was inoculated onto leaves, the differential reactions varied, depending upon the slight difference in physiological condition of the leaves. Therefore, application of toxin to intact leaves cannot circumvent the variability seen following fungal inoculation in a given genotype.

When the toxin was applied to suspension-cultured cells, cell growth was inhibited in cultures of both resistant and susceptible cultivars. Differential reactions between resistant and susceptible tobacco cultivars were apparent, however, in terms of cell viability, which was judged by staining with phenosafranin. Cells of cultures of a resistant cultivar survived toxin treatments better than those of a susceptible cultivar. In the case of susceptible cultivars, almost all the cells were dead after the 24-hr treatment with 3% AT toxin. These findings indicate that so-called field tolerance among tobacco cultivars is expressed at the cultured cell level. One of the advantages of using cultured cells is that they are quite uniform and that their physiological state can be controlled. Therefore, treatment of cultured cells with AT toxin could be a new and reliable evaluation method for resistance to tobacco brown spot.

One of the disadvantages of the suspension-cultured cells, however, is that it takes more than 6 mo to establish a suitable fast-growing culture. This causes problems, particularly when one wants to select resistant strains out of a large number of segregating progenies, which is a typical case in plant breeding. Treating cultured protoplasts provides an acceptable alternative. Although freshly isolated protoplasts from resistant and susceptible cultivars responded similarly to AT toxin, older protoplasts that had initiated cell wall formation exhibited a clear difference similar to the extent of the resistance of the parental line. These results agree with the reaction of suspension-cultured cells that have well-formed cell walls. These data suggest that the expression of AT toxin resistance requires the cell wall.

Since the range of tobacco cultivars used in this study was not large, we are now repeating this work using a large number of tobacco cultivars as well as wild species of *Nicotiana* to further confirm the validity of this method for evaluating resistance to tobacco brown spot.

There have been few reports indicating a clear correlation in the resistance between cultured cells and whole plants. Deaton et al (2) reported that the resistance to the tobacco black shank pathogen (*Phytophthora parasitica* Dast. var. *nicotianae* (B. de Haan) Tucker) was expressed among callus tissue cultures of tobacco when the cultures were challenged by the pathogen. Hartman et al (3) observed clear correlations between the responses of bean calli to the culture filtrate of *Pseudomonas syringae* pv. *phaseolicola* (Burkh.) Young et al and that of whole plants. They emphasized the usefulness of tissue culture assays in practical breeding.

As reviewed by Daub (1), various tissue culture systems have been used in combination with appropriate selective agents, such as purified phytotoxins or crude culture filtrates, to select resistant cell lines and eventually resistant regenerates. The toxin-preparation procedure described here is not complicated, and large numbers of protoplasts can be isolated and readily cultured to whole plants in tobacco. Although the primary concern of this report was to establish an efficient and reliable selection system for brown spot resistant genotypes, this system can also be applicable for selecting AT toxin-resistant cell lines in a given cultivar, as has been partly demonstrated by Thanutong et al (11).

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LITERATURE CITED

1. Daub, M. E. 1986. Tissue culture and the selection of resistance to pathogens. Annu. Rev. Phytopathol. 24:159-186.
2. Deaton, W. R., Keyes, G. J., and Collins, G. B. 1982. Expressed resistance to black shank among tobacco callus cultures. Theor. Appl. Genet. 63:65-70.
3. Hartman, C. L., Secor, G. A., Venette, J. R., and Albaugh, D. A. 1986. Response of bean calli to filtrate from *Pseudomonas syringae* pv. *phaseolicola* and correlation with whole plant disease reaction. Physiol. Mol. Plant Pathol. 28:353-358.
4. Kohmoto, K., Hosotani, K., Otani, H., and Nishimura, S. 1981. Partial purification of host-specific toxin (AT toxin) produced by tobacco pathotype of *Alternaria alternata*. Ann. Phytopathol. Soc. Jpn. 47:384.
5. Kodama, M., Nishimura, S., and Kohmoto, K. 1984. Isolation of host specific toxin of *Alternaria alternata* tobacco pathotype. Ann. Phytopathol. Soc. Jpn. 50:410.
6. Linsmaier, E., and Skoog, F. 1965. Organic growth factor requirements of tobacco tissue culture. Physiol. Plant. 18:100-127.
7. Lucas, G. B. 1975. Brown spot. Pages 267-295 in: Disease of Tobacco. 3rd ed. Biological Consulting Association, Raleigh, NC.
8. Mikami, Y., Nishijima, Y., Iimura, H., Suzuki, A., and Tamura, S. 1971. Chemical studies on brown-spot disease of tobacco plants. Part I. Tenuazonic acid as a vivotoxin of *Alternaria longipes*. Agric. Biol. Chem. 35:611-618.
9. Nagata, T., and Takebe, I. 1971. Plating of isolated tobacco mesophyll protoplasts. Planta 99:12-20.
10. Stavely, J. R., Pittarelli, G. W., and Lucas, G. B. 1971. Reaction of *Nicotiana* species to *Alternaria alternata*. Phytopathology 61:541-545.
11. Thanutong, P., Furusawa, I., and Yamamoto, M. 1983. Resistant tobacco plants from protoplast-derived calluses selected for their resistance to *Pseudomonas* and *Alternaria* toxins. Theor. Appl. Genet. 66:209-215.
12. Widholm, J. 1972. The use of fluorescin diacetate and phenosafranine for determining viability of cultured plant cells. Stain Tech. 47:189-194.