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Cell wall appositions and plant disease resistance: Acoustic microscopy of papillae that block fungal ingress

(cytology/host response/parasite-host interaction/primary penetration)

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ABSTRACT **Plant cells react to localized stress by forming wall appositions outside their protoplasts on the inner surface of their cellulose walls. For many years it has been inferred that appositions elicited by encroaching fungi, termed "papillae," may subsequently also deter them and thus represent a disease-resistance mechanism. Recently, it has been shown that preformed, oversized papillae, experimentally produced in coleoptile cells of compatible barley, *Hordeum vulgare*, can completely prevent direct entry of *Erysiphe graminis* f. sp. *hordei* that ordinarily penetrates and causes disease. To discover how these papillae may function, acoustic microscopy was used to contrast their *in vivo* elastic properties with those of ineffective normal papillae and contiguous cell wall. Raster and line scans showed intense acoustic activity at sites of preformed papillae; scans in selected focal planes identified this activity with the papillae, not with subtending cell wall. Minimal acoustic activity was found in normal papillae. It is suggested that some wall appositions could serve in disease resistance as viscoelastic barriers to mechanical forces exerted by the special penetration structures of advancing pathogenic fungi.**

Living plant cells rapidly react to localized stress by directly apposing substances onto the inner surface of their cellulose walls. Because such wall appositions (1) could, by healing, forestall death of injured cells (2), their timely formation could also contribute to the survival of whole plants when injury from pathogen penetration and subsequent infection are imminent. It has even been suggested that wall appositions elicited by fungi, termed "papillae" (3), may represent a disease-resistance mechanism because in some cases they seem to intercept invading pathogens (for review, see ref. 2).

In the past, papilla function has usually been judged from after-the-fact correlations, often taken from spent or preserved parasite–host systems, which have thus been equivocal (2). Recently, however, some workers have turned to evaluations of living systems, and only very lately has the process been examined experimentally.

In the most recent report (4), part of an extended series of studies done in our laboratories (5–14), we showed that oversized, preformed papillae experimentally produced in coleoptile cells of compatible barley (*Hordeum vulgare*) can prevent both entry and establishment of an obligate parasite, *Erysiphe graminis*, that ordinarily gains easy access unimpeded by normal papillae and causes a powdery mildew disease.

This controlled assessment of papilla function has set the stage for direct analytical work in which comparisons between the totally effective preformed and ineffective normal papillae could reveal how wall appositions can stop fungi from invading plant cells. The challenge now is to devise new or adapt already existing analytical techniques that can yield reliable physical and chemical data on cells.

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Acoustic microscopy represents a new capability for visualizing certain physical properties, not dependent upon refractive index, of cells and tissues (15). Point-by-point properties of structures can be evaluated nondestructively as they react *in situ* to periodic stress waves. With acoustic microscopy one may view structures within living samples and gain detailed information about their elasticity, density, or viscosity. This information is unobtainable by any other technique. Thus, the acoustic microscope seemed uniquely suited for estimating the "strength" of papillae, as they repel or yield to attacking parasitic fungi.

The goals of this study were simple and direct: (*i*) to contrast the acoustic activity of effective (resistant) papillae with that of the ineffective (nonresistant) and with that of adjacent cell wall, and (*ii*) to assess the potential for acoustic microscopy in cytological research on intact living specimens.

MATERIALS AND METHODS

Parasite–Host. *Erysiphe graminis* D. C. f. sp. *hordei* Em. Marchal (race B7) was maintained on compatible barley, *Hordeum vulgare* L. cv. "*Proctor*," as described (8).§

Specimens for examination were prepared by published methods (see protocol B, ref. 4); in fact, some were prepared simultaneously during the earlier study on papilla function. Briefly, host coleoptiles were excised from barley seedlings 7 days after sowing and readied as described by Takamatsu *et al.* (16). These preparations were floated on 0.01 M $Ca(NO_3)_2$ in petri dishes, inoculated with freshly harvested conidia in a spore-settling tower, and incubated at 18°C in the dark for 9 hr. Then, the preparations were incubated for 15 hr on a 1:1 (vol/vol) mixture of 0.01 M $Ca(NO_3)_2$ and 0.01 M sodium phosphate buffer at pH 7.5; this procedure results in the formation of oversized papillae. Next, the coleoptiles were returned to a fresh 0.01 M $Ca(NO_3)_2$ solution, the superficial fungal structures were wiped off with a moist cotton swab, and the tissues were inoculated again (challenge) with fresh fungal conidia. Twelve hours later (about +38 hr), the preparations, bearing preformed, oversized papillae that, when challenged, effectively blocked fungal ingress, were ready for microscopic examination. Concurrent controls of normal papillae were prepared either by inoculation and incubation for 14 hr on 0.01 M $Ca(NO_3)_2$ or by incubation for 25 hr on 0.01 M $Ca(NO_3)_2$ followed by inoculation and a further 12-hr incubation.

§ Under standard conditions, when conidia (propagules) of *E. graminis* contact barley leaves and coleoptiles they germinate within 1–2 hr. One germ tube enlarges apically to form an appressorium (attachment structure) which is mature when a lateral extension, or "hook," appears at its tip. About 10.5 hr after inoculation, a thin penetration peg grows from the hook through the host cell wall. One hour later, the peg enlarges at its tip to form a haustorial central body, thereby completing penetration.

Light Microscopy. Preparations were mounted on glass slides over 0.01 M $Ca(NO_3)_2$ and areas of interest were identified by using Zeiss interference-contrast optics. Photographs were recorded on Kodak Tri-X Pan film at ×160 or ×256 and appropriately enlarged.

Acoustic Microscopy. The basic principles of the scanning acoustic microscope have been described (17–19), but a brief summary is in order. A highly convergent acoustic beam that raster scans across an object in an immersion fluid [in this instance, 0.01 M $Ca(NO_3)_2$], is used. The reflected signals are displayed on a synchronized cathode ray tube and recorded electrically and photographically, as appropriate. The acoustic beam is focused from a transceiver lens consisting of a polished hemispherical depression in a sapphire rod. A piezoelectric transducer converts acoustic waves to and from the electrical signals that are either received from the high-frequency generator or transmitted to and imaged on the cathode ray tube monitor.

The experimental microscope used in this study was a mechanically scanned (60 Hz) system operated in a 50-nsec pulsed-reflection mode at a frequency of about 375 MHz; it used the amplitude of the reflected signal exclusively. Raster scanning produced a two-dimensional display that was recorded in a single 400-line, 7-sec scan; gray-scale photographs at approximately ×150 to ×200 were obtained, in this case, with Polaroid type 107 Land film. Alternatively, individual line traces could be displayed and recorded from a conventional oscilloscope as the image was line-scanned at any desired specimen location. Under the conditions used, point resolution of the instrument ranged between 1 and 2 μm.

For this study, the biological specimens were prepared in Ithaca, NY, and shipped or hand-carried, while appropriately incubated, via connecting airline flights to Malibu, CA, where the subsequent microscopy was done. Replicate observations were made on three separate occasions.

RESULTS

The parasite–host system under examination was fully compatible with the protocol for acoustic microscopy. Although the area of attention was near the upper surface of the cell (Figs. 1 and 2), well within the focal depth of the lens, the full structure of papillae could be followed into the lumen of the cell

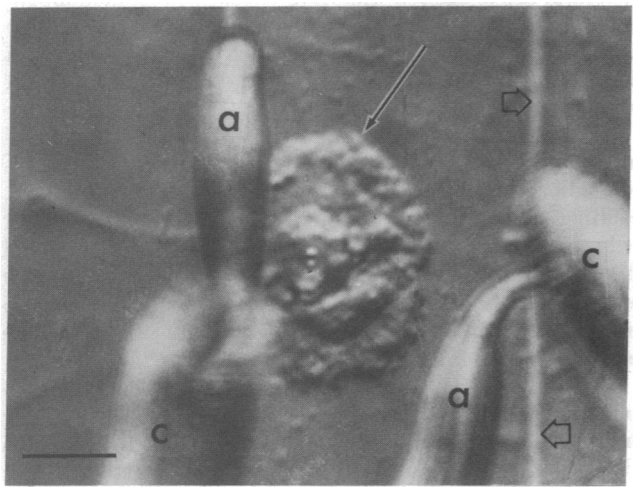

FIG. 1. Preformed papilla (thin arrow) produced 9–24 hr after inoculation and during incubation over 0.01 M $Ca(NO_3)_2$/0.01 M Na phosphate buffer. Also shown are two conidia (c), from a challenge inoculation, each with its appressorium (a), and the anticlinal side walls (thick arrows) of the columnar epidermal cells. Superficial fungal structures of the primary, inducing inoculum had been wiped away. (Interference-contrast light microscopy; bar = 10 μm.)

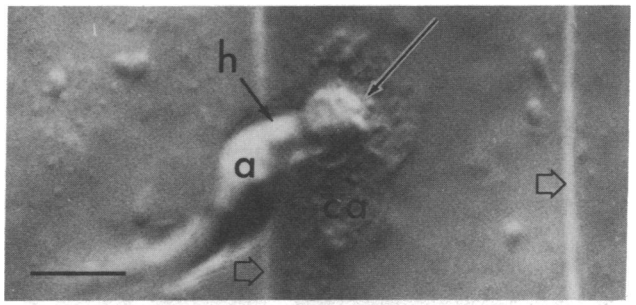

FIG. 2. Details of a penetration site showing a normal papilla (thin arrow), formed during continuous incubation over 0.01 M $Ca(NO_3)_2$, an appressorium (a) with its hook (h), and the host cell side walls (thick arrows) and cytoplasmic aggregate (ca) that precedes papilla formation. (Interference-contrast light microscopy; bar = 10 μm.)

(Fig. 3). Because the preparations routinely were floated on 0.01 M $Ca(NO_3)_2$ and often were submerged briefly during photomicrography, the need for immersing them during acoustic analyses presented no problems. A number of specimens examined in the acoustic microscope were subsequently compared photo-optically, 3 days after their return to Ithaca, with others of the same age, some of which had been shipped to Malibu and others that had not, and were found to be fully viable and responsive. Although considerable cellular detail was revealed by the acoustic microscope, attention was given only to the cell wall and attached appositions.

Preformed, oversized papillae were much more acoustically active (elastic, viscous, or dense) than both their normal counterparts formed in the presence of only 0.01 M $Ca(NO_3)_2$ and contiguous areas of cell wall. The design of the instrument is such that the more optically bright structures in the video scans are more acoustically active. The line scan in Fig. 3*i*, which traverses 12 cells, shows three levels of response. The lowest level of peaks results from reflections off anticlinal side walls of the host; the next level, from its convex outer periclinal walls; and the highest peak, from the preformed papilla. A number of scans (not shown) done above and below the latitude indicated in Fig. 3*h* clearly showed that the intense reflections were coincident with the site of the papilla. (This correlation proved to be of great assistance because one could quickly locate preformed papillae by locating intense peaks in line scans as the object was rapidly translated across the fixed lens.) The line scan in Fig. 4*b*, was typical of those taken along the periclinal wall of a cell with a papilla. One-for-one association between this tracing and optically bright areas in Fig. 4*a* are difficult because the video system was tuned for highest gain (maximal contrast, minimal gray scale) at the time in order to compare accurately regions of maximal reflectance in wall and papilla. By carefully recording line scans in selected focal planes it was determined that the very pronounced acoustic activity, of any given papilla site, was exclusive to the papilla with little contribution from the subtending cell wall. Most, but not all, preformed papillae exhibited centers with comparatively lower reflectance near the cell surface and thus appeared toroidal in some views (Figs. 3 *a–f* and 4*a*).

Little to no acoustic activity, as determined from both video and line scans, was associated with normal papillae (Fig. 5).

By directly comparing acoustic micrographs of preparations from which superficial fungal structures (conidia, appressoria, germ tubes, etc.) were removed with those that were left intact, it was evident that these structures also exhibited minimal acoustic response and thus had little effect on the registered acoustic patterns.

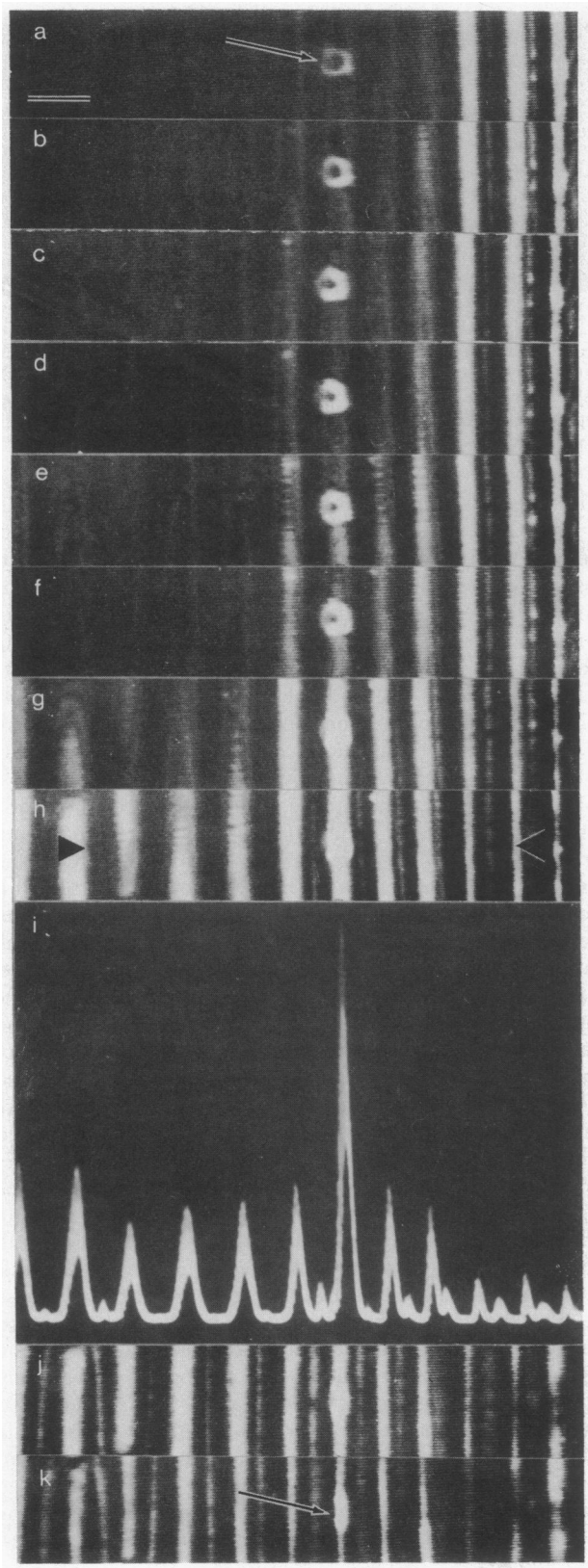

FIG. 3. (*a–h, j,* and *k.*) Acoustic micrographs, taken at 10 different focal planes separated by ≈3 μm, of a single preformed papilla (thin arrow) attached to the inner surface of the outer periclinal wall of the host cell. Note that the acoustic response of the papilla, which is beneath the wall, is much stronger than that of the cell wall, even at a number of focal planes (*a–f*) above the specimen. (*i*) Single line scan, left to right, across the coleoptile in the same focal plane shown in *h* and at the latitude indicated by the solid black triangles. (Bar = 50 μm.)

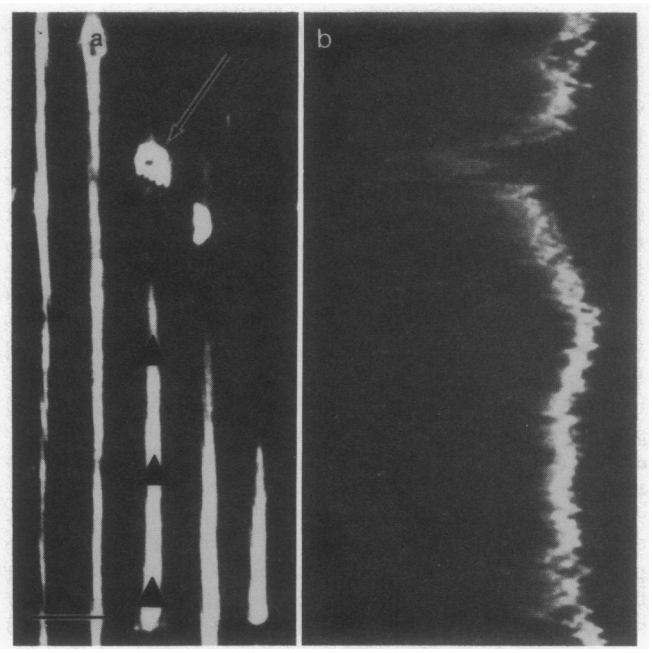

FIG. 4. (*a*) Acoustic micrograph of a preformed papilla (thin arrow) similar to that shown in Fig. 3. (*b*) Line scan bottom to top, along the crest of the periclinal wall, to which the papilla is attached, in the approximate path indicated by the black triangles in *a* (Bar = 50 μm.)

DISCUSSION

Traditionally, two distinct models, which correspond to long-held views on how fungal pathogens penetrate directly into plant cells (20), have been offered to explain localized, even subcellular, disease-resistance mechanisms. One model relates to the chemical composition of an area and its potential for regulating passage of injurious substances or for neutralizing injurious degradation processes. The other model derives from an area's physical properties (elastic moduli?) and its potential for resisting mechanical force.

A number of investigators have sought to relate wall appositions, particularly papillae, to the former model (for review, see ref. 2), but the small sizes of papillae (normally 5–15 μm in diameter), their low incidence in the cells, and their close structural ties to the cell wall have virtually precluded meaningful cytochemical and biochemical analyses. With respect to the latter model, Bushnell (21) micrurgically removed a single papilla from a cell and surmised that it was "hard" but could conclude little about its physical strength.

The present study suggests that the problem and the instrument appear to be ideally suited for each other. In the parasite–host system used, wall appositions are discrete structures, routinely identified by light microscopy, that are located just below the upper surface of the specimens, within the focal depth of the acoustic lens. Because the work can be done *in vivo* and *in situ*, and individual analyses can be done quickly (<5 min), numerous experimental problems that would otherwise cloud biological interpretations can be obviated.

The results clearly indicate that preformed, oversized papillae, experimentally produced and tested to be completely impregnable, are more acoustically active (i.e., elastic) than both their normal counterparts and contiguous areas of cell wall. But a number of important questions remain unanswered.

We do not yet know to what measure the oversized papillae are more acoustically active. Although advances continue with respect to the makeup, accuracy, and interpretation of the acoustic image (19, 22–25), and the instrumentation for quantitative analyses is being developed (26), precise quanti-

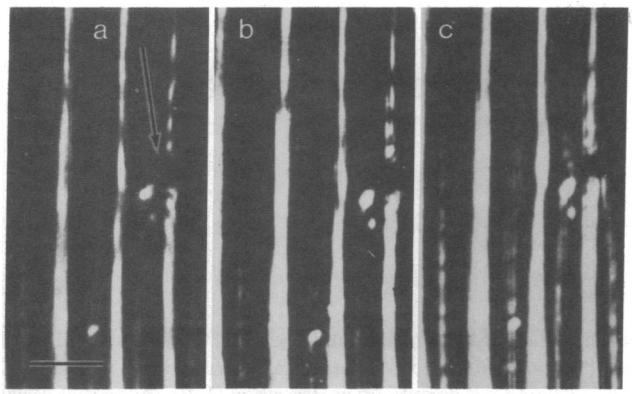

FIG. 5. Three acoustic micrographs, at different focal planes ≈3 μm apart, of a coleoptile with normal nonresistant papillae. Little to no acoustic activity was associated with the sites of these papillae, such as at the end of the arrow in *a*, where a papilla was identified by using bright-field light microscopy. (Bar = 50 μm.)

fication of the echoes from complex structures will require some additional developments.

We do not yet know when, during formation, the papillae become more elastic and how long this quality persists. Using micrurgical techniques devised for removing intact papillae from living cells (11), we have found that freshly excised normal papillae are firm and hard, almost impenetrable to glass needles. There is also some tentative evidence that appositions initially are soft and harden with time (2), but hardness alone may not be important in this instance. Acoustic microscopy now affords a means for examining the real-time development of individual papillae and for assessing performance with respect to specific physical properties.

We have not yet examined, at high resolution, the acoustic substructure of preformed papillae. Sometimes they exhibit a concentric ring pattern when viewed from above at right angles by light microscopy (Fig. 1; ref. 4) as well as laminations when viewed in cross sections by transmission electron microscopy. Information about the relative acoustic activity of these regions and of peripheral cell wall areas could help answer some of the questions that have arisen with respect to the diminished fungal penetration that occurs in regions beyond the papilla's edge but within 10 μm of it (4), as determined by light microscopy. In this regard, we also cannot account exactly for the toroidally shaped echoes that are often emitted by subsurface regions of preformed papillae (Figs. 3 *a–f* and 4*a*). Provisionally, we regard the low acoustic activity in the centers of these images to result from a hemispherical core region, often seen in transmission electron microscopy, that is equal in size and hardness—as reckoned by micromanipulation with glass needles—to normal papillae.

The final unanswered and paramount question concerns the heart of the matter—disease resistance at the subcellular level. Is increased elastic strength the sole contributor or are there other features, less mechanical in nature, that confer resistance? Only thorough analyses can offer answers to this question, analyses for which some instrumentation is not yet a reality. Even such answers will not detract from the essential conclusion of this presentation—that preformed, oversized papillae, known to block fungal ingress, are more elastic, viscous, or dense than the typically vulnerable normal papillae. This being so, the precise physical and chemical nature of these experimentally generated large papillae remains a question of great interest and one to be more fully explored.

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