Contact with Membrane Grooves Induces Wheat Stem Rust Uredospore Germlings to Differentiate Appressoria But Not Vesicles

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ABSTRACT

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The capacity of uredospores of the wheat stem rust fungus, *Puccinia graminis* f. sp. *tritici* (races 32 and 56), to develop infection structures in response to contact stimuli was reexamined. Uredospores were germinated on a variety of membranes that were scratched by rubbing them with steel wool or carborundum, and examined for nuclear division and differentiation. These responses were compared with those obtained on the membranes after a heat shock or stimulation with acrolein. The results showed that germlings underwent one round of nuclear division (four nuclei) and formed appressoria abundantly in the grooves on scratched sheets of polystyrene, polyethylene, or aluminum foil. The infection structures did not develop further. Differentiation of appressoria also occurred on scratched glass and cellulose, but the frequency of response was low. Complete infection structures (appressorium, peg, vesicle, and infection hypha) developed when the germlings were stimulated by heat shock or by acrolein (1.5×10^{-9} M), but the appressoria were located randomly on the surfaces. These structures had at least eight nuclei. We concluded that contact stimuli serve to position appressoria over stomata, but development of the vesicle may require other factors, apparently from the host.

Additional key words: differentiation, infection structures, rust fungi, *Triticum aestivum*.

Germlings of the wheat stem rust fungus, *Puccinia graminis* f. sp. *tritici*, can be induced to form infection structures by a chemical fraction extracted from the fungus itself (1,4). The active principle in the fraction is acrolein (8), but the role of acrolein in pathogen development has not yet been demonstrated.

Differentiation also can be induced by a volatile fraction extracted from wheat leaves which is active when combined with cell wall fragments or cuticular phenols in agar (5). The latter findings suggest that differentiation of wheat rust uredospore germlings occurs in response to the chemical composition of the surface of the host (8,10,14) and may include some that are located specifically in the region of the stomata (5).

In nature, the wheat stem rust (WR) uredospore germling locates and enters the stomata of a host with great accuracy (7,11), but domains of chemical activity that are so precisely localized have yet to be demonstrated. Instead, contact stimuli would provide a simpler concept, at least to start differentiation, as is the case with the bean rust fungus, *Uromyces phaseoli* (13). The present study was made to reexamine the possibility that WR germlings might initiate differentiation in response to contact stimuli.

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MATERIALS AND METHODS

Wheat rust fungus uredospores. Uredospores of the wheat rust fungus, *P. graminis* f. sp. *tritici* races 32 and 56, were collected by shaking the spores from leaves of wheat, *Triticum aestivum* L. 'Little Club.' The spores were stored up to 1 wk at 4 C before use. For race 32, the wheat was grown in a controlled environment chamber at 18 C and a 16-hr day. Race 56 was obtained from greenhouse-grown plants.

Chemicals. Collodion (a 40% solution in a mixture of alcohol and ether [1:3, v/v]) was purchased from Merck (Darmstadt, West Germany). Mithramycin and acrolein were purchased from Serva Feinbiochemica (Heidelberg, West Germany). The hydrophilic polycarbonate membranes (Nuclepore Corp., Pleasanton, CA, 94566 USA) used were unperforated (40-gauge, 25 mm diameter). Sheets of ordinary polyethylene and high density polyethylene were cut from bags purchased from local supply houses. Polystyrene petri dishes were scored when that plastic was used. Aluminum foil sheets were cut from rolls of aluminum foil. Cellulose sheeting was a gift from G. Hänβler (Institut für Biologie III, RWTH Aachen).

Cytological procedures. The membranes (about 0.5 cm square) were scratched before cutting them to size by rubbing the surface firmly with a fine (grade 0) steel wool without causing the surface to crease. Grooves of various depths and widths were produced. Oil-collodion membranes were prepared as described by Maheshwari et al (10); it was not possible to scratch them with steel wool.

Membranes were placed in small open plastic petri dishes (previously lightly misted with water to hold the membrane in place), and dusted with uredospores. The uredospores on the membrane were misted lightly with water, the dishes were covered, and incubated overnight (about 16 hr) at 18 C.

WR spores were induced to differentiate by heat shock as described by Maheshwari et al (10). When acrolein was used, the membranes bearing the spores were placed in the inner well of a modified Conway dish. Then 1.0 ml of an aqueous solution of acrolein (1.5×10^{-9} M) was pipetted into the outer well and the dish was sealed.

After germination, the membranes were transferred to slides, two drops of aniline blue-lactophenol stain were added with a cover glass, and infection structures were observed with a light microscope at ×100 or ×160. Counts of appressoria were made by scoring 200 germ tubes on each of two membranes. Experiments were repeated twice. For convenience, germ tubes were considered to have differentiated if a terminal swelling with a cross wall was present. Data in the table are averages of two separate determinations, each based on counts of more than 200 germ tubes.

Nuclear division in the appressoria was also monitored. Nuclei were observed by using the mithramycin procedure described by Staples and Hoch (12).

Scanning electron microscopy. Leaf pieces and polyethylene sheets were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 48 hr and postfixed in 1.5% osmium tetroxide in the same buffer for 1 hr. They were washed three times in cacodylate buffer containing 5% sucrose for a total of 30 min after each fixation. Tween-20 at a final concentration of 0.005% was used in all fixative and washing solutions. The specimens were dehydrated through an ethanol series and dried by the critical-point method (2) by using absolute ethanol as the intermediate fluid and carbon dioxide as the transition fluid. Temperature in the bomb was not allowed to exceed 38 C. Specimens were then mounted on stubs with aluminum paint, coated 4 min with gold-palladium in a vacuum evaporator, and stored in a desiccator.

All specimens were examined and photographed in a Cambridge Stereoscan, model Mark 2A, scanning electron microscope.

RESULTS

Differentiation on scored membranes. Surface scratches in all of the membranes that were tested induced germlings to form appressoria, at least to some degree (Table 1). Polystyrene was the most efficient membrane. None of the membranes induced WR to differentiate unless they were scratched. The low percentage of differentiation that occurred in germlings on membranes which were not scored could usually be attributed to errant scratches. Oil-collodion membranes, included for comparison with the literature (10), as expected were not inducive. The races did show some differences in frequency of response to the various membranes; however, these did not seem to us to be significant.

Infection structures induced by grooves in the membranes generally consisted of an appressorium and a short outgrowth from it (Fig. 1). Cross walls were present between the germ tube and the appressorium (Fig. 2). The appressoria generally were elongate with the axis of the groove.

Differentiation induced by stimulators. Development of vesicles was not achieved unless the germlings were shocked by heat (Fig. 3), or when acrolein (1.5×10^{-9} M) was present in the incubation chamber. For race 32, acrolein induced 52.4 ± 17.9% of the germ tubes to differentiate on polyethylene when the data for scratched and unscratched membranes were combined. Data for scratched and unscratched membranes separately determined were not significantly different. Heat shock induced 77.9 ± 4.5% to differentiate, and the appressoria generally were spherical.

Differentiation induced on wheat leaves. WR germinated on wheat leaves was examined by scanning electron microscopy for comparison of the infection structures with those on scored membranes (Fig. 4). The morphology of the appressoria developed over the stomata was very similar to that of appressoria over scratches.

Nuclear condition. Almost all of the appressoria induced on scratched membranes had four nuclei (Figs. 5 and 6). Additional nuclei were seldom present. At least eight nuclei were present in the infection structures induced by acrolein or heat. After 16 hr of incubation, these nuclei generally were present in the vesicles or infection hyphae. Thus, development of appressoria induced by grooves in membranes ceased after one round of nuclear division.

Location of appressoria. Appressoria were located in the grooves of scratched membranes when infection structures were induced without chemicals or heat shock (Fig. 2), just as they were located over the long axis of the stomatal opening. The elongated axis of the appressorium was oriented parallel with the groove and the germ tubes were oriented perpendicularly to it in a striking manner.

In contrast, infection structures induced chemically or by heat shock were located randomly on the scratched membranes (Fig. 3). The rather spherical appressoria were located both in the grooves and between them. Similarly, the infection structures were located randomly on heat-shocked wheat leaves (Fig. 7). The vesicles and

TABLE 1. Effect of scratching on the capacity of several membranes to induce wheat stem rust uredospores to differentiate infection structures[a]

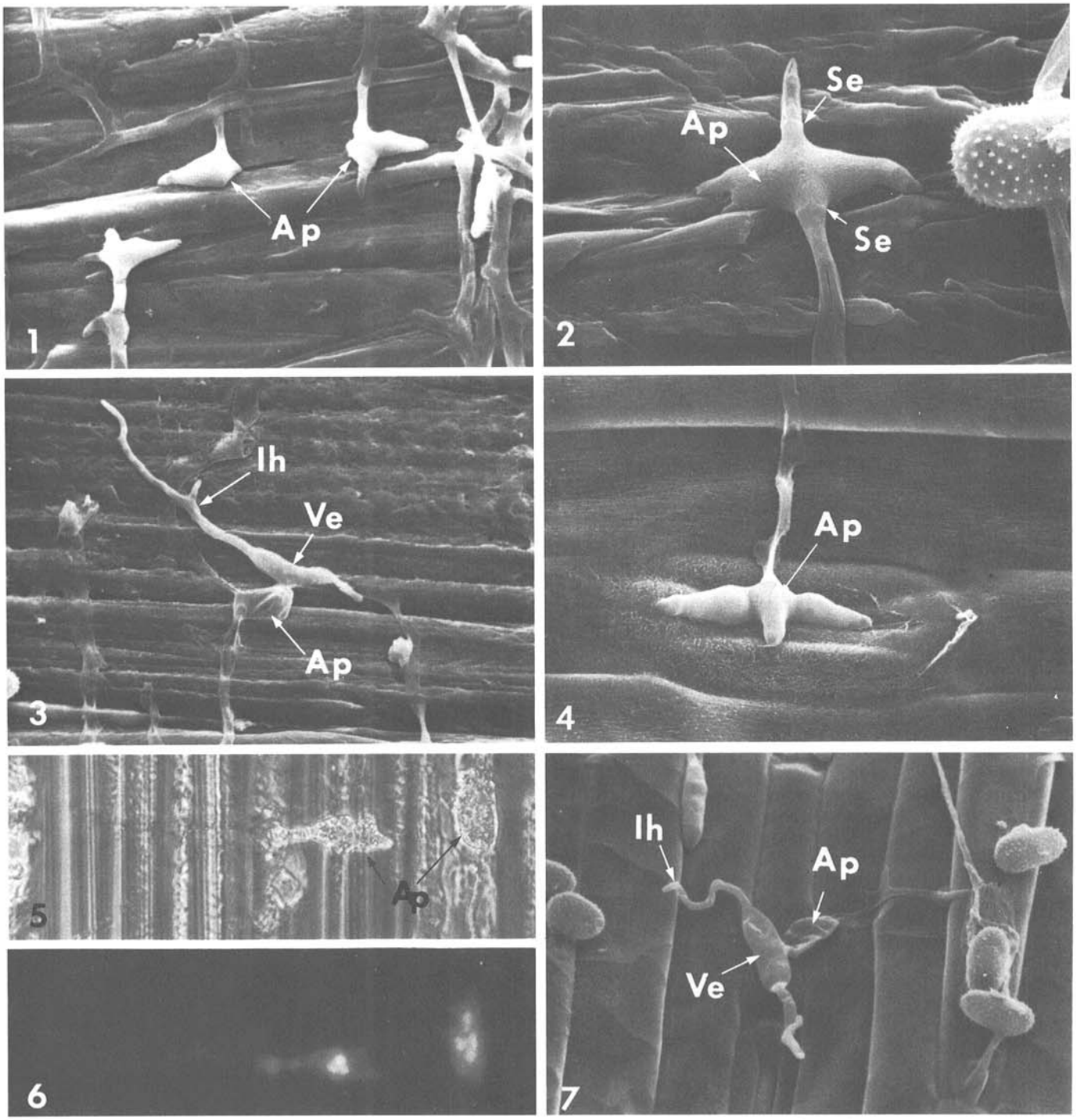
		Race 32		Race 56	
Membrane	Scratched	Appresoria (%)	Standard deviation	Appressoria (%)	Standard deviation
Polystyrene	+	68.5	10.7	60.5	2.5
	−	0.3	0.3	0	−
Polyethylene	+	43.5	4.7	14.3	9.5
	−	0	−	0	−
Aluminum	+	27.5	5.6	−	−
	−	0	−	−	−
HD-polyethylene[b]	+	21.0	5.6	29.0	0.6
	−	2.9	1.7	2.5	0.7
Glass	+	8.0	0.9	7.0	1.6
	−	0	−	0	−
Nucleopore	+	6.4	1.7	9.3	0.3
	−	0	0	−	−
Cellulose	+	5.5	1.5	18.5	4.9
	−	0.4	0.9	−	−
Oil-collodion[c]	na	0	−	0	−

[a] Data are percentages of germ tubes which contained appressoria.
[b] High-density (HD) polyethylene.
[c] It was not possible to scratch oil-collodion.

infection hyphae were oriented without regard to the axis of the grooves (Figs. 3 and 7). However, the germ tubes of these heat- or chemically-stimulated germlings on wheat were oriented perpendicularly with the groove axis, indicating that contact stimuli controlled their orientation.

DISCUSSION

A number of workers have studied differentiation of uredospores of the wheat stem rust fungus (WR) germinated on various membranes. Dickinson (3) was one of the earliest to study the effect



Figs. 1-7. 1, Scanning electron micrograph showing position in the grooves of appressoria (Ap) formed by germlings of wheat stem rust (WR) (race 32) uredospores germinated on a sheet of polyethylene scored with steel wool. Only Ap developed. ×710. **2,** Scanning electron micrograph of an Ap which was developed in the groove by a WR uredospore germling (race 32) germinated on a sheet of scratched polyethylene. Note the elongate shape of the Ap and its location in the groove of the scratch. The appressorium is separated from the germ tube by a septum (Se). ×1,560. **3,** Scanning electron micrograph of a heat-shock-induced infection structure produced by a WR uredospore germling, race 32, germinated on a scratched polyethylene sheet. Note the rather spherical shape of the collapsed Ap and diagonal direction of growth of the vesicle (Ve) and infection hyphae (Ih). ×530. **4,** Scanning electron micrograph of a differentiated WR uredospore germling (race 32) on a leaf of wheat (cultivar Little Club) showing the Ap located over a stomate. Note the elongate shape of the Ap. ×1,200. **5,** Phase-contrast photomicrograph of WR uredospores germinated on a scratched sheet of polystyrene. The Ap has developed in the grooves. Appressorium (Ap). ×530. **6,** Photomicrograph of WR uredospores germinated on a scratched sheet of polystyrene, stained with mithramycin, and photographed by epifluorescence microscopy to show the four nuclei in the Ap. ×530. **7,** Scanning electron micrograph of WR (race 32) uredospores germinated on a heat-shocked leaf of wheat (cultivar Little Club). The micrograph shows the infection structures that developed after induction by a heat shock. Note that Ap were not located over the stomata. Also note the shape of the collapsed Ap, and the orientation of the Ve and Ih. ×610.

of surfaces systematically. He published several figures showing complete infection structures which included the appressorium, vesicle, and infection hypha. The structures were developed on "paraffin-wax, collodion membranes" supplemented with cell wall materials or protein extracted from wheat leaves. The structures contained only four nuclei. The frequency with which these structures were found was not stated.

These studies apparently influenced Maheshwari et al (10) who adapted the collodion membrane system invented by Dickinson (3). However, while they showed that it was an excellent system for many rust fungi, they also found that the oil-collodion membrane would not induce WR uredospores to differentiate. Instead, they discovered that either a temperature shock or a germination stimulator extracted earlier from WR uredospores by Allen (1) and by French et al (4) induced differentiation of WR efficiently. Later, Macko et al (9) also extracted an active fraction from WR uredospores and proved that the active principle was acrolein. While this research did show that a surface was not essential for differentiation of WR germlings, it did not explain why in nature the development of infection structures is associated so efficiently with the position of the stomata.

In a study of inoculation procedures, Rowell and Olien (11) reported briefly that WR developed appressoria when germinated on polyethylene sheets scratched with a pin. They did not provide data, but stated that "... appressoria were formed abundantly in the scratches...." The nuclear condition of the appressoria was not examined. The spores were sprayed onto the membrane surface in a mixture of Mobilsol 100 and paraffin oil (1:1, v/v), which on wheat leaves caused a significant dislocation of appressorium away from the stomata without greatly reducing the numbers of infection structures that were developed. While it contributed significantly to the development of spray procedures, the study left uncertain the problem of how germ tubes locate stomata.

More recently, Grambow and co-workers (5,6) prepared a volatile extract from wheat leaves that, when used together with either a phenolic cuticular fraction or a cell wall fraction, stimulated differentiation by WR. These workers proposed that WR may locate stomata on host leaves in response to the biochemical environment at the entrance to the stomatal cavity. This suggestion was reinforced when Wynn (14) reported that WR failed to recognize the stomatal image on polystyrene leaf replicas, a technique that had been used with good success for differentiation of the bean rust fungus.

The early studies by Rowell and Olien (11), and the near impossibility of demonstrating highly localized areas of chemical activity on a leaf surface, led us to inquire whether physical contact stimuli could be recognized by WR germlings at least to position the appressorium. Our present finding that WR germlings develop appressoria on a series of scratched membranes demonstrates that WR appressoria can be produced solely in response to contact with grooves in the surface. The appressoria were located in the groove of the scratch (Table 1, Fig. 1).

It seems reasonable that purely contact stimuli participate in the induction of appressoria. The typical ridge that stimulates appressorium development is relatively deep and sharp-edged compared with ridges typically involved with orientation of the germ tube. The involvement of local surface charge or static electricity in appressorium induction cannot be ruled out; however, the fact that smaller ridges induce orientation and not differentiation makes extremely subtle any participation of surface charges in the induction process. At our present level of understanding, stimulation by contact offers a simpler and experimentally more useful concept.

Regarding the nature of the groove in the plastic, it has been disappointing that use of the polystyrene membrane leaf surface replica technique employed successfully by Wynn (14) to induce differentiation of the bean rust fungus failed to induce differentiation of WR germlings. The reason seems to be that the groove formed in the plastic at the stomatal opening is insufficiently distinct or large enough for WR to respond. Because wheat leaf stomates close when leaf surface replicas are prepared, perhaps successful use of this technique with WR hinges on the preparation of images of open stomates.

Only appressoria that contained four nuclei were produced by WR on the scored membranes. As reviewed above, however, complete infection structures are easily stimulated to form, and some chemical factor may be required to complete the infection structures once the germ tube has located the stomatal opening and produced the appressorium and peg. Such a chemical factor may include a reduced partial pressure of CO_2 such as may be found in the substomatal cavity (15), or various chemical stimuli from the host (6). Failure of WR to develop vesicles on scratched membranes in the closed system used here (the Conway dish) leaves the role of endogenous fungal stimulators (eg, acrolein) uncertain. If endogenous acrolein were important as an inducer of the vesicle, its formation should have occurred in the closed dishes.

We do not know the reason for the apparent differences between membranes in efficiency of induction of appressoria. Possibilities include differences in the number of effective grooves per area of membrane, plasticity of the membranes and surface hardness. There is much that we need to know yet about the effects of the physical dimensions of features in the membrane surface which provide a suitable stimulus for the induction of infection structures by WR uredospore germlings.

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