Extracellular Proteins in the Intercellular Space of Stem Rust–affected Wheat Leaves

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Histological studies have shown that phenotypic expressions of resistance of wheat (*Triticum aestivum* L.) against the stem rust fungus (*Puccinia graminis* f. sp. *tritici* Eriks. & Henn.) differ considerably at the cellular level, depending on the resistance genes and avirulence genes that are involved in host and parasite, respectively (Rohringer and Heitefuss 1984). Some of the gene interactions are strictly localized to the particular host cell that is invaded by the fungus. From this it can be concluded that products of these genes are nondiffusible and may be restricted to the haustorial/host cell interface. Other gene interactions do not appear to be so localized; in these cases it is possible that one or both determinants of the incompatible reaction (i.e., the products of the resistance and avirulence genes) are diffusible and could be present in the intercellular space of the infected tissue. If this is correct, then a possible search strategy for these substances could include recovery of soluble material from the intercellular host space in which large amounts of fungal hyphae are present.

A procedure for this purpose is available. It has been used for the recovery of various substances, including cell-wall-associated enzymes from noninfected and infected plant tissues, such as stems or leaves. Briefly, tissues are detached, vacuum-infiltrated with water or buffer, and then gently centrifuged to yield "intercellular washing fluid" (IWF). In a most interesting plant pathological study, use of this technique led to the recovery of gene-specific elicitors of chlorosis and necrosis from tomato leaves infected with compatible races of *Cladosporium fulvum* Cke. (deWit and Spikman 1982; deWit et al. 1984). We have used a similar technique (Rohringer et al. 1983) to obtain IWF from primary leaves of wheat, healthy or infected with one of four races of the stem rust fungus. The races had been

selected to represent widely differing avirulence gene combinations. Plants were maintained at 19°C and harvested 4 days after inoculation. The first objective was to determine whether IWFs from these four host/parasite combinations differ in their protein composition.

Two-dimensional separation by isoelectric focusing–gradient polyacrylamide gel electrophoresis of "native" IWF proteins (175 μg/gel) and staining with Coomassie Brilliant Blue (CBB) yielded reproducible patterns of at least 58 well-defined "spots" representing the major proteinaceous constituents in these fluids. At least nine of these were infection-related, i.e., they were not seen in gels in which IWF proteins from noninfected leaves had been separated. Six of these proteins were present in all fluids from infected leaves, regardless which of the four races of the fungus had been used for inoculation. Three additional infection-related proteins were not detected in all fluids from infected plants. However, two of these three were near the limit of detection and may have been present in all fluids from infected leaves. With the methods used in this study, CBB-stainable proteins from stem rust–affected leaves did not show much variability with respect to the fungal race used for inoculation.

More variability between races was seen when the separated IWF proteins were examined for affinity to concanavalin A (Con A). For most Con A–binding proteins, the method of detection we used (Hawkes 1982) is much more sensitive than CBB or silver staining. At a loading of 175 μg total protein per gel, 18 Con A–binding proteins were detected in IWF from noninoculated leaves, including six prominent β-D-xylosidase isozymes (Holden and Rohringer 1985b). The number of detected Con A–binding proteins in fluids from inoculated leaves ranged at this loading from 40 to 61, depending on the race used for inoculation. Since infection did not affect number or electrophoretic properties of endogenous β-D-xylosidases, these isozymes served as convenient internal standards to take account of slight run-to-run variations in separation patterns and to serve as "landmarks" in comparing Con A–binding proteins between samples. The patterns of Con A–binding proteins were highly reproducible, both between replicate gels of the same IWF samples and between samples of fluids obtained in different trails involving the same host/parasite combination.

Many of the infection-related Con A–binding proteins had similar electrophoretic properties, regardless from which host/parasite combination the IWF was obtained. However, several of them varied between races, including the prominent glycoproteins in the

upper-left-hand quadrants of the nitrocellulose membrane replicas illustrated in Figure 1. At the present time, it is not known whether all of these conspicuous differences between races can be attributed to variations in the relative amounts of glycoproteins, i.e., whether all of them were present in each IWF from inoculated leaves, but some of them perhaps in amounts close to, or below, the

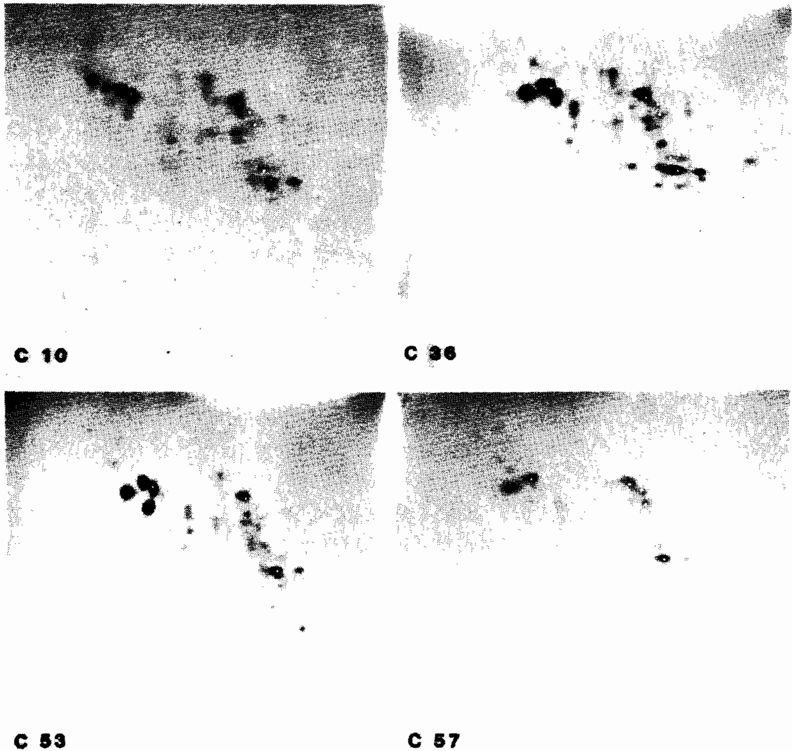

Figure 1 Con A-binding proteins in intercellular washing fluids from wheat leaves infected with races C10, C36, C53, or C57 of the stem rust fungus. Proteins (175 μg/gel) were separated by isoelectric focusing from cathode (left) to anode (right), followed by polyacrylamide gel electrophoresis (top to bottom) under nondenaturing conditions, transferred to nitrocellulose membranes, assayed for activity of β-D-xylosidase, and reprobed with Con A and horseradish peroxidase. Endogenous β-D-xylosidase isozymes (white squares) serve as internal markers to facilitate comparison of Con A-binding protein patterns. Most of the Con A-binding proteins are infection-related and many of them are likely to be of fungal origin, including those in the upper-left-hand quadrant of the nitrocellulose membrane replicas. Note the differences in location of Con A-binding proteins between the samples.

limit of detection. Gross protein composition is known to affect the pH gradient in isoelectric focusing, but it is unlikely that this contributed to the observed differences in the electrophoretic separation of Con A–binding proteins, since the patterns of CBB-stainable proteins and β-D-xylosidase isozymes were similar or identical, respectively, between races.

We had shown previously (Holden and Rohringer 1985a) that many of the infection-related Con A–binding proteins in IWF most likely originated from the fungus, including some that corresponded in location to those in the upper-left-hand quadrants of nitrocellulose membrane replicas shown in Figure 1. Presumably, they were excreted by the fungus or had been loosely bound to the fungal walls. Although electron micrographs gave no indication that fungal cells were damaged during preparation of IWF, we cannot exclude the possibility that fragile hyphal tips may have been broken during this procedure and may have released intracellular proteins. If many of the infection-related Con A–binding proteins in IWF are extracellular products of the fungus, it should be of interest to determine the glycoprotein composition of the fungal wall. That fungal walls in general are more complex than those of higher plants is well known, but little information has been available on the cytochemistry of the intercellular hyphal walls of the stem rust fungus.

We have studied these walls by electron microscopy (Chong et al. 1985 and unpubl.), using various staining methods and gold-bound lectins as cytochemical probes, and we have shown that walls of these hyphae consist of at least four layers. The two outside layers contain relatively small amounts of substances with vicinal hydroxyl groups, but either or both layers have an affinity to a variety of lectins, including Con A, *Bandeiraea* I lectin, *Ricinus communis* agglutinin, soybean lectin, arabinogalactan protein (AGP), *Lotus tetragonolobus* lectin, and horseshoe crab lectin; affinity to wheat germ lectin was confined to the inner two layers, indicating that chitin may be localized there. Evidently, the outer two hyphal wall layers contain glycosubstances with α-bound glucose and/or mannose, α- or β-bound galactose or *N*-acetylgalactosamine, and α-bound fucose. In addition, glucuronic acid may also be present. That some of the bound sugars or sugar derivatives are β-linked is indicated by the affinity of AGP to these layers. With present techniques it is impossible to confirm these cytochemical data by direct chemical analysis, since no method is available to obtain sufficiently large amounts of intercellular hyphae for this purpose. Although we know from ultrastructural studies that germ tube walls differ from

intercellular hyphal walls, it is interesting to note that glycosubstances containing glucose, mannose, and galactose (Joppien 1976), fucose (Kim et al. 1982), and glucuronic acid (W.K. Kim and R. Rohringer, unpubl.) have been isolated from purified walls of germ tubes.

DISCUSSION

Our cytochemical studies indicate that the outer two layers of intercellular hyphal walls of *P. graminis* f. sp. *tritici* are very complex because they bind Con A as well as lectins that have an affinity to galactose, fucose, and glucuronic acid. Many Con A-binding proteins were recovered from the intercellular space of wheat leaves infected with this fungus; most of these were likely of fungal origin and many of them differed between samples, depending on the race of the rust fungus used for inoculation.

Races are defined according to their virulence/avirulence gene combinations. They probably also differ with respect to genes that have nothing to do with virulence or avirulence, and products of these genes may be present in the intercellular space, possibly in addition to avirulence gene products. Tracking down the putative avirulence gene products in this complex mixture by comparative analysis would be very laborious and time-consuming, even if they can be detected with present methods. Potentially more rewarding approaches would include bioassays or affinity assays with which presumed physiological or molecular biological effects of the determinants could be detected. Suitable bioassays may involve observation of host or parasite necrosis or determination of the activity of enzymes that are likely involved in the production of cell chlorosis or necrosis. Alternatively, affinity assays may be used, on the basis of the assumption that determinants of incompatibility from host and parasite bind to one another and that this is part of the mechanism of gene-specific recognition. In this approach, the methods of analysis would be similar to those used in cytochemistry with gold-bound lectin probes.

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