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Glycerol generates turgor in rice blast

Many plant pathogenic fungi are able to penetrate the cuticles of their host plants by elaborating specialized cells known as appressoria[1,2]. The morphology and development of appressoria have been well studied[2], but little is known about how these cells are able to breach the tough plant surface. We have now found that the appressoria of rice blast fungus (*Magnaporthe grisea*) use glycerol to generate pressure which ruptures plant cuticles.

Rice blast is the most serious disease of cultivated rice, the staple food for one-third of the world's population[3,4]. The fungus forms heavily melanin-pigmented appressoria that generate enormous turgor. The pressure is applied as a physical force to break the rice leaf cuticle[5,6]. Turgor can be as great as 8.0 MPa (modal value, 6.0 MPa)[5], equivalent to 40 times the pressure in a car tyre and is, to our knowledge, the highest recorded in any living organism[5].

We extracted the contents of appressoria and biochemically characterized them, searching for a metabolically compatible solute responsible for generating the hydrostatic pressure. We grew spores of *M. grisea* in water drops on hydrophobic plastic membranes and allowed them to form appressoria. These generated full turgor over a period of 24–48 h. Gas–liquid chromatography showed that the most abundant solute in appressoria is glycerol.

Glycerol is generated rapidly during germination and germ-tube elongation (Fig. 1a). Here it probably contributes to plasma membrane biosynthesis during initial fungal growth[7]. Glycerol levels decrease at the onset of appressorium formation but rise sharply during turgor generation. This coincides with the collapse of the conidium and germ tube, and concentration of the cytoplasm within the unicellular appressorium (Fig. 1b). Intracellular glycerol concentration is considerably higher at this time (48 h), being contained in a small volume.

We estimate the mean internal volume of an appressorium as 64 μm^3 assuming that the cell is hemispherical with an internal radius of 3.1 μm ($n = 100$, s.d. = 0.5). The mean concentration of glycerol in an appressorium rose from 0.50 M at 24 h of development to 3.22 ± 0.40 M after 48 h. This is a conservative estimate because not all of the cell volume is available for glycerol accumulation. The osmotic potential generated by this concentration of glycerol would be −8.7 MPa at 20 °C (assuming that glycerol is an ideal solute).

Glycerol solutions deviate from ideal at high concentrations. The maximum concentration of glycerol for which osmotic potential can be assayed psychrometrically[8] is 2.0 M, but by extrapolation we estimate that 3.22 M glycerol would generate an osmotic potential of −5.8 MPa. This represents a minimum osmotic potential because living appressoria contain other solutes. The appressorium forms in a drop of water, so the turgor produced is at least 5.8 MPa.

To validate these estimates we incubated appressoria in a series of glycerol solutions of varying molarity and measured the frequency of cytorrhysis[5] (cell collapse). The frequency was dependent on external glycerol concentration. A concentration of 1.75 M glycerol (−3.7 MPa) caused the collapse of 52% of appressoria (data not

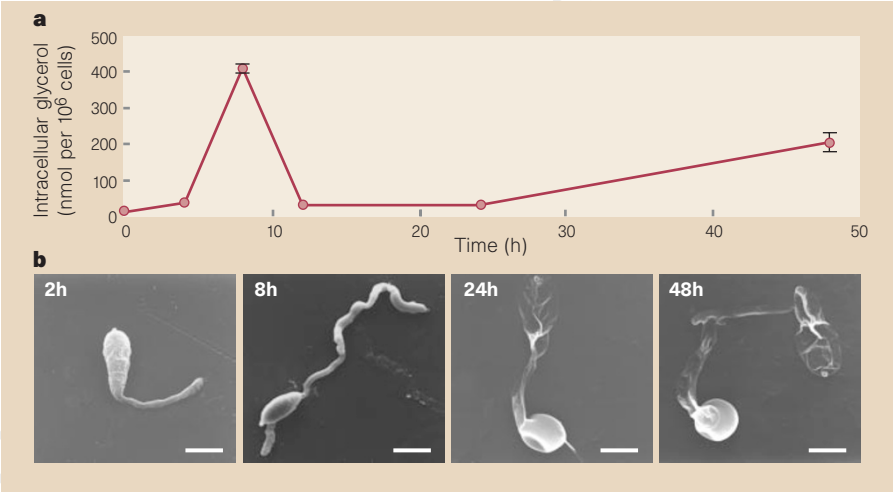

Figure 1 Intracellular glycerol increases during *M. grisea* appressorium turgor generation. **a,** Change in glycerol contents of extracts made from germinating conidia and developing appressoria with time, determined enzymatically using a glycerol-specific assay (Boehringer). **b,** Low-temperature scanning electron microscope images of developing appressoria at corresponding times. Appressoria were allowed to form in water drops on polytetrafluoroethylene (PTFE-Teflon, DuPont) membranes. As the appressorium develops turgor the conidium and germ tube collapse[6]. Cytoplasm is present only in the unicellular appressorium. Scale bars, 20 μm.

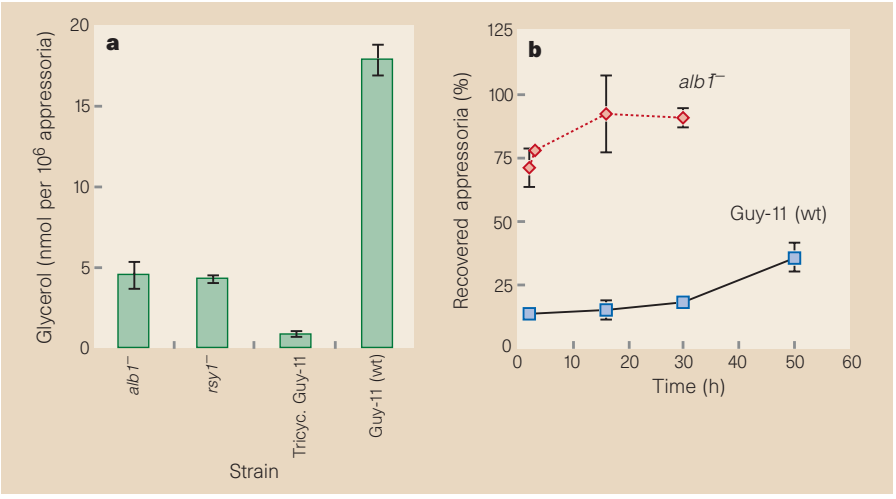

Figure 2 Non-melanized appressoria are permeable to glycerol. **a,** Intracellular glycerol levels in appressoria of *alb1*⁻ and *rsy1*⁻ mutants[9]; and of strain Guy-11 treated with tricyclazole, compared with wild-type untreated appressoria. We measured intracellular glycerol concentration after 24 h. **b,** We allowed appressoria to form in water for 24 h on PTFE membranes and then replaced the water with 4 M aqueous glycerol. We determined the rate of cytorrhysis and the proportion of appressoria that had recovered from cytorrhysis at each time point. Mean (± s.d.) values from 100 appressoria in two independent experiments are represented. Melanized and non-melanized appressoria recovered fully after 60 min incubation in water (not shown).

shown). This supports the link between turgor and glycerol accumulation but also implies that the melanized wall is largely impermeable to glycerol.

Appressoria of *M. grisea* are heavily melanized[9,10] and it has been shown genetically[11] that non-melanized appressoria fail to generate turgor and are non-pathogenic[5]. We found much lower levels of intracellular glycerol in appressoria from non-melanized strains carrying single gene mutations at *ALB1* and *RSY1* (Fig. 2), genes encoding enzymes required for dihydroxynaphthalene–melanin biosynthesis[10]. We found a similar reduction in glycerol accumulation after treatment of *M. grisea* with tricyclazole, a melanin biosynthesis inhibitor[12,13] (Fig. 2a). Thus, melanin biosynthesis is required for efficient glycerol accumulation.

In cytorrhysis experiments we found that *alb1*$^-$ mutant appressoria collapsed in hyperosmotic solutions of glycerol but quickly recovered (in under 1 min) and instead became plasmolysed[5]. This indicates that the non-melanized wall is permeable to glycerol and after initially causing cytorrhysis, glycerol diffuses through the cell wall and induces plasmolysis of the appressorial protoplast. This is in marked contrast to wild-type melanized appressoria which showed only limited recovery from cytorrhysis even after 48 h incubation in hyperosmotic glycerol (Fig. 2b). Maintenance of the enormous glycerol concentrations within appressoria is likely to be a consequence of the reduced permeability of melanized cell walls to glycerol preventing rapid leakage of the solute.

Several important plant pathogens form appressoria[1,2] and although secretion of enzymes may aid cuticular degradation[2], mechanical infection of plant tissues is probably widespread. The infinite solubility and metabolic compatibility of glycerol therefore provides a simple and durable mechanism for plant infection which may be widely applied by pathogenic fungi.

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Reptile relationships turn turtle...

Turtles are so anatomically bizarre that their affinities with other reptiles remain contentious. Wilkinson *et al.*[1] showed how even the extensive morphological information amassed by Rieppel and deBraga[2,3] provides only weak support for their view that turtles are advanced diapsid reptiles, rather than descendants of primitive anapsid reptiles, as conventionally thought. But there seem to be errors in Rieppel and deBraga's data matrix, many involving turtles or their putative 'anapsid' relatives[4,5]. I have corrected and reanalysed the data (see 'Incorrectly coded characters', overleaf), and find that Rieppel and deBraga's data actually support, rather than challenge, the traditional view.

The modified data yield a tree where diapsid monophyly is restored and turtles are related to anapsid pareiasaurs (Fig. 1a). This result is consistent with other recent analyses[4,5]. In particular, the tree is almost identical to that proposed in another detailed phylogenetic analysis of the entire Reptilia[6] (Fig. 1b). The large impact of these apparently minor corrections to Rieppel and deBraga's data set is not surprising.

In their phylogeny (Fig. 1 in ref. 2) almost all of the characters interpreted as supporting turtle–diapsid affinities (those diagnosing clades 3 to 7) also occur in all or many anapsid 'parareptiles', or are absent (presumed reversed) in turtles. Thus, only a slight modification to the data caused turtles to shift from deep within diapsids (as lepidosaur relatives) to deep within parareptiles (as pareiasaur relatives).

My results, together with those of Wilkinson *et al.*[1], emphasize the importance of evaluating just how strongly a preferred tree is supported over alternatives. Rieppel and deBraga have identified a surprisingly strong phylogenetic signal linking turtles and advanced diapsids. However, the conventional views regarding

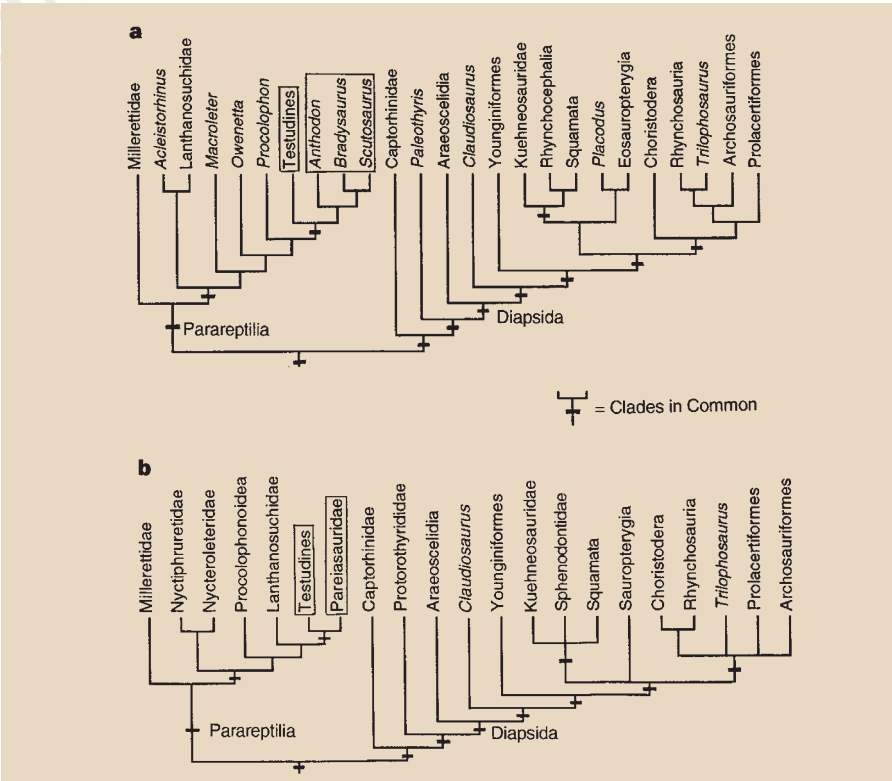

Figure 1 Alternative reptilian phylogenies. **a,** The single most parsimonious tree (length 763 steps, consistency index 0.511, retention index 0.695), based on an analysis of the data matrix of Rieppel and deBraga[2,3], after corrections detailed in footnote. Turtles (Testudines) are nested within 'anapsid' parareptiles, as the nearest relatives of pareiasaurs (*Anthodon*, *Scutosaurus*, *Bradysaurus*), and diapsid monophyly is restored. **b,** Phylogeny obtained from an independent study of higher-level reptile phylogeny[6]. Note the close similarity to **a**. Clades in common are indicated by horizontal bars. In both trees, diapsids are monophyletic and turtles are nested within parareptiles.