Catastrophic wall rupture during conidial germination of a genetically tagged mutant of *Glomerella graminicola*

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Video microscopy was used to examine a genetically tagged mutant (T30) of *Glomerella graminicola* whose conidia have a propensity to burst during the germination process. Before the germ tube is produced, the cell wall ruptures and the cytoplasm is extruded. The bursting takes place in less than 0.1 second. Bursting can be prevented by adding osmotica to the germination medium. By phase-contrast microscopy of alkali-washed conidia, we found that each ruptured conidium had a single gaping hole that was oriented parallel to the long axis of the spore. The holes were more frequent in the middle region of the conidia than at the apices. A mathematical model for stress based on conidial shape indicated that conidia ruptured where the stress on the wall was greatest. The microscopic observations on the orientation and distribution of the rupture sites are consistent with the hypothesis that T30 conidia have weaker walls than the wild-type. Comparative tests of resistance to mechanical breakage support this hypothesis.

INTRODUCTION

Glomerella graminicola (anamorph *Colletotrichum graminicola*) is an economically important pathogen of corn worldwide (Bergstrom & Nicholson 1999). Moreover, it is a useful model system for studying morphogenesis of a filamentous ascomycete (Epstein & Nicholson 1997, Epstein, Lusnak & Kaur, 1998, Perfect *et al*. 1999). On the host, falcate-shaped conidia are produced on the plant surface, are dispersed in rain-splash, and serve as the inoculum for new infections (Bergstrom & Nicholson 1999). Previously, we transformed G. *graminicola* with a plasmid (pUC-ATPH) and screened the transformants microscopically for developmental mutations (Epstein *et al*. 1998). The falcate conidia of the mutant strain T30 ruptured when incubated in either V-8 broth or potato dextrose broth. Those T30 conidia that did not rupture germinated but produced hyphae with terminal or intercalary swollen regions. When T30 conidia were incubated in media amended with a variety of osmotica, including 0.3 M KCl, 30 mM polyethylene glycol 10000, or 50 mM sucrose, they did not rupture and the hyphae had wild-type morphology. We demonstrated that the T30 phenotype resulted from a single insertion of plasmid DNA. Confirmation of insertional mutagenesis and consequent gene tagging was based on: (1) meiotic co-segregation of the mutant phenotype with the plasmid DNA as demonstrated by antibiotic resistance on selectable media and, in selected progeny, by the presence of a hybridizing band on Southern blots probed with plasmid DNA; and (2) plasmid rescue from the mutants followed by targeted gene inactivation of the wild-type with the rescued plasmid.

In this study, we used computer-enhanced video microscopy to visualize the rupturing process in T30 conidia of *G. graminicola*. The orientation and distribution of rupture sites was analyzed with a mathematical model based on spore shape. Cell wall strength was tested by mechanical breakage. All these findings support the hypothesis that mutant conidia have weaker cell walls than the wild-type.

MATERIALS AND METHODS

The wild-type strain M1.001 (M1) and the insertionally mutagenized strain T30 were described previously (Epstein *et al*. 1998). T30 was single-spore purified and the conidia were stored in silica gel at 4 °C (Johnston & Booth 1983). To produce falcate conidia, Petri dishes with oatmeal agar (Johnston & Booth 1983) were uniformly seeded with inoculum. Cultures were incubated at 24° for 14 d under continuous fluorescent lighting (approx. 50 μE m^{-2} s^{-1}).

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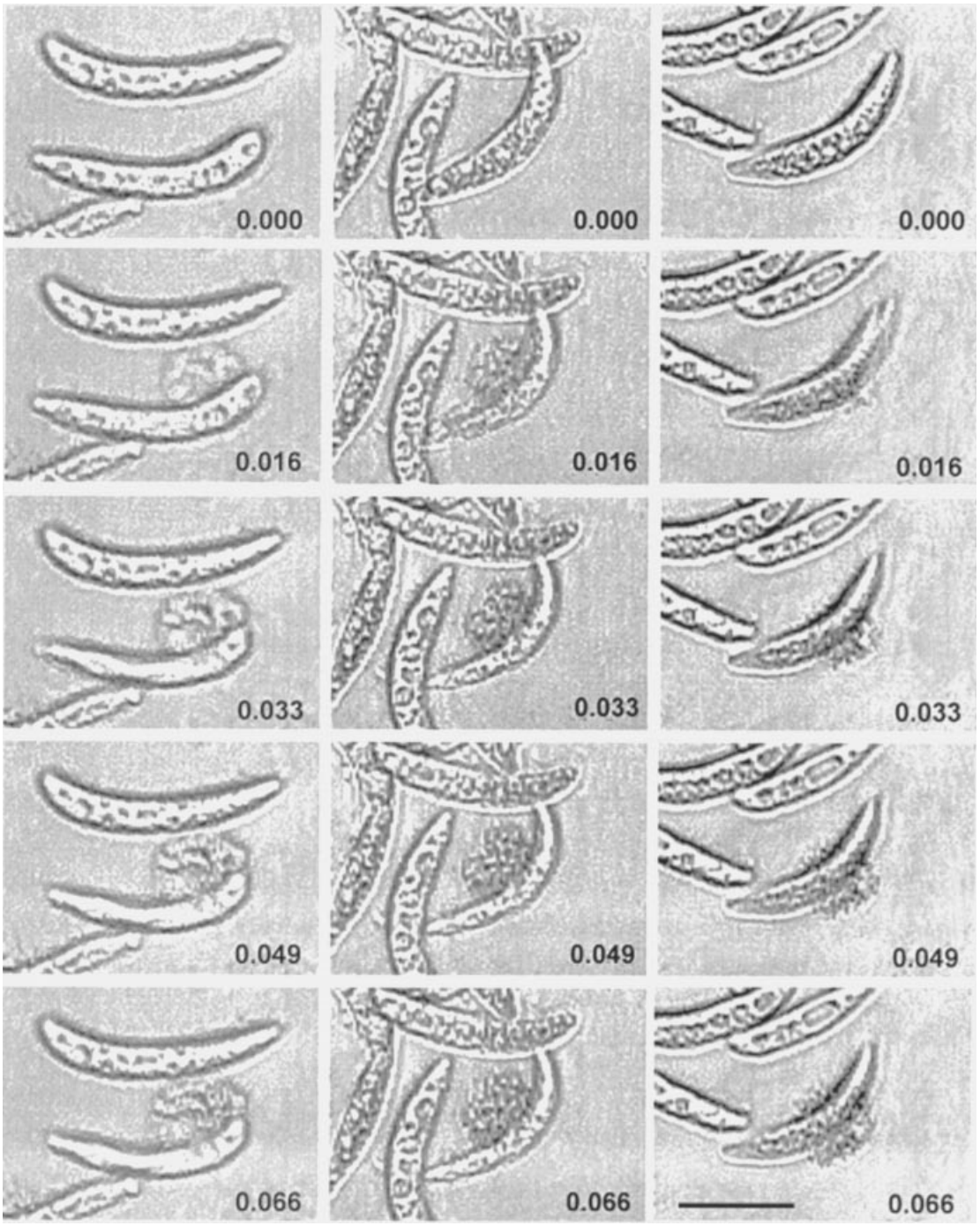

Fig. 1. Each column shows a sequence of images from video microscopy of a rupturing conidium of an insertionally mutagenized strain of *Glomerella graminicola*. The mutant T30 conidia were incubated at 28 °C in a 1:4 dilution of potato dextrose broth. The conidium in the following column burst after incubation for approximately the following hours: left column after 6 h; middle column after 8 h; and right column after 4 h. The conidium in the left and middle column burst on the concave side of the spore whereas the conidium in the right column burst at the spore-substratum interface. The numbers in the right hand corner indicate the seconds after the first frame of each sequence. Bar = 25 μm.

Conidia were collected and washed in sterile, distilled and deionized water, and washed twice in water by centrifugation for 2 min at 16000 ***g*** (Epstein *et al*. 1998).

For video microscopy of rupturing conidia, 2×10^4 conidia per 100 μl droplet of a 1:4 dilution of potato dextrose broth were incubated at 28 ° on multi-well slides (Carlson Scientific, Peotone, IL) in moist chambers. After incubation for 3.5 to 7.5 h, the excess liquid was gently pipetted away, the well was ringed with petroleum jelly, and a cover slip was applied. During microscopic observation, the conidia were maintained at 28 ° by warming the microscope stage with a hair drier connected to a rheostat to deliver the required amount of warm air. Slides were incubated on the microscope stage for a maximum of an hour. Conidia were viewed with an Olympus

Vanox-S microscope fitted with a bright field 40X objective lens and a 10 × eyepiece.

Video images obtained with a Hamamatsu C2400–07 camera were enhanced with an Argus-10 image processor (Hamamatsu Photonic Systems, Bridgewater, NJ), and displayed on a 30 cm, black and white monitor (Sony model PVM-122). Rupture sequences were videotaped in real time with a S-VHS recorder (JVC model BR-S822). Videotaped sequences were played on a variable tracking player (JVC model BR-S525U) and individual images were captured in 8-bit grey scale with an Imascan/Chroma frame grabber (Imagraph, Chelmsford, MA).

To quantify the loss of spore volume after the burst, we traced an enlarged image of the pre-burst spores at '0' time (Fig. 1, upper row) and the post-burst spores at 0.066 seconds (Fig. 1, bottom row). The percentage volume lost by each spore during the burst was calculated as

$$100 \times \{1\text{-}[L_{\mathrm{POST}}(\Sigma W^2_{i\,\mathrm{POST}})/L_{\mathrm{PRE}}(\Sigma W^2_{i\,\mathrm{PRE}})]\},$$

where L = length of the spore, and W_i = width at the ith of nine evenly spaced locations along the length of the spore; this assumes that the cross-sections of the conidia before and after rupture were circular. When we assumed that the conidia were circular in cross section before rupture and flat post-rupture, the formula was calculated as

$$100 \times \{1 - (4/\pi^2)\,[L_{\mathrm{POST}}(\Sigma W^2_{i\,\mathrm{POST}})/L_{\mathrm{PRE}}(\Sigma W^2_{i\,\mathrm{PRE}})]\}.$$

Conidia were examined microscopically before and after rupture. Conidia were suspended at a concentration of 5×10^5 ml^{-1} of a 1:4 dilution of potato dextrose broth, and incubated at 28 ° on a rotary shaker at 200 rpm. After incubation for either 2 h (pre-burst) or 18 h (post-burst), the conidia were collected and then concentrated by centrifugation at 9600 ***g*** in 0.01 % Tween 20. The cytoplasm was extracted from the spores by incubation in 2:1 (95 % EtOH: 2N KOH) at 85 ° for 10 min. The extraction was repeated two more times. Extracted walls were examined with a 40X phase contrast objective and recorded by video microscopy.

To quantify the position of the rupture sites along the longitudinal axis, 87 conidia in which the entire holes were visible were selected and recorded by video microscopy. For each conidium, we recorded four pairs of x, y coordinates of pixels: x_{a1}, y_{a1} and x_{a2}, y_{a2} at the apices and x_{m1}, y_{m1} and x_{m2}, y_{m2} on either side of the conidium at its widest point. Four pairs of x, y coordinates for each hole were recorded in the same way. The x, y coordinates of the midpoint of each hole were computed as the mean of the x, y coordinates of the four points that defined the hole. Then, each flattened spore was modeled as an ellipse that was transformed so that its major axis formed a circular arc through the apices and its sides passed through x_{m1}, y_{m1} and x_{m2}, y_{m2}, respectively. The width to length ratio and the lateral displacement to length ratio were computed for each of the 87 ellipses obtained from the individual spores. The single model conidium shown in Fig. 3 was constructed by transforming an ellipse so that its major axis formed a circular arc and its width to length ratio and the lateral displacement to length ratio were the means of the 87 ellipses. Using the single model conidium, three regions with equal area were computed from area integrals. The hole in each actual conidium was projected onto the single model conidium using the same plane transformation that transforms the individual conidium's ellipse onto the single ideal ellipse. Holes that mapped outside the single ideal ellipse were moved just inside the boundary at the same relative distances from the apices. To quantify the position of the rupture sites along the transverse axis, an additional 126 ruptured conidia were videotaped, without regard to whether there was a frontal or side view of the hole. We constructed a line through the midpoint of the hole across the width of the spore. Assuming that the cytoplasm-free conidia were flat, we divided the line into thirds, and classified each hole according to its position on the concave, middle or convex side of the spore.

To quantify the location where germ tubes emerged, 2×10^4 conidia per 100 µl droplet of a 1:4 dilution of potato dextrose broth amended with 0.3 M KCl were incubated at 28 ° on multi-well slides in moist chambers. After 18 h, slides were prepared for microscopic examination on a Nikkon Microphot – SA. To quantify the position of germ tube emergence, a line was constructed between the two apices of each conidium with the tip of one apex as 0 and the other apex as 10. Based on the model conidium described above, three regions with approximately equal surface area were defined as follows: the two apices were between 0 and 2 and between 8 and 10; the two subapical regions were between 2 and 3.5 and between 6.5 and 8; and the middle region was from 3.5 to 6.5. The location of germ tube emergence was recorded for a total of 334 conidia with a total of 540 germ tubes.

To compare the mechanical strength of the walls in the mutant versus the wild-type conidia, conidia were harvested from Petri dishes and washed as indicated above. The concentration of cells was determined with a hemacytometer. In a 2 ml tube, 3×10^8 conidia in 400 µl water were mixed with 800 µl of 0.5 mm glass beads and shaken in a Mini-Beadbeater (Biospec Products, Bartlesville, OK) for 40 s, and then again for another 20 s. Conidia were recovered from the glass beads by repeatedly suspending the beads in water and collecting the supernatant. Then, the conidia were concentrated by centrifugation and the concentration of unbroken conidia was quantified in a hemacytometer. The percentage of conidial breakage was calculated for each of two replicates, and the experiment was repeated.

Means ± SD are shown in the text. The distribution of holes and points of germ tube emergence were compared by chi-square analysis. Cell breakage in the two strains was analyzed with an analysis of variance. All experiments were performed on at least two occasions with similar results.

RESULTS

Without osmotic amendment to the gemination medium, approximately 80 % of the T30 conidia ruptured and approximately 10 % of the conidia germinated after a 2–14 h incubation at 28 °C. Conidial rupture was not synchronous in the T30 population but always occurred before germ tube emergence. In the wild-type, no conidial rupture during germination was ever observed. We observed approximately twenty T30 conidia in the process of rupturing at various magnifications and recorded three rupturing conidia on

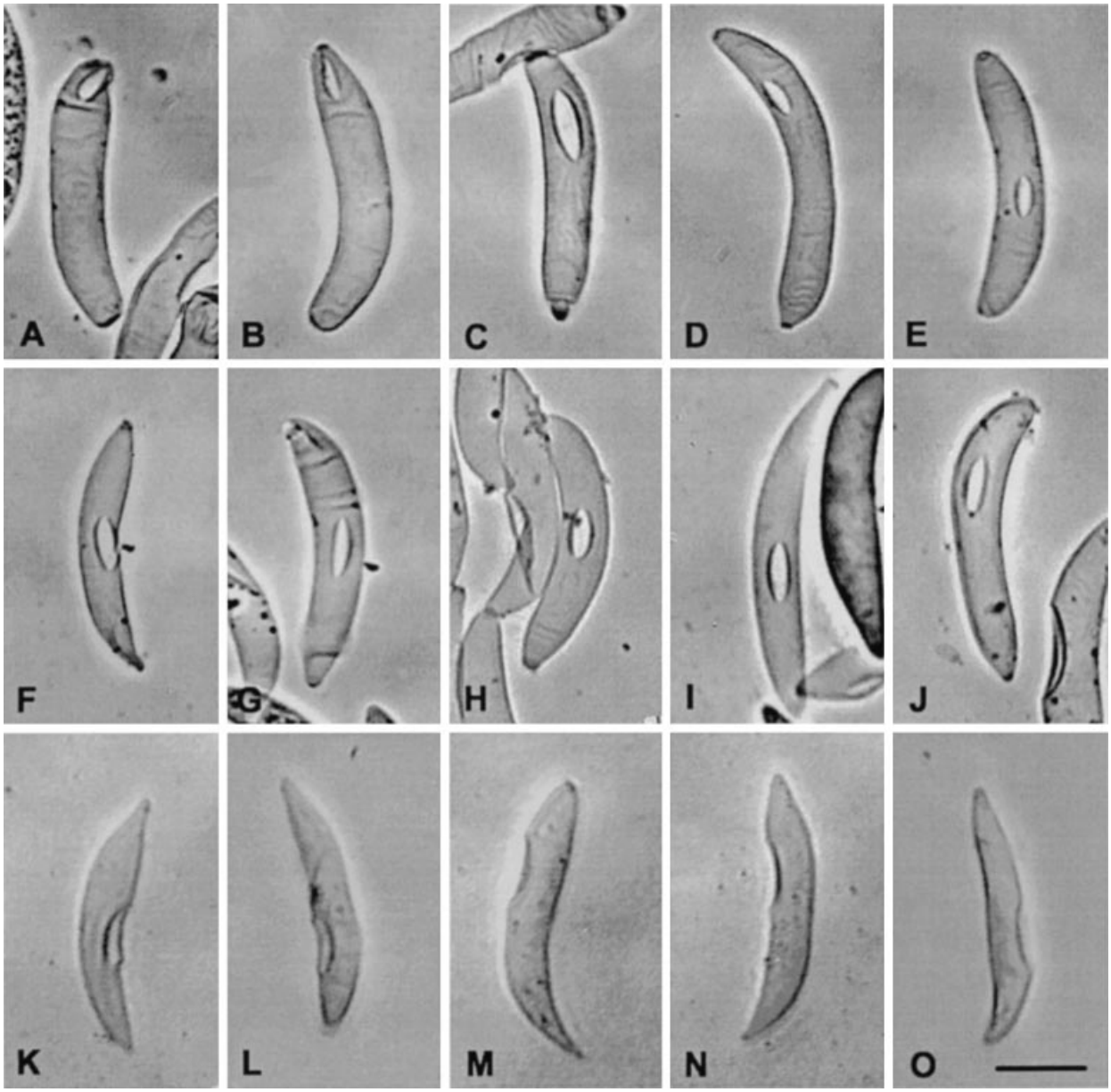

Fig. 2. Phase contrast microscopy of conidia of *Glomerella graminicola* T30 after they ruptured while incubated for 18 h at 28 °C in a 1:4 dilution of potato dextrose broth. After incubation, the conidia were extracted in KOH to remove the cytoplasm. **A–O**, Rupture sites in a variety of locations on the conidial surface are shown. Bar = 12 μm.

videotape (Fig. 1 left column, middle column and right column). Before rupture, no intracellular changes were observed. In addition, no changes in the spore volume were detected (data not shown). During conidial rupture, cytoplasm spewed rapidly and forcibly from what appeared to be a small perforation in the wall. In all three rupture sequences, the bulk of the expelled cytoplasm passed from the interior to the exterior of the spore within 0.07 s. After bursting, the overall volume of the spore decreased substantially. If we assume that the conidia remained circular in cross section after rupture, then we obtain a lower bound of a 48 ± 13 % decrease in volume during the burst. However, if we assume that the conidia became flattened after rupture, then we obtain an upper bound of a 79 ± 5 % decrease in volume.

To characterize the tears in the ruptured conidum walls, 'pre-burst' conidia and 'post-burst' conidia were examined. The former were incubated for only 2 h (0 % burst) and the latter for 18 h (approx. 80 % conidia burst). Cytoplasm was extracted from the spores with KOH, and the cells were examined by phase contrast microscopy. No perforations were observed in the conidial walls of the pre-burst conidia. In contrast, a single gaping hole was observed in the walls of most post-burst conidia (Fig. 2). These long holes (length:width ratio = 4.1) had a fusiform (or spindle-like) outline with the long axis consistently oriented parallel to the long axis of the conidium. The holes had a mean length of 7.1 ± 1.9 μm and a middle mean width of 1.8 ± 0.5 m (n = 87). As a point of reference, the chemically-extracted conidia were 35.2 ± 3.6 m long and had a flattened width of 6.7 ± 1.0 μm (n = 87).

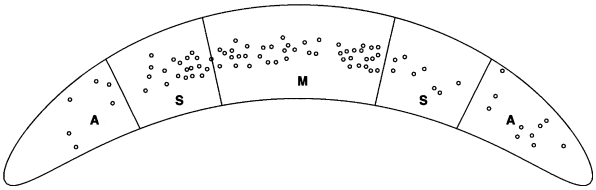

Fig. 3. Schematic of the distribution of holes from 87 ruptured *Glomerella graminicola* T30 conidia projected onto a model conidium. For this analysis, conidia were selected in which the full view of a hole was visible, i.e. conidia with rupture sites on the concave or convex side were not analyzed. When two holes were mapped to the same location, one of the holes was shifted laterally to allow visualization. To assess the longitudinal distribution of the holes on the conidial surface, the model conidium was divided into three regions with equal surface area: A, containing the two apical regions; S, containing the two sub-apical regions; and M, the middle region.

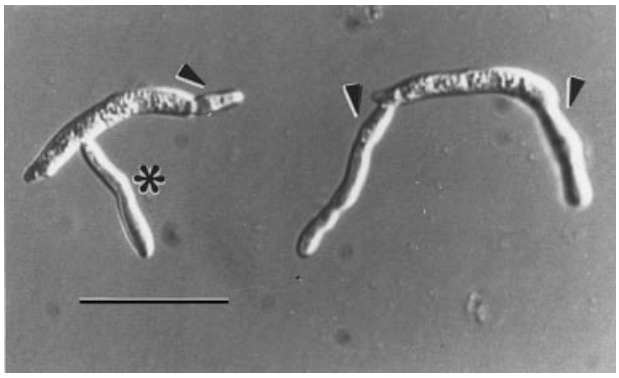

Fig. 4. Differential interference contrast microscopy of germinated conidia of *Glomerella graminicola* T30 incubated for 10 h at 28 °C in a 1:4 dilution of potato dextrose broth with 0.3 M KCl. Arrows point to germ tubes that emerged from the apical regions. An asterisk marks a germ tube that emerged from the middle region. Bar = 25 μm.

The tears occurred over the surface of the spores (Figs 2–3, Table 1): longitudinally from the middle to the apices, and transversely from the convex to the concave side. However, the location of tears was not random and more tears per unit area occurred in the middle of the spores than near the spore apices (Table 1, Fig. 3). In contrast, germ tubes were most likely to emerge from the apical regions (Fig. 4, Table 1). A statistical analysis confirmed that bursting holes and germ tube emergence sites had a significantly different spatial distribution ($P = 0.001$).

We constructed a model, based on the shape of the spores, that explains the orientation and position of the tears. The model assumes that the strength of the wall is uniform in all directions and at all points on the surface of the spore, and that the thickness of the wall is constant throughout the length of the spore. Micrographs from freeze-substituted conidia of *G. graminicola* (Mims *et al*. 1995) are consistent with the assumption that wall thickness is constant. If a solid that is the shape of a falcate conidium is cut by two slightly-separated, parallel planes that are perpendicular to the longitudinal axis, the portion of the solid between the planes is approximately cylindrical. In a cylinder, stress on the wall in the transverse direction $\sigma_T = rP/t$ in which r is the cross-sectional radius, P is the pressure, and t is the thickness of the spore wall (Carpita 1985, Nobel 1983). In contrast, in the longitudinal direction $\sigma_L = rP/2t$ (Money & Harold 1993). Thus, the stress in the transverse direction is approximately double the stress in the longitudinal direction. Walls tear in the direction of greatest stress, resulting in a lengthwise slit (Fig. 2). It follows from the preceding formulas that stress is greater where the radius is greater and that rupture sites would occur more frequently in the middle of the conidum where the radius is larger than at the apices where the radius is smaller (see Discussion).

As a test of the hypothesis that T30 conidia have weaker walls than the wild-type, freshly harvested conidia were shaken with glass beads. The T30 conidia were more susceptible to mechanical breakage than the wild-type. After shaking for 40 seconds, 49 ± 3 % of the T30 conidia were broken whereas only 8 ± 1 % of the wild-type conidia were broken ($P = 0.003$).

DISCUSSION

Glomerella graminicola strain T30 is a genetically tagged mutant whose conidia have a propensity to burst during the germination process (Epstein *et al*. 1998). Before there is any evidence of germ tube formation, the conidial wall rips open and the cytoplasm spills into the medium (Fig. 1). The videotaped images of rupturing conidia illustrate that the cytoplasm within conidia is under considerable pressure. Money & Howard (1996) demonstrated that the average turgor pressure within the end cells of ungerminated conidia

Table 1. For *Glomerella graminicola* T30 conidia, the locations of either the rupture site in burst conidia or of germ tube emergence in non-ruptured conidia[a].

Three regions on the conidium surface with approx. equal area	Location of the rupture site in the conidial wall, %[b,c]	Location of germ tube emergence, %[c,d]
Middle	49	24
Sub-apical	32	22
Apical	18	54

[a] Conidia were incubated for 18 h at 28 °C in a 1:4 dilution of potato dextrose broth with or without 0.3 M KCl; conidia incubated without 0.3 M KCl ruptured whereas conidia incubated with 0.3 M KCl did not rupture and produced germ tubes.

[b] After extraction of the cytoplasm in alkali, 87 conidia were examined by video microscopy.

[c] The distribution of the holes was not uniform ($P = 0.002$, $\chi^2 = 13$, D.F. = 2). Similarly, the location of germ tube emergence was not uniform ($P < 0.001$, $\chi^2 = 101$, D.F. = 2). There were highly significant differences ($P = 0.001$) between the location of the rupture sites and the location of germ tube emergence ($\chi^2 = 44$, D.F. = 2).

[d] A total of 334 conidia containing a total of 540 germ tubes were examined microscopically.

of a non-melanized strain of *Magnaporthe grisea* was approx. 1.4 MPa after a 2-h incubation.

The orientation and distribution of the tears in the conidial walls of *G. graminicola* strain T30 can be explained biophysically. Our finding that the split in the conidial walls is oriented in the longitudinal direction is analogous to findings on pressure-induced bursting of sporangiophores of *Phycomyces* (Roelofsen 1959). Squeezing the sporangiophore tube causes it to split along its length parallel to the longitudinal axis, despite the fact that the wall microfibrils are mainly oriented in the transverse direction. Castle (1937) used an engineering principle to explain this paradox: tensile stress in the wall of a cylinder exposed to uniform internal pressure is about twice as great in the transverse direction as in the longitudinal direction. This principle also has been invoked in the plant physiology literature (Carpita 1985, Nobel 1983). Thus, in a cylinder under pressure, fibers in the transverse direction tear, which results in a longitudinal split. The same formulas for wall stress indicate that for the tapered conidia of *G. graminicola*, the stress on the wall is greater in the middle region of the spore, where the radius is greater, than at the apices, where the radius is smaller. Thus, the model predicts that cell rupture would occur more frequently in the middle region of the spore than at the spore apices, in agreement with our observations.

Since germ tube emergence in other fungi is accompanied by localized lysis of the spore wall (Hess *et al*. 1975), one could postulate that T30 conidia rupture because they overproduce wall-lytic enzymes during the incubation process, particularly at the site of germ tube emergence (Bartnicki-Garcia & Lippmann 1972, Wessels *et al*. 1990). The finding that wall ruptures occur most frequently in the middle of the conidia (Fig. 3) whereas germ tubes emerge most frequently from the apices (Table 1, Fig. 4) speaks against this possibility. Also, there is no evidence of greater chitinase activity in the conidia of the T30 mutant than in the wild type (Amnuaykanjanasin & Epstein, unpubl.). In addition, the long fusiform shape of the rupture is more indicative of a physical split in the fabric of the wall than of localized enzymatic erosion.

The evidence collected suggests that the catastrophic bursting of germinating conidia of T30 is due to a physical weakness of the cell walls. This contention is supported by the observation that T30 conidia break more rapidly than wild-type conidia when shaken with glass beads, and is further supported by the finding that conidial walls in T30 have approx. 25 % less *N*-acetylglucosamine than the wild type (Amnuaykanjanasin, Epstein, & Labavitch, unpubl.). In addition, the turgor pressure in T30 conidia was not greater than in wild type conidia (Epstein *et al*. 1998). Thus, we postulate that during germination the intrinsically weak conidial wall of T30 cannot withstand the turgor pressure that develops inside the conidium. Although the disrupted gene in T30 has not yet been identified, the T30 mutant indicates that there is a single gene whose function is essential for maintenance of cell wall integrity in *G. graminicola* conidia. Preliminary evidence suggests that wall integrity of multiple cell types is affected.

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