Incompatibility Conditioned by the *Mla* Gene in Powdery Mildew of Barley: The Halt in Cytoplasmic Streaming

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ABSTRACT

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Cytoplasmic streaming stopped in host epidermal cells of barley under attack by *Erysiphe graminis* f. sp. *hordei* when host-parasite incompatibility was conditioned by the *Mla* gene. The cytoplasm halted 1–3 hr before the host cells collapsed hypersensitively. These events in tissues partially isolated from coleoptiles were viewed at 1- to 4-hr intervals by direct observation, or continuously by time-lapse cinephotomicrography. The hypersensitive collapse of cells usually occurred 18–26 hr after inoculation when the primary haustorium was partly formed. The halt in cytoplasmic streaming that preceded this collapse was, in turn, preceded by a 0.5-hr period in which host cytoplasm accumulated in small amounts near the haustorium. Organelles within this cytoplasm moved in a localized, restricted fashion. The results indicate that an early metabolic or structural change associated with gene-for-gene incompatibility interferes with cytoplasmic movement.

As part of a survey of the events associated with primary infection in powdery mildew of barley, Bushnell and Bergquist (2) noted briefly that cytoplasmic streaming stopped 1–3 hr before host cells lost turgor in a host-parasite combination in which the *Mla*

gene conditioned incompatibility. This observation was neither documented nor discussed in their paper. The observations were made in epidermal tissues partially isolated from coleoptiles and mounted so that living host cells could be observed during the early stages of pathogenesis. In these specimens, they found that host cells collapsed 16–21 hr after inoculation.

Because the incompatibility conditioned by the *Mla* gene was expressed early and decisively in the coleoptile tissue, I chose the *Mla*-coleoptile system for further descriptive work to learn if it might be suitable for investigations of the biochemical causes of

incompatibility. The halt in cytoplasmic streaming was found to be a consistent feature of incompatibility conditioned by the *Mla* gene. This paper documents this finding and describes cytoplasm as viewed by light microscopy before and after movement stops.

MATERIALS AND METHODS

Culture CR3 of race 3 of *Erysiphe graminis* (DC) Mérat f. sp. *hordei* was used in combination with two nearly isogenic barley (*Hordeum vulgare* L.) lines that differ in reaction to culture CR 3. Algerian/4*-(F14) Man. R contains the *Mla* gene for resistance to culture CR3; Algerian/4*-(F14) Man. S contains the *Mla* gene for susceptibility to culture CR3. The lines are here termed "AlgR" and "AlgS," respectively. These lines were developed by Moseman (8) who kindly supplied both culture CR3 and the barley lines.

All observations of infected tissue were of epidermal tissues partially isolated from barley coleoptiles generally prepared as in earlier studies (2,3). The coleoptiles were grown and inoculated as described by Johnson et al (4). Tissues were inoculated after they were mounted in support wafers so that the inoculated zone was restricted to an observation hole, 1.6 mm in diameter (~2 mm^2).

Spores were applied with a settling tower, usually at 20–40 spores per square millimeter. Generally, 40–55% of spores produced haustoria so that tissues usually had 10–25 haustoria per square millimeter. The spores were applied 2–5 hr after the end of the daily 16-hr light period in the chamber used to grow coleoptiles and inoculum. The inoculated tissues were kept in an incubator for 12–17 hr in the dark at 17.5 ± 1.0 C and 95% R.H. The tissues were then placed on a laboratory bench at 20 ± 1 C for observation until 23–30 hr after inoculation when they were returned to the dark incubator until a final observation at 36–48 hr after inoculation. All tissues were maintained on 0.01 M Ca(NO$_3$)$_2$ supplied to the uninoculated side of the mounted epidermis.

To help identify cells for repeated observations of cytoplasmic behavior, the entire inoculated zone of each mount was photographed at ×60 magnification using Polaroid (Polaroid Corp., Cambridge, MA 02139) Type 107 film. Thirteen to 17 hr after inoculation, approximately five cells per mount were marked on the photograph for subsequent series of observations. In the case of inoculated tissues, the cells were selected to contain a young haustorium, to be entirely within the borders of the observation hole and, with respect to the set of five cells, to be widely distributed across the observation hole. In the case of uninoculated tissues, the cells were selected on the photograph of each mount to occupy the same approximate locations as those of the corresponding selected cells in inoculated tissues.

The amount of cytoplasmic movement was monitored by direct observation in 50 μm segments at each end of the cell, and usually in the case of infected cells, also in a 50 μm segment centered on the haustorium. These segments were each examined at ×300 by bright-field microscopy by focusing up and down while searching for signs of cytoplasmic movement. If no movements were seen, the search was usually discontinued after about 10 sec. Movement was rated on the following subjective index of streaming:

0. No movement.
1. Organelles moving in vibratory fashion, indistinguishable from Brownian motion.
2. Organelles showing traces of translational movement in strands or sheets of cytoplasm, or intact cytoplasmic strands showing traces of wiggling.
3. Abundant organelles moving sluggishly in one or more strands, or small numbers of organelles moving rapidly in widely spaced strands or sheets.
4. Abundant organelles moving rapidly in one or more thin strands or sheets.
5. Abundant organelles moving rapidly in one or more thick strands or sheets.

Whether or not the host cell had collapsed also was noted at each observation time. In large experiments involving up to 80 cells at 2- to 4-hr intervals, each round of observations required 0.5–1.5 hr. In smaller experiments involving up to 32 cells at 1-hr intervals, each round required 25–40 min. The indicated time of observation is the average of individual observation times within the round.

Time-lapse cinephotomicrographs were taken following procedures described earlier (3,14). Exposures were made at 3-sec intervals; the exposure time was 0.05 sec. For photographing time-lapse sequences, a Reichert Zetopan (American Optical, Buffalo, NY) microscope was used with a ×40 objective lens and a ×5 eyepiece with differential interference contrast. To enhance contrast of cytoplasm, the condenser aperture was partially closed. The time-lapse sequences were analyzed by projecting them at 1–24 frames per second with an L-W MKV Model 224A photo optical data analyzer (L-W International, Woodland Hills, CA 91367). For phase or differential interference contrast microscopy of halted cytoplasm at high magnifications, tissues were removed from mounts and placed in water under a coverslip and then photographed using ×63 or ×100 objective lenses.

RESULTS

Direct observations. Cell collapse, which was readily visible in mounted tissues, occurred only after the haustorium was partly formed and clearly visible in the infected cell. Generally, the central body of the haustorium was present, but the fingerlike haustorial lobes were only beginning to form. Although the majority of cells collapsed in the period 20–27 hr after inoculation, some cells collapsed as early as 17 hr after inoculation; others collapsed 27–36 hr after inoculation.

The amount of hypersensitive cell death varied among the sets of inoculated tissues that were used to observe behavior of cytoplasm. Among all experiments, 43–99% of the cells that contained a haustorium had died by 48 hr after inoculation. This variation, which was typical of results in other experiments, has not been eliminated by any of several varied environmental regimes (W. Bushnell and S. Carr, *unpublished*).

Since there were 20–50 haustoria in each mount of tissue, each mount potentially could have 10–50 dead haustorium-containing cells, depending on the frequency of hypersensitive cell death. Since the inoculated 2 mm^2 of each mount typically contained about 150 epidermal cells, the haustorium-containing dead cells constituted 7–33% of the total number of cells. However, one or more cells adjacent to each haustorium-containing cell also frequently died, sometimes doubling the total number of dead cells.
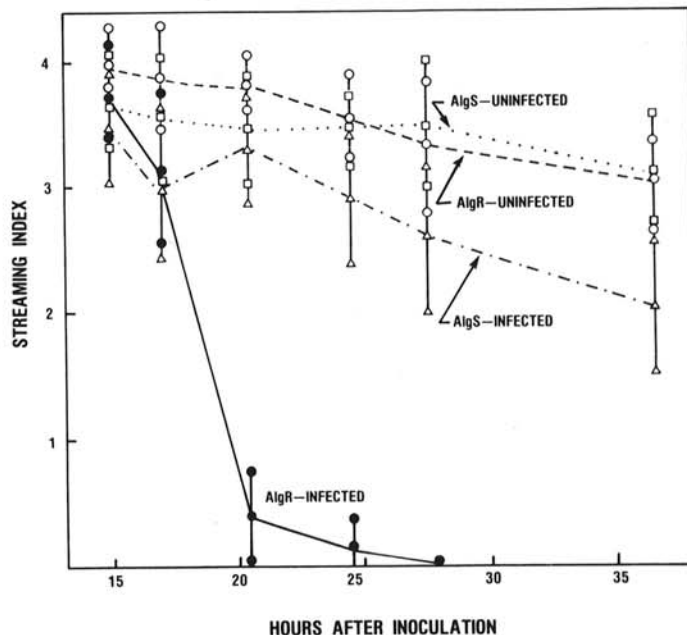

Fig. 1. The cytoplasmic streaming index in barley epidermal tissues uninfected and infected with *Erysiphe graminis* f. sp. *hordei*. Near-isogenic lines AlgS (compatible) and AlgR (incompatible) differ at the *Mla* locus for compatibility with the fungus. For streaming index, see Materials and Methods. Confidence intervals, $\pm t_{.05}s\bar{x}$ (10).

Patterns of cytoplasmic streaming in the two nearly isogenic barley lines, AlgR and AlgS, are shown in Fig. 1. In this experiment, 19–20 selected cells of infected and of noninfected tissues of each line were rated subjectively for cytoplasmic movement. Each cell was observed at 2–4 hr intervals from 15.0–27.5 hr after inoculation and again at 36.5 hr. (In this experiment, cytoplasmic movement was rated at each end of each cell, but not in the haustorial region). Although streaming indices decreased slightly over the course of the experiment, the two uninfected lines and the infected AlgS consistently showed high rates of cytoplasmic activity. Occasionally, movement was absent in one end of a cell at a given observation time, but in these cases, movement was seen in the other end (with only one exception). However, in infected AlgR, average cytoplasmic indices dropped abruptly to very low levels by 20.5 hr after inoculation, and dropped to 0 by 27.5 hr. The decline in cytoplasmic activity seemed to precede cell collapse as streaming indices were 0.4 or less at 20.5 hr and thereafter, whereas only six and 12 of the 19 observed cells

had collapsed at 20.5 and 24.5 hr, respectively. Results similar to those of Fig. 1 were obtained in a second experiment (not shown).

To learn more about the decline in cytoplasmic activity, infected cells of AlgR were observed hourly 16–29 hr after inoculation. In two experiments, a total of 45 cells was observed. Fifteen of the cells collapsed during the experimental period, all within the period 21–29 hr (average 23.3 hr). The average streaming index for the 15 cells, plotted in relation to time before cell collapse (Fig. 2), declined to less than 1.0, 2 hr before cell collapse, and was only 0.2, 1 hr before collapse. The data can also be expressed by the number of cells that were nearly quiescent (index less than 1.0) a given maximum number of hours before cell collapse. Thus, four cells were nearly quiescent for 3 hr before collapse, five for 2 hr, and five for 1 hr. One unusual cell was quiescent for 8 hr before collapse. Omitting the latter, the average time of near-quiescence was 1.9 hr.

Streaming activity also declined in observed infected cells of AlgR that did not collapse. Thus, in one set of hourly observations in which about 50% of observed infected cells collapsed (a relatively low rate of collapse), the streaming index in the remaining 50% of cells declined to 1.0 by 24 hr after inoculation and remained low thereafter (Fig. 3). This suggested that incompatibility had inhibited streaming without inducing cell collapse. However, the streaming index only declined as the number of collapsed cells on test mounts began to increase (Fig. 3).
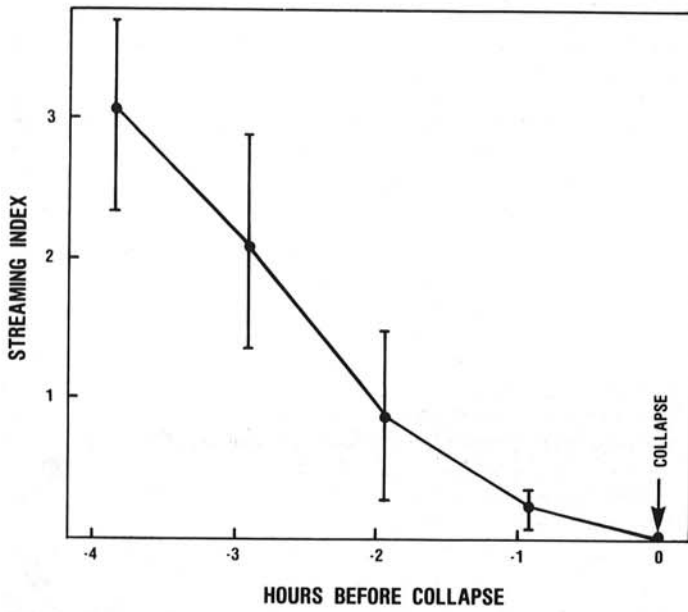

Fig. 2. Streaming index 0–4 hr before hypersensitive cell collapse in incompatible epidermal tissues of AlgR under attack by *Erysiphe graminis* f. sp. *hordei*, race 3. Average for 15 cells. Average time of collapse was 23.3 hr after inoculation. Confidence intervals, $\pm t_{.05} s_{\bar{x}}$ (10).
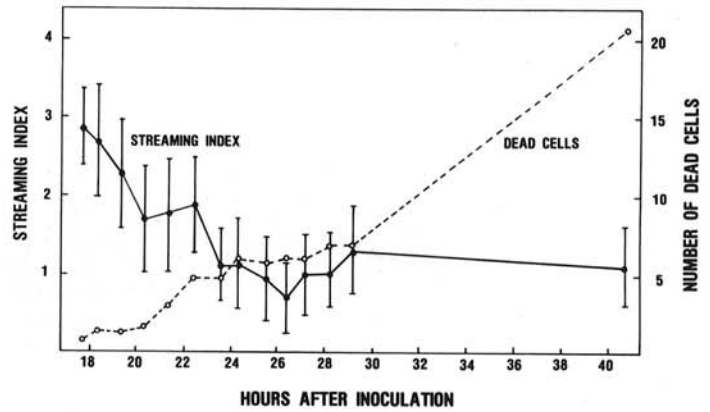

Fig. 3. Streaming index in infected cells which remained alive in incompatible tissue 41 hr after inoculation, and the average number of dead cells in the 2 mm² inoculated zone of each tissue mount. Incompatible epidermal tissue of AlgR was inoculated with *Erysiphe graminis* f. sp. *hordei*, race 3. Confidence intervals, $\pm t_{.05} s_{\bar{x}}$ (10).

TABLE 1. Timing of cytoplasmic events in time-lapse sequences in relation to the hypersensitive collapse of barley epidermal cells. AlgR (incompatible) under attack by *Erysiphe graminis* f. sp. *hordei*, race 3

Mount	Cell	Haustorium[a]	Long distance streaming stopped- (Min before localized movement stopped)	Localized movement slowed- (Min before localized movement stopped)	Localized movement stopped- (Min before cell collapsed)	Host cell collapsed- (Hr:min after inoculation)
A	1	+	21	9.5	71	19:02
B	1	+	14	3.5	148	20:23
C	1	?[b]	18	3.8	62	20:43
	2	+	27	3.0	57	20:51
	3	+	...	1.2	125	21:38
D	1	+	6	3.7	182	20:54
	2	+	29	18.7	186	20:54
E	1	+	...	...	111	21:01
F	1	+	15	5.1	121	21:12
G	1	+	10	5.4	93	21:27
H	1	−[c]	(20+[d])	3.9	52	21:48
	2	+	37	10.1	137	23:26
I	1	+	52	...	308	26:45
	Avg		23	6.2	127	21:32

[a] + = Haustorium present; − = haustorium not present.
[b] Cell was immediately adjacent to cells 2 and 3, each of which had a haustorium.
[c] Cell was immediately adjacent to cell 2, which had a haustorium.
[d] Value not included in average.

This raised the alternative possibility that dead cells were releasing substances that inhibited streaming in neighboring cells as a secondary result of hypersensitive cell death. To test these alternatives has not been practical so far because the number of noncollapsing cells in most trials has been low and variable. Nevertheless, the sluggish streaming activity has been seen consistently in noncollapsed infected cells in AlgR at 24 hr after inoculation both by direct observation and in time-lapse sequences.

Time-lapse cinephotomicrography. To avoid the period of generally sluggish cytoplasmic activity in infected AlgR at 24 hr after inoculation and later, time-lapse sequences were started by 18 hr after inoculation. The plan was to record a period of normal cytoplasmic activity preceding the 1–3 hr period of quiescence expected if cells were to collapse. Filming was discontinued if cytoplasmic movement had not stopped by 24 hr after inoculation. With this procedure, a halt in cytoplasmic streaming and cell collapse were recorded in about half of the filmings attempted. The timing of events for all successful filmings is in Table. 1

Stages of cytoplasmic activity following infection. Three distinct stages in behavior of cytoplasm were evident in viewings of the time-lapse films projected at 12–24 frames per second. These were:
Long-distance streaming. Strands of cytoplasm were frequently visible in the normal cell—strands in which organelles moved while the strands themselves frequently moved sideways. These strands were sometimes parallel to the longitudinal axis of the cell, but often stretched diagonally across the cell. Strands and organelles sometimes were seen to move across the entire time-lapse frame (180 μm), or to move 60–90 μm before disappearing from the plane

of focus. Such long-distance movement was typical of uninfected cells, of infected AlgS cells, and also of infected AlgR cells before incompatibility was expressed. This movement was typical of that reported for vacuolate higher plant cells (6,7).
*Localized movement of organelles.* An eventual halt in cytoplasmic streaming was preceded by a transition period in which long-distance streaming was replaced by localized, but vigorous, movement of organelles and cytoplasm. Usually a mass of cytoplasm and organelles accumulated near the haustorium, occupying a segment of the host cell 22–110 μm long (avg 48 μm). This cytoplasm was sometimes located on only one side of the haustorium. In any case, the cytoplasmic mass did not have sharply defined borders (Figs. 4 and 5). As viewed at 18–24 frames per second (54–72 times the original speed), the movement of organelles resembled Brownian motion at normal speed in that little net translational movement occurred in any one direction. The localized movement continued for 10–53 min preceding the complete halt in cytoplasmic movement (Table 1).
Cytoplasmic quiescene. The localized movement of organelles slowed over a 6.2-min period (Table 1) and stopped completely 52–308 min (avg 127 min) before cells collapsed (Table 1). In the quiescent period that followed, the organelles showed no vibratory movement, suggesting that the cytoplasm had lost fluidity. Rarely, a single organelle moved rapidly 50–80 μm across the field of view in an irregular trajectory, possibly in the vacuole. The quiescent cytoplasm had a grainy appearance at low and medium magnifications whereas organellelike bodies were visible in a wide variety of sizes and shapes at high magnification (Figs. 6 and 7). Although some were probably mitochondria, none of the bodies was identified; and none other than mitochondria has been seen in abundance in normal, living cytoplasm.

The period of quiescence was followed by abrupt collapse of host cells. This was clearly evident in the time-lapse sequences as the shading imparted by the corrugated, dry tissue surface changed as
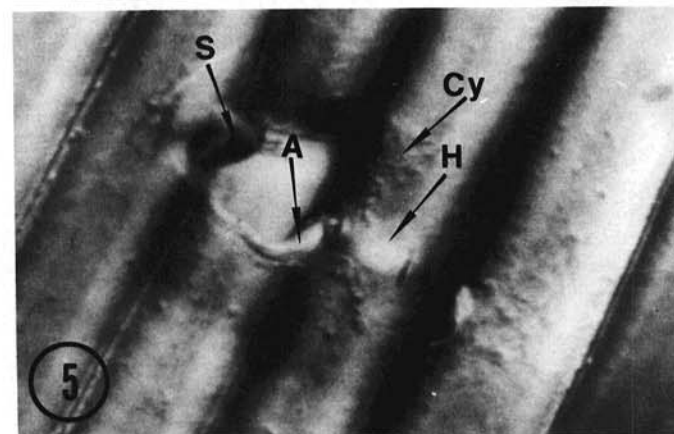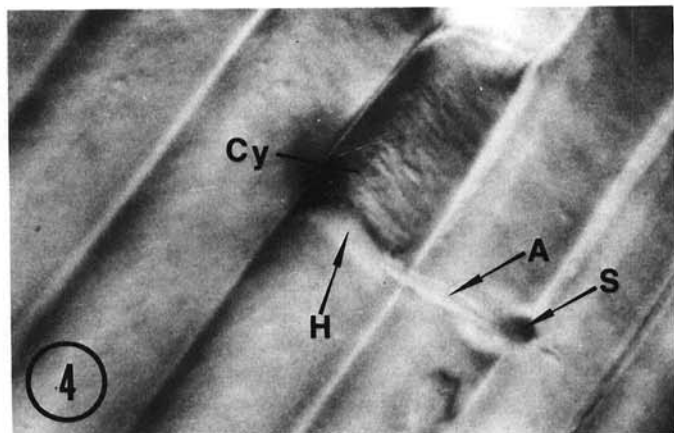
Figs. 4 and 5. Cytoplasm with restricted organelle movement that accumulated near a haustorium in an incompatible barley line (AlgR) under attack by *Erysiphe graminis* f. sp. *hordei*. Selected photomicrographs from two time-lapse sequences which ended in host cell collapse. **4,** 1 hr 40 min before cell collapse; from mount G, cell 1 of Table 1. **5,** 2 hr 32 min before cell collapse; from mount H, cell 2 of Table 1. Cell orientation was oblique for uniformity in interference contrast. ×400. A = appressorium; Cy = cytoplasm which accumulated in haustorial region; H = haustorium; and S = spore (out of focus).
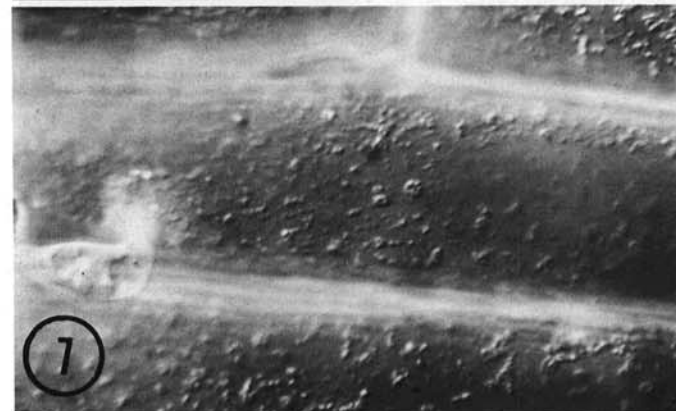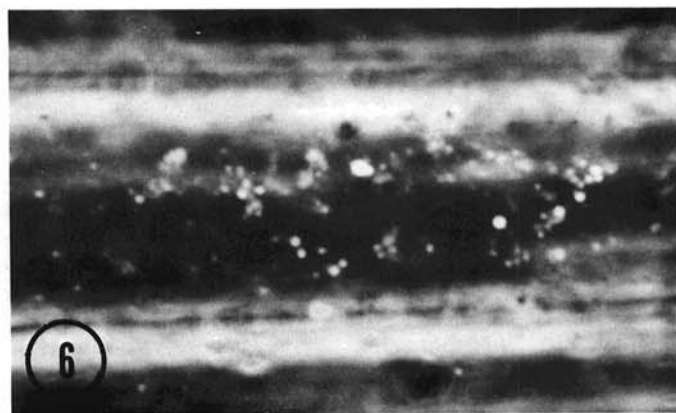
Figs. 6 and 7. Organelles in quiescent cytoplasm before hypersensitive cell collapse. Epidermal cells of AlgR, 24 hr after inoculation with *Erysiphe graminis* f. sp. *hordei*, race 3. Organellelike bodies in varied shapes and sizes lined the inner surface of the outer wall of the epidermal cells. **6,** Phase contrast; **7,** differential interference contrast, ×850.

the cell wall folded. The collapse phenomenon is under separate study, but has always been preceded by collapse of the host nucleus, and usually is followed by collapse of the haustorium (W. Bushnell and S. Carr, *unpublished*).

DISCUSSION

The present results show that a halt in cytoplasmic movement is a consistent event preceding hypersensitive host cell collapse when incompatibility is conditioned by the *Mla* gene. The time-lapse data, combined with data from direct observations at 1–4 hr intervals, show that the halt usually occurred 1–3 hr before cells collapsed, and that there was virtually no cytoplasmic movement thereafter.

The quiescent cytoplasm appeared by light microscopy to be at least partly in the form of irregular deposits on the cell wall. The lack of Brownian motion of organelles in the quiescent cytoplasm suggests that the cytoplasm may have gelled or coagulated. Whether the cytoplasm was irreversibly altered in the quiescent state is not known.

Instead of a change in the physical properties of cytoplasm, the halt in cytoplasm could result from a direct inhibition of the actin-myosin interaction, which requires ATP and is now suspected to provide the motive force for cytoplasmic streaming in higher plants (12,13). Thus, a fungal metabolite such as cytochalasin B or phalloidin could be interfering directly with the driving mechanism as suggested by Palevitz (9) with respect to parasite-induced cytoplasmic aggregates. More likely, in view of the eventual collapse and death of the cell, is the possibility that the ATP supply is cut off through interference with oxidative metabolism in the cell.

Since the halt in cytoplasmic streaming was the first visible sign of incompatibility, and preceded cell collapse by 1–3 hr, the halt in streaming, could itself, be a cause of cell collapse. Thus, maintenance of the semipermeability of the plasma membrane could be prevented by the halt in streaming. However, it is not known whether the membrane begins to lose semipermeability when streaming stops (or earlier), or changes only shortly before cell collapse. Walls of cells begin to collapse visibly only after the last trace of turgor is gone. We need to determine by osmotic measurements when, in relation to the quiescent period, the plasma membrane begins to lose semipermeability and cells begin to lose turgor.

During the transition period in which long-distance cytoplasmic movement was lost, but cytoplasm continued to move locally, small amounts of cytoplasm accumulated in parts of the host cell near the haustorium. This cytoplasm did not have sharply defined borders and was not as compact or abundant as the well-defined cytoplasmic aggregate that is associated with wall penetration at about 12 hr after inoculation (1,2). As noted by Bushnell and Bergquist (2), the aggregation phenomenon and the associated deposition of the papillae were completed several hours before the first sign of aberrant cytoplasmic behavior due to *Mla* incompatibility. The transition period may be a time in which properties of cytoplasm were changing or in which the driving forces of cytoplasmic movement became limiting. Some of the restricted movement may have been due to Brownian motion.

The results here with the *Mla* gene in barley parallel those of Tomiyama and co-workers (5,11) who described a halt in cytoplasmic streaming that preceded hypersensitive cell death caused in potato tuber tissue by *Phytophthora infestans*. The sequence of events they studied was more telescoped in time than mine, as cytoplasmic movement stopped 26–48 min after the extrapolated time of host wall penetration (5). Cells lost their normal ability to take up neutral red 10–30 min after streaming stopped (a criterion used for cellular dysfunction since cell collapse apparently could not be seen in the mounted tissues). This was followed by granular degeneration of cytoplasm (11). Thus, the halt

in cytoplasmic streaming was an early sign of incompatibility, preceding cell death, much as in the present study.

The halt in cytoplasmic streaming in potato cells infected with the late blight pathogen occurred within 1 hr after penetration, following a period of enhanced cytoplasmic activity, which was associated with wall penetration. This is evident in the movie film titled "The reaction of the potato plant cell to the infection by the incompatible and compatible races of *Phytophthora infestans*," which was produced by K. Kitazawa, H. Inagaki, and K. Tomiyama in 1972 and kindly supplied by K. Tomiyama, Plant Pathology Laboratory, Faculty of Agriculture, Nagoya University, Nagoya 464, Japan. In that film the enhanced cytoplasmic activity in both compatible and incompatible host-parasite combinations usually resembled the well-defined cytoplasmic aggregation that, as noted earlier, is associated with penetration of barley cell walls by *Erysiphe graminis*. Kitazawa et al (5) concluded that cytoplasm accumulates more actively around infection sites in incompatible host-parasite combinations than in compatible ones. However, the accumulated cytoplasm did not exhibit restricted organelle movement corresponding to that seen in the cytoplasm that accumulated near powdery mildew haustoria during the transition period before all movement halted. Regardless of this difference, the work of Tomiyama and co-workers together with the work reported here suggest that a halt in cytoplasmic streaming is a general event preceding hypersensitive cell death in gene-for-gene incompatibility.

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