if not identical (Fig. 3). The spectra show absorbances at 2800 to 3000 cm^{-1} and at 1465 cm^{-1} characteristic of methylene groups ($-CH_2-$), and at ~1720 cm^{-1}, characteristic of six- and seven-membered ring ketones, as is expected for an oxidized homogentisic acid polymer.

The synthetic and extracted pigments were stable to acid hydrolysis. Hydrolysis of the extracted pigment, in 6*N* HCl at 110°C for 20 hours, did not yield any nitrogenous ninhydrin-reactive components. Amino acid analysis of the extracted bone, on the other hand, showed that the bone matrix was typically collagenous. The infrared spectrum of the extracted pigments did not contain any absorption bands that could be attributed to amino acid or peptide components. It has been suggested that ochronotic pigment is covalently associated with protein or proteoglycan components of the connective tissue (*5*). The pigment isolated in the articular cartilage of Harwa, however, was readily extractable in dilute alkali and, as indicated above, was free of protein or amino acid components.

Normal human skin and cartilage contain metalloenzymes, homogentisic acid polyphenol oxidases, which appear to be responsible for the oxidation of accumulated homogentisic acid not normally metabolized. However, neither the chemical nature of the extract of the ochronotic pigment nor the mechanism of its biosynthesis is known. We have not, therefore, attempted a more complete characterization of either the synthetic or extracted pigments.

However, the above data show that the solubility and properties of the spectra of the extracted pigment from Harwa's cartilage are nearly identical with those of the synthetic pigment produced from the oxidative polymerization of homogentisic acid. We can thus conclude that the black pigment found in Harwa's joints and bones was derived from homogentisic acid and that Harwa, living at about 1500 B.C., suffered from the heritable disorder of ochronosis. The common observation of black pigments in Egyptian mummy cartilages suggests that this autosomal recessive disorder was of frequent occurrence in ancient Egypt, probably a result of frequent intrafamilial marriage.

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Mechanically Induced Wall Appositions of Plant Cells Can Prevent Penetration by a Parasitic Fungus

Abstract. *Localized, paramural wall appositions resembling appositions commonly induced by fungal attack, were induced in kohlrabi (*Brassica oleracea *L. gongyloides) by mechanical wounding (bending) of the root hairs before the hairs were inoculated with zoospores of a compatible parasitic fungus. The appositions were effective in preventing fungal penetration at the wound sites, which shows that wall appositions can prevent fungal ingress into plant cells.*

All living tissues respond characteristically to wounding. Plant tissues may respond morphologically by producing new cells and modifying existing cells at the periphery of the injured region, thereby containing the damage. The accompanying metabolic changes include increased respiration and altered phenolic and protein metabolism. Because these and other wound-healing responses closely resemble plant disease-resistance reactions, it is widely held that wound healing is a major disease-resistance mechanism (*1*).

Individual plant cells rapidly respond to both wounding and fungal attack by accumulating a mass of seething cytoplasm (cytoplasmic aggregate) at the site and depositing localized, amorphous, heterogeneous masses of materials paramurally—between their plasmalemmas and cell walls (*2*). The paramural depositions were termed wall appositions (*3*) and were postulated to operate as a defense mechanism against fungal ingress, although the evidence is not conclusive (*2*). By mechanically inducing wall appositions before inoculation and determining whether or not a fungus can penetrate them, I have obtained evidence that this widespread plant cell reaction can prevent fungal penetration.

The parasitic fungus *Olpidium brassicae* (Wor.) Dang. was maintained in roots of *Brassica oleracea* L. *gongyloides*, kohlrabi, as described previously (*4*). Zoospores of this fungus encyst on root hairs and initiate penetration 2.25 hours later by means of penetration tubes which grow through the cell walls into the host cells. The parasite protoplasts are then injected through the tubes into the host cytoplasm. Each cyst requires about 15 minutes to penetrate, and a synchronized population of cysts will normally complete penetration between 2.5 and 3.0 hours after inoculation. Host cytoplasmic aggregates and wall appositions are always associated with penetrations, and most appositions are initiated after the penetration tubes are fully extended (*4*).

Wall apposition formation was induced mechanically in hydroponically cultured kohlrabi seedlings (*4*). The seedlings were first taken from their growth-supporting salt solution and suspended in a moist chamber. Fluids adhering to the root axes bent the hairs near their bases and held them flat against the roots. Localized wall appositions (Fig. 1A) were deposited by cytoplasmic aggregates (*4*) along creases (wounds) in the walls where the hairs were bent. Thirty minutes later the roots were returned to the solution for an additional 30 minutes; deposition continued even though the root hairs straightened out in the solution. This procedure was then repeated with the roots of each seedling receiving a final incubation of 60 minutes in the solution, to ensure both a high frequency of well-formed appositions and complete termination of cytoplasmic aggregates before the next step. Some roots were then inoculated immediately with a suspension of *O. brassicae* zoospores (*4*), while others were heat-shocked at 41°C for 20 minutes (*5*) and then inoculated. Heat-shocking was done to inhibit new apposition formation (*5*) in response to pene-

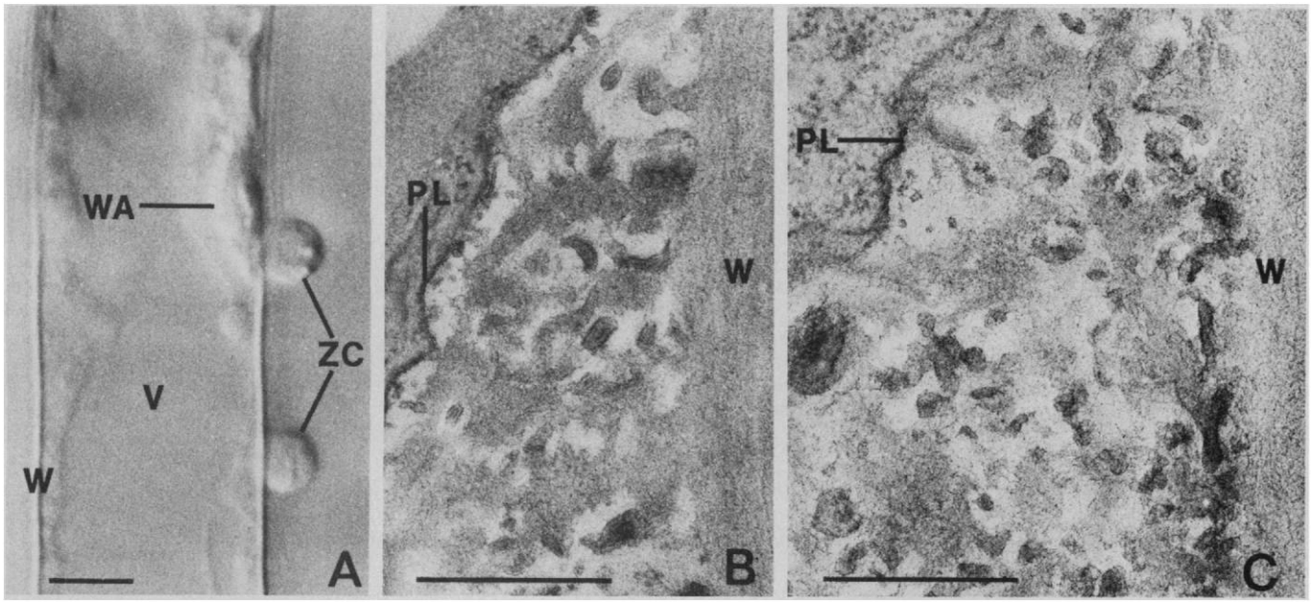

Fig. 1. Micrographs of wall appositions in kohlrabi root hairs. (A) A pair of cysts, typical of those from which the data in Table 1 were compiled, as seen in the living state by means of interference contrast optics. This photomicrograph, taken about 2 hours after the inoculation of unshocked root hairs with zoospores of *O. brassicae*, shows the pair at the time of selection, before visible interactions had occurred. The root hair vacuole (*V*), root hair wall (*W*), mechanically induced wall apposition (*WA*), and zoospore cysts (*ZC*) are indicated. (B and C) Electron micrographs showing portions of heterogeneous wall appositions deposited between the plasmalemma (*PL*) and cell wall (*W*) by kohlrabi root hairs. Note the ultrastructural similarities between the mechanically induced (B) and the *O. brassicae*–induced (C) appositions. Scale bars, 0.5 μm.

tration at the encounter sites and thereby to determine whether the mechanically induced appositions alone were sufficient to prevent penetration. The heat shock was applied before inoculation to avoid direct damage to the fungus; host cells recovered cytoplasmic streaming between 4 and 8 hours after the heat shock (*5*).

During the inoculation periods, some of the zoospores encysted over the appositions, whereas most of them encysted more than 10 μm from the nearest apposition since wall appositions subtended only a small portion of the root hair surface area. Seventy-five minutes after inoculation and before host penetration began, the inoculated roots were mounted on microscope slides, and sets of two neighboring cysts (Fig. 1A), one of which was located over an apposition, were selected by means of interference contrast optics (*4*). Since comparisons were made between cysts over appositions and those > 10 μm from appositions, and both cysts in each pair were located on the same root hair, most variables except wall apposition sites were avoided. To ensure a representative sample, no more than one pair was selected from any one root hair and usually only two to five pairs were selected on a given day. The experiment was repeated until sufficient data were accumulated. The data in Tables 1 and 2, obtained by interference contrast microscopy of the fresh, living, preselected cyst pairs, were taken 5 hours after inoculation to allow the parasites extra time to penetrate (*4*).

For electron microscopy, roots with mechanically induced appositions were fixed 4 hours after inoculation in 0.08*M* Pipes (1,4-piperazinediethanesulfonic acid) buffer, *p*H 8.0 (*6*), containing 5 percent glutaraldehyde. Roots without mechanically induced appositions were fixed in the same solution 2.5 hours after inoculation. All roots were subsequently fixed in 2 percent OsO_4 in 0.18*M* Pipes buffer, *p*H 6.8, dehydrated in acetone, and embedded in Spurr's medium (*7*). Thick (50 μm) transverse sections were cut from the embedded roots, wall appositions were selected from these sections by light microscopy, and the sections were remounted and trimmed for thin sectioning (*8*). Thin sections were mounted on Formvar-carbon coated grinds, stained with uranyl acetate and lead citrate, and viewed in a Philips EM 200.

The mechanically induced appositions of kohlrabi root hairs were ultrastructurally similar to those induced by the fungus (Fig. 1, A and B). Both were composed of paramural, variously shaped areas of graded texture and electron density with interspersed membranous elements, a common appearance of wall appositions (*2*). This ultrastructural similarity between mechanically and fungal-induced kohlrabi appositions provided additional direct evidence that they represented a single plant cell response to localized perturbation (*2*). Lesemann and Fuchs (*9*) previously reported comparable *O. brassicae*–induced wall appositions in kohlrabi root epidermis.

Although, as expected (*4*), the majority of cysts not located over appositions penetrated successfully, few of those located over appositions did (Table 1). Thus, the appositions were highly effective in preventing penetration by *O. brassicae*. Furthermore, the appositions were equally effective in preventing penetration into both shocked and unshocked root hairs. Thus, neither synergism between mechanically and fungal-induced appositions nor an effect of heat shock on appositions was responsible for the induced resistance; the appositions were innately resistant to fungal ingress. In addition to fewer successful penetrations, cysts over appositions appeared to produce 27 percent fewer penetration tubes than their counterparts over apposition-free host wall regions. This statistically significant difference ($P = .05$) (*10*) could reflect either inhibition of tube initiation or the difficulty of detecting penetration tubes in the appositions. The data show that most cysts over appositions initiated, but did not complete, penetration. The four cysts which penetrated appositions did so through peripheral areas of the appositions not more than 1 to 2 μm thick; different regions in appositions may vary in their capacity to resist penetration.

To determine whether or not the inhib-

Table 1. Effects of mechanically induced wall appositions and heat shock on the incidence of various events at selected, paired encounter sites (potential or actual penetration sites) between zoospore cysts of *O. brassicae* and root hairs of kohlrabi, *B. oleracea*.

Treatment prior to inoculation	Cyst pairs	Cysts over appositions*		Cysts > 10 μm from appositions		
		Penetration tubes	Penetrations	Fungal-induced appositions	Penetration tubes	Penetrations
Apposition induction only	21	14	2	19	19	18
Apposition induction plus heat shock	20	12	2	2	18	16
Total	41	26	4	†	37	34

*It was impossible to determine reliably whether or not new appositions were induced by cysts over mechanically induced appositions. †This total would have no meaning.

Table 2. Effects of mechanically induced wall apposition proximity and of heat shock on the incidence of various events at selected, paired encounter sites between zoospore cysts of *O. brassicae* and root hairs of kohlrabi, *B. oleracea*.

Treatment prior to inoculation	Cyst pairs	Cysts 2 to 4 μm from appositions			Cysts > 10 μm from appositions		
		Fungal-induced appositions	Penetration tubes	Penetrations	Fungal-induced appositions	Penetration tubes	Penetrations
Apposition induction only	22	16	18	16	20	20	18
Apposition induction plus heat shock	20	3	16	15	2	19	18
Total	42	*	34	31	*	39	36

*This total would have no meaning.

itory effect of wall appositions on penetration was confined to the appositions, penetrations by cysts 2 to 4 μm from appositions and their paired counterparts ten or more micrometers from appositions were compared (Table 2). The experimental procedure was similar to that of the previous experiment. No significant difference ($P = .5$) (*10*) in successful penetration occurred in this experiment; the inhibitory effect of appositions is highly localized. As before, results with heat-shocked and unshocked host cells were similar.

There was evidence that the localized, temporary bending of the host walls during mechanical wounding did not cause the induced resistance observed in this study. First, the dimensions of the structures involved indicated that few penetration tubes would directly contact the previously creased wall regions: the mechanically induced appositions were circular or elongate, 4 to 12 μm in diameter and up to 5 μm thick in side view, and the penetration tubes about 0.5 to 1.0 μm in diameter, whereas wall creases in bent root hairs were approximately 1 μm wide. Second, most cysts over appositions were observed not to be located directly over a central, more highly refractile portion of wall appositions, presumed to directly underlie the temporary wall creases.

Vance and Sherwood (*11*) recently reported results, on inhibition by cycloheximide of fungal-induced apposition formation, which they believed implicated appositions in disease resistance. However, as the authors themselves pointed out, production of antifungal compounds not associated with wall appositions, as well as other possible antifungal processes requiring protein synthesis, were not eliminated.

Current knowledge of the formation of wall appositions (*2*) suggests that the deposition process results not only in paramural depositions, but also in localized alterations in their bounding structures (plasmalemma and cell wall). It follows that resistance occurring only at the site of the appositions would be attributable to the appositions themselves, to the accompanying alterations in the host plasmalemma and cell wall, or both. It seems reasonable to conclude from the present results that fungal ingress was prevented as a consequence of wall apposition formation.

In previous studies, *O. brassicae*–induced kohlrabi wall appositions were shown not to be responsible for certain penetration failures by the fungus, even though such failures were associated with large, hemispherical appositions formed in advance of penetration tubes (*4, 5*). The mechanically induced appositions in the present study resembled fungal-induced kohlrabi wall appositions in overall size, shape, refractility, and ultrastructure, but differed from them in ability to prevent penetrations. This different function probably reflects a difference in chemical structure or composition. Because the fungal-induced appositions were only a few minutes old when they were challenged by the fungus, whereas the mechanically induced appositions were 4 to 6 hours old, these chemical and functional differences could be time dependent. Alternatively, mechanically and fungal-induced appositions could be chemically and functionally different at their inception. Insofar as the wall appositions induced either mechanically or by the fungus represent the same cellular reaction (*2*), the present results suggest that compatible host cells could ward off fungal invasion by producing appositions. The possibility that instances of natural incompatibility are effects of appositions (*2*) still needs testing.

It is interesting that a widespread plant cell response which heals localized wounds is also capable of deterring fungal attack. A measure of economy could be achieved by combining both functions into one set of sequential, cellular processes, that is, localized paramural deposition of materials. This demonstration of the plant disease resistance potential of wall appositions should stimulate work on their induction and composition, because the resulting information could be of practical value in efforts to enhance plant resistance to fungal diseases.

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Host Control of Tumor Growth

Abstract. *A humoral factor (molecular weight less than 60,000) that was present in the ascitic fluid of mice bearing intraperitoneal tumors and in pleural effusions from human cancer patients was found to promote the growth of a murine tumor and to suppress cell-mediated tumor immunity. However, the hosts that had recovered from the immunosuppressive state produced a serum factor that could neutralize the immunosuppressive effect.*

After years of intensive investigation, the mechanisms for the induction of a primary tumor remain unresolved. However, there is an increasing body of evidence that the immune system may play an important role in retarding tumor growth. It has even been postulated that immune mechanisms were evolved primarily as a natural defense against neoplasia (*1*).

Recently, we have been studying the immune response of C57BL/6 mice to FBL-3 cells, a syngeneic leukemia induced by the Friend virus (*2*). This tumor line shows an unusual growth behavior: intraperitoneal inoculation of as few as 1×10^1 cells produces progressive tumor growth and eventually kills the hosts (progressors); in contrast, subcutaneous inoculation of up to 50×10^6 FBL-3 cells produces transient tumor growth with subsequent complete regression (regressors). The regressors then resist the intraperitoneal challenge of up to 1×10^6 FBL-3 cells. Thus, the growth of FBL-3 cells in vivo can be easily controlled by varying the route of inoculation, and it appears that this tumor might be used for studying the various factors which may determine the fate of tumor growth, and the host immune status.

A distinct feature of the growth of FBL-3 cells in the progressors and the regressors is that the intraperitoneal growth is ascitic in form and the subcutaneous growth is localized. One explanation is that factors present in the ascitic fluid promote tumor growth. We have investigated this possibility.

Ascitic fluid was collected from progressors and separated from tumor cells by centrifugation at 500*g* for 10 minutes, then at 100,000*g* for 50 minutes. The fluid was added to tissue culture medium or inoculated into mice to study its effect on the growth of FBL-3 cells in vitro and in vivo. We also inoculated mice with pleural effusion obtained from a human patient with breast carcinoma, and with benign ascites fluid from C57BL/6 mice that had been inoculated intraperitoneally with pristane (*3*).

Figure 1A shows that ascitic fluid almost doubled the growth rate of FBL-3 tissue culture cells in vitro. Figure 1 shows that subcutaneous tumor growth could be detected in the control animals given no ascitic fluid or benign ascites fluid at day 5 after inoculation, the tumor reached peak size at days 15 to 20, then regressed and became undetectable at days 30 to 40. In contrast, in the experimental animals given ascitic fluid or breast carcinoma effusion, the tumors grew much faster and many animals died of progressively growing tumors. Some tumors did eventually regress, but much

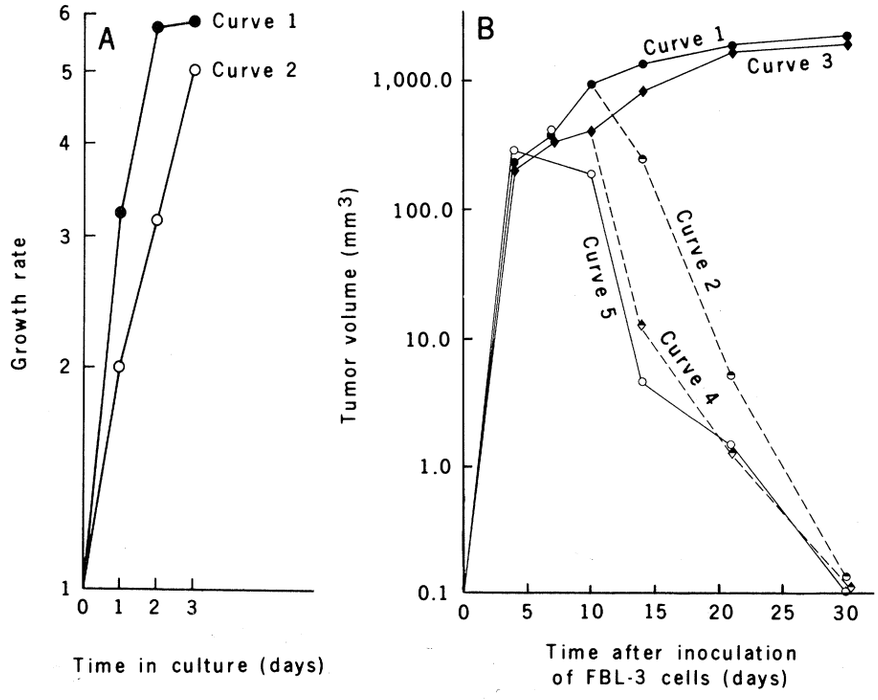

Fig. 1. Effect of FBL-3 ascitic fluid, breast carcinoma pleural effusion, and benign ascites fluid on the growth of FBL-3 cells (A) in vitro and (B) in vivo. (A) Curve 1, 10 percent ascites fluid added to FBL-3 cells grown in RPMI 1640 (Gibco) containing 20 percent fetal bovine serum. Curve 2, FBL-3 cells grown in RPMI 1640 containing 30 percent fetal bovine serum; no ascites fluid added. (B) FBL-3 cells (1.5×10^7) were inoculated subcutaneously into C57BL/6 mice on day 0; the mice then received three intraperitoneal inoculations of 0.3 ml of undiluted ascites fluid or pleural effusion at 3-day intervals (on days 0, 3, and 6). The control groups received tumor cells, and then either benign ascites fluid or no further treatment. The experiments were repeated three times or more with the same results. The incidence of development of progressively growing tumor (progressor) in both the group treated with ascites fluid and the group treated with malignant pleural effusion was significantly higher than the control group ($P \leq .01$ by the χ^2 test). Of the 18 rats that were treated with undiluted ascites, 14 became progressors (curve 1), and four became regressors (curve 2). Of the 15 mice that were treated with pleural effusion, six became progressors (curve 3), and nine became regressors (curve 4). Curve 5, control groups.