Targeted Cell Wall Degradation at the Penetration Site of Cowpea Rust Basidiosporelings

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Basidiospore germlings of the cowpea rust fungus (*Uromyces vignae*) penetrate the epidermal cell wall of the nonhost plant *Vicia faba*. In order to characterize the wall structure of the penetration site, leaves were high pressure frozen, freeze substituted, and embedded in appropriate resins. With antibodies against epitopes present in pectin, polygalacturonic acid, xyloglucan, and callose, we studied the modification of these wall components during infection. The density of epitopes was determined at the penetration site and compared with noninfected areas of the epidermal wall. Along the fungal penetration hypha, a zone of the plant wall, 0.2 μm wide, exhibited a reduced density of pectin and xyloglucan epitopes. A similar reduction of epitope density was also found for xyloglucan after treatment of sections from noninoculated plants with cellulase and xylanase and for pectin after treatment with pectinase. The density of polygalacturonic acid epitopes remained unchanged in the outer layer of the epidermal wall, but increased over the inner layer. A high density of polygalacturonic acid epitopes was found over a collarlike wall apposition produced by the plant cell along the penetration hypha. These results indicate that the fungus degrades the plant cell wall at the penetration site and that the plant cell secretes new wall material into this area to form the wall apposition.

The plant epidermis is the first barrier that must be breached by fungal pathogens that penetrate directly, i.e., without using wounds, stomata, or other natural openings. The epidermal surface, the cuticle, may be smooth or, as a result of wax crystals that form on the cutin layer, quite rough. The cuticle layer that contains the cutin polyester varies in thickness and extends into the epidermal wall below (Jeffree 1996). Similar to the cuticle, the epidermal wall has characteristics typical for each plant species. It consists mainly of a cellulose-xyloglucan framework and fibrous proteins embedded in a pectin matrix (Carpita and Gibeau 1993). Fungi have adapted to these variable conditions and use different means to overcome this barrier. The rice blast fungus, *Magnaporthe grisea* (Hebert) Barr, produces a well-developed appressorium containing a wall layer of melanin. High turgor pressure

within this pigmented infection structure supports the progress of the penetration hypha (Howard et al. 1991). The infection structures of bean rust basidiospores, on the other hand, appear quite delicate (Gold and Mendgen 1991). The germ tube is short, and the appressorium exhibits little differentiation and is not separated from the vacuolated germ tube by a septum. Therefore, we assume that turgor pressure in these infection structures does not surpass the values typically observed in hyphal tips of other fungi (Money 1995). This suggests that although turgor pressure may support penetration, enzymes seem to play a major role. However, since *Uromyces* is a biotrophic pathogen fed by haustoria that the fungus produces within living plant cells, enzyme secretion should be very restricted during the penetration process (Deising et al. 1995). Therefore, this study was undertaken to investigate if and how far the wall at the penetration site of *Uromyces vignae* Barclay shows signs of enzymatic degradation.

With conventional staining methods for ultrathin sections, a modified electron opacity at the penetration site has historically been taken to indicate localized degradation of the epidermal wall by plant pathogens such as *Ustilago maydis* (DeCandolle) Corda, *Colletotrichum lagenarium* (Pass.) Ellis & Halst., and endophytic fungi (Snetselaar and Mims 1993; Stein et al. 1993; Viret and Petrini 1994). Cytochemical techniques, such as periodic acid–chromic acid–phosphotungstic acid (Roland et al. 1972) and periodic acid–silver methamine (Thiéry 1967), revealed the modification of polysaccharides at the penetration site of *Colletotrichum* (O'Connell and Bailey 1986). However, these methods cannot discriminate between different types of polysaccharides (Ruel and Barnoud 1985). More specific results have been obtained with lectins, enzymes, or antibodies coupled to colloidal gold, allowing several cell wall constituents to be recognized with high spatial resolution (Bendayan and Benhamou 1987; VandenBosch 1991). With this technology, degradation of pectic components and cellulose has been detected within plant tissue infected by perthotrophic pathogens (Benhamou et al. 1990; Nicole et al. 1995; Nicole and Benhamou 1993). In order to study cell wall degradation during the earliest stages of infection at the penetration site of a biotrophic fungus, we chose the cowpea rust pathogen (*U. vignae*) and the nonhost plant broad bean (*Vicia faba* L.), because penetration occurs here with higher efficiency, compared with the host plant *Vigna sinensis* (L.) Savi ex Hassk. (Xu and Mendgen 1991). In this combination, host specificity is expressed several hours after penetration events. Using monoclonal antibodies against pectin, polygalacturonic acid, and xyloglucan, we demonstrated

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Fig. 1. Fine structure of penetration site. **A**, Epidermal wall of *Vicia faba* consists of a smooth, evenly stained cuticle (c) and two discernible wall layers below. Outer wall layer (ol) exhibits darker, inner wall layer (il), lighter, staining. ($\times 50,000$). **B**, Penetration hypha (ph) of *Uromyces vignae*. Appressorium broke away during processing. Diameter of hypha is constricted at site of cuticle penetration. Cuticle is modified (arrows) and exhibits electron-dense material ($\times 60,000$). **C**, Median section of penetration site. Plant cell wall is modified in a zone along penetration hypha (ph). Areas with high and low electron density occur irregularly. A small papilla (pa) occurs around penetration hypha ($\times 34,000$). **D**, Tangential section of penetration hypha (ph). Pockets (p) with light and dark contents are numerous in plant wall close to penetration hypha ($\times 44,000$).

Fig. 2. Immunocytochemical characterization of the penetration site. **A**, Pectin epitope distribution (Jim 7) in an area of noninoculated epidermal wall. (g = Golgi body, $\times 47,000$). **B**, Pectin epitope distribution (Jim 7) adjacent to penetration site of *Uromyces vignae*. (ph = penetration hypha, $\times 50,000$). **C**, Epitopes in fucosylated xyloglucan (CCRC-M1) over epidermal wall away and close to penetration site of *U. vignae*. (ph = penetration hypha, $\times 25,000$). **D**, Polygalacturonic acid epitope distribution (Jim 5) over noninoculated epidermal wall. (ol = outer layer of epidermis, il = inner layer, $\times 31,000$). **E**, Polygalacturonic acid epitopes (Jim 5) along penetration hypha (ph) of *U. vignae*. (ol = outer layer of epidermis, il = inner layer, wa = wall apposition, $\times 46,000$). **F**, Epitopes in β -1,3-glucan over wall (fw) of penetration hypha (ph) and over papilla (pa), but not over additional wall apposition (wa) ($\times 37,000$).

localized modifications of the plant epidermal cell wall that are similar to those that result from the application of enzymes to uninoculated tissue.

RESULTS

The broad bean cuticle was about $0.12 \pm 0.03 \mu\text{m}$ thick. The plant cell wall below was $0.5 \pm 0.2 \mu\text{m}$ thick and consisted of an outer, more-electron-dense layer, and an inner, less-electron-dense layer (Fig. 1A).

At the penetration site of *U. vignae* (Fig. 1B, C), appressoria broke away during the freezing process. However, the penetration hypha was well preserved. The cuticle around the penetration hypha had changed its structure after contact with the fungus. The otherwise homogenous cuticle had been transformed into material with increased electron opacity at the edge that contacted the penetration hypha (Fig. 1B). Analysis of serial sections of penetration sites indicated that the penetration hypha had the smallest diameter at the level of the cuticle and the hypha widened during further passage of the epidermal wall (Fig. 1C). Along the penetration hypha, the epidermal wall exhibited areas with high and low electron density (Fig. 1C). In a tangential section of the penetration site, such areas or pockets appeared empty or filled with darkly staining material and surrounded the fungus (Fig. 1D).

Judging from slight modifications in staining intensity, the plant cell wall along the penetration hypha appeared modified over an area at least $0.2 \mu\text{m}$ wide. This area, excluding the dark and light pockets, was evaluated for cell wall components with monoclonal antibodies, and compared with non-infected areas of the epidermal wall. The density of epitopes, as indicated by the number of gold particles, was determined for the three major components of the plant cell wall. Epitopes present in pectin were evenly distributed over the epidermal wall (Fig. 2A). Along the penetration hypha, in a $0.2\text{-}\mu\text{m}$ -wide area, the number of pectin epitopes was reduced to about 50% (Figs. 2B and 3). A similar result was obtained with the monoclonal antibodies that recognize primarily a fucosyl-containing epitope present in xyloglucan (Fig. 2C). Compared

with the noninfected epidermal wall, the number of epitopes was reduced to about 45% (Fig. 3) in the immediate vicinity ($0.2 \mu\text{m}$) of the penetration hypha.

In the noninfected epidermis, epitopes present in polygalacturonic acid were abundant in the outer epidermal wall and scarce in the inner epidermal wall (Fig. 2D). At the penetration site (Fig. 2E), epitope density remained unchanged in the outer layer of the epidermis, but increased dramatically over the inner epidermal layer and reached similar high values over a new wall layer that reached along the penetration hypha into the cell (Fig. 3).

Antibodies that recognize β -1,3-glucans labeled the fungal wall and the papilla but not the additional wall layer called wall apposition (Fig. 2F).

To find out if the number of epitopes from the three major constituents of the epidermal wall could be modified in the absence of the fungus by the activity of enzymes, sections from high-pressure-frozen, noninoculated samples embedded in acrylic resin were treated with enzymes and probed with appropriate antibodies, and the number of gold particles was determined in each variant. Pectinmethylesterase removed most pectin epitopes in both layers of the epidermal wall (Figs. 4A and 5). The same treatment did not affect epitopes from galacturonic acid in the outer layer of the epidermal wall, but uncovered a high number (Fig. 5) of such epitopes in the inner layer (Figs. 4B and 5). In addition, fibrillar material became obvious in the inner layer of the epidermal wall (Fig. 4B). Cellulase, xylanase, and a combination of both enzymes had comparable effects on both layers of the epidermis. Cellulase (compare Figure 4C and D), xylanase (compare Figure 4C and E) and a combination of cellulase and xylanase (compare Figure 4C and F) reduced to a similar extent the number of epitopes present mainly in xyloglucan (Fig. 6).

DISCUSSION

We show that the homogenous structure of the cuticle is modified at the penetration site of the fungus, probably as the result of enzyme activity. In the epidermal wall below, pectin and xyloglucan are degraded in a zone, at least $0.2 \mu\text{m}$ wide, along the penetration hypha of *U. vignae*. We were not able to discriminate a gradient of cell wall degradation within this zone because the density of detectable epitopes was not high enough to divide this zone into a number of smaller regions. In spite of this problem, a reduction in the number of epitopes of these wall components by more than 50% could be measured and suggests enzyme activities at this site. Commercially available xylanase and pectinase applied to sections similarly degraded the epidermal wall. The polygalacturonic acid epitopes in the plant wall are altered in a highly localized manner along the penetration hypha. While the outer layer of the epidermis remained unchanged after infection, the inner layer exhibited a dramatic increase in numbers of these epitopes. A similar increase in the density of polygalacturonic acid epitopes was also obtained after treatment with a commercial pectinesterase. Obviously, enzymes applied to sections have effects comparable to the activity of the enzymes probably secreted by the basidiosporelings during wall penetration.

A deposition of new wall material occurs in the zone of the wall apposition. This apposition differs from the papillae induced by the fungus immediately upon wall penetration (Xu

Fig. 3. Comparison of noninfected cell wall area and modified area, $0.2 \mu\text{m}$ wide, along penetration hypha. Density of pectin (Jim 7) and xyloglucan (CCRC-M1) epitopes is shown over both outer and inner layers of cell wall. Density of polygalacturonic acid (JIM 5) epitopes is given for outer layer, inner layer, and wall apposition.

and Mendgen 1994) by its composition and staining quality. Papillae are characterized by high amounts of callose. In contrast, the additional wall apposition produced during fungal growth within the plant cell contains a high amount of polygalacturonic acid epitopes. Pectin epitopes were not detected in the apposition (H. Xu and K. Mendgen, unpublished result). These results show that the plant cell deposits wall layers with different major components around the penetration site. We assume that Golgi bodies accumulating around the penetration site (Xu and Mendgen 1994) are involved in the secretion of the polygalacturonic acid.

The deposition of cell wall material around fungal structures is not restricted to the nonhost situation (Heath 1995; Skalamera and Heath 1996). In a compatible interaction between *U. vignae* and *V. sinensis*, polygalacturonic acid is part of an encasement around ineffective or degenerating intracellular hyphae. Further major components of the encasement were pectin, xyloglucan, and arabinogalactan (Stark-Urnau and Mendgen 1995). The deposition of wall constituents around penetrating fungal hyphae has also been observed in roots immediately after penetration by hyphae of *Fusarium oxysporum* Schlechtend.:Fr. (Rodriguez-Galvez and Mendgen

Fig. 4. Effect of enzyme treatments on epitope distribution shown in acryl-embedded, noninoculated material. **A**, Treatment of sections with pectinmethylesterase and subsequent probing with monoclonal Jim 7 results in very low number of pectin epitopes ($\times 60,000$). **B**, Treatment of sections with pectinmethylesterase and subsequent probing with Jim 5 reveals very high number of epitopes for polygalacturonic acid over epidermal wall. Arrows indicate fibrillar material ($\times 60,000$). **C**, Distribution of xyloglucan before enzyme treatment as detected with CCRC-M1 ($\times 60,000$). **D**, Cellulase treatment reduces epitopes in xyloglucan ($\times 60,000$). **E**, Xylanase treatment reduces epitopes in xyloglucan ($\times 60,000$). **F**, Cellulase and xylanase reduce epitopes in xyloglucan ($\times 60,000$).

1995). Also, in the symbiotic interaction of arbuscular mycorrhizal fungi, such as *Glomus versiforme* and *Gigaspora margarita*, an interfacial zone is produced that reflects the composition of the host cell wall (Balestrini et al. 1996). Currently, it remains difficult to differentiate the pathogenic from the mutualistic interaction because the sequence and dynamics of the deposition of the various wall components are not known. Deposition of high amounts of callose, however, does seem typical of the pathogenic interaction.

Targeted deletion of genes in plant pathogenic fungi coding for cell-wall-degrading enzymes has not yet been able to demonstrate the importance of such enzymes during development of pathogens (Schauwecker et al. 1995; Walton et al. 1995). Perhaps, genes not involved during infection were deleted. During saprophytic growth, fungi produce a series of wall-degrading enzymes with overlapping activities. It is difficult to determine which enzymes are active or needed during cell wall penetration (Mendgen et al. 1996). Results presented by Köller et al. (1995) suggest that cutinase isoforms of *Alternaria brassicicola* (Schwein.) Wiltshire involved in pathogenicity are produced only during pathogenic development in contact with the leaf.

The targeted deletion of cutinase genes of *Fusarium solani* (Mart.) Sacc. f. sp. *pisi* (F. R. Jones) W. C. Snyder & H. N. Hans. resulted in a controversy (Rogers et al. 1994; Stahl et al. 1994) since, depending on the infectivity assay used, different results were obtained. However, the targeted secretion of cutinase was observed below appressoria of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. (Podila et al. 1995). The way fungi degrade host walls or wall appositions seems to follow complicated schemes. The dikaryotic urediospores of *U. viciae-fabae* secrete a number of cell-wall-degrading enzymes in a highly regulated manner. Cellulase pro-

duction occurs already during appressorium formation (Heiler et al. 1993), pectinmethylesterases are secreted during infection hypha development, and polygalacturonate lyase is produced during differentiation of haustorial mother cells (Deising et al. 1995). Thus, the fungus seems to be adapted to the specific infection conditions found during stomatal penetration and subsequent host cell penetration. We are not yet able to follow the secretion of cell-wall-degrading enzymes by the basidiosporelings used in this work because of the small amount of spores available.

We also show in this paper that high pressure freezing and freeze substitution preserved the penetration site of a fungus with a high degree of quality. The amount of ice crystal formation was minimal and separation of the cytoplasm from the walls did not occur. We do not know why appressoria were regularly lost during processing. We had this problem with the epoxy resin and the acrylic resin used. However, embedding in two different resins allowed cytochemical and morphological studies. We have evidence now for a targeted, highly regulated degradation of plant wall material and, at the same time, deposition of new wall material. Numerous fungal enzymes may be coordinated during the degradation process of the plant wall and the different types of wall appositions (Walton 1994). It seems difficult to imagine that a single wall-degrading enzyme could play a major role during this process upon which further fungal development would rely. A single isoform of any of these various enzymes may only modify slightly the outcome of an infection, such as affecting the rate of progress of the penetration hypha. Schäfer (1994) defined this situation as a change in pathogen virulence.

MATERIAL AND METHODS

Plant material and fungal isolate.

Broad bean, *Vicia faba* var. Hedosa (Rijks Zwaan, The Netherlands), was grown at $20 \pm 2^\circ\text{C}$ and illuminated during

Fig. 5. Epitope density after treatment of noninoculated cell walls with pectinmethylesterase. Number of pectin (Jim 7) epitopes over inner layer decreases but polygalacturonic acid (Jim 5) epitopes increase.

Fig. 6. Epitope density in xyloglucan (control) over both layers of epidermis and after treatment of noninoculated cell walls with cellulase, xylanase, and a combination of both enzymes.

16-h photoperiods with 11,000 lux. The cowpea rust fungus, *Uromyces vignae* Barclay, race CPR-1, was a gift from M. C. Heath, Toronto, Ontario, Canada. Basidiospores were obtained from teliospores as described earlier (Xu and Mendgen 1991). The expanded primary leaves of 14-day-old broad bean plants were inoculated with basidiospores of the rust fungus under high humidity conditions.

Tissue preparation for electron microscopy.

Six to 10 h after inoculation, leaf pieces (2 mm in diameter) were vacuum infiltrated with 8% (vol/vol) methanol in water, high pressure frozen in a Balzers HPM 010 apparatus (Mendgen et al. 1991), and stored in liquid nitrogen. Cutting the leaves into pieces, vacuum infiltration at 40 mm Hg pressure, and freezing took between 5 and 15 min. Specimens were freeze substituted for 24 to 48 h at -90°C in acetone with 2% osmic acid and embedded in either Epon/Araldite resin (Knauf et al. 1989) or LR White (The London Resin White Co. Ltd., Basingstoke, Hampshire, UK). Serial sections, 65 to 75 nm thick, cut with a diamond knife, were collected on copper slot grids for morphological observations or on nickel slot grids for immunocytochemical studies (Xu and Mendgen 1994).

Antibodies.

Monoclonal antibodies Jim 5, binding to polygalacturonic acid, and Jim 7, binding to pectin, were from P. Knox, Leeds, UK (Knox et al. 1991). Monoclonal antibody CCRC-M1, binding to a terminal α -(1 $\rightarrow$ 2)-linked fucosyl-containing epitope, primarily present in dicot xyloglucan and as a minor component of rhamnogalacturonan (Puhlmann et al. 1994), was from M. Hahn, Athens, GA. The specificity of the polyclonal antibody to β -1,3-glucans (#CH-11-512; Cambridge Research Biochemicals, Northwich, Cheshire, UK) has been characterized (Northcote et al. 1989).

Sections from material embedded in epoxy resin were used for the detection of the different wall constituents at the penetration site. For monoclonal antibody labeling, sections were first treated with a blocking buffer of 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS; 10 mM Tris-HCl, 150 mM NaCl, pH 7.4) for 15 min at room temperature, then incubated with hybridoma culture supernatants diluted 1:1 with TBS for 16 h at 4°C , washed with TBS for 10 min, and incubated for 1 h at room temperature with goat anti-rat IgG (Jim 5 and Jim 7) or goat anti-mouse IgG + IgM (CCRC-M1) secondary antibodies, coupled to 15 nm of colloidal gold (from Plano Planet, Marburg, Germany), diluted 1:10 with TBS. Labeling specificity was controlled by replacing the primary antibody with buffer or with monoclonal antibodies directed against carbohydrate epitopes from rust fungus infection structures (Mendgen et al. 1988). For polyclonal antibody labeling, sections were treated as previously described (Xu and Mendgen 1994).

Enzyme treatment to degrade the epidermal wall.

Sections from material embedded in LR white were used for incubation with enzymes and subsequent detection of wall constituents. Pectinmethylesterase (EC 3.1.1.11; Sigma, Deisenhofen, Germany), was dialyzed in 2×500 ml TBS at 4°C for 24 h and diluted to contain 12 mg of protein per ml. The sections were floated on the enzyme solution, diluted 1:100 in

TBS, for 1 h at room temperature. After being washed in TBS, the sections were treated with antibodies (Jim 5 or Jim 7) as described. Cellulase (EC 3.2.1.4, Fluka, Buchs, Switzerland) 1 mg/ml in 50 mM sodium acetate buffer, pH 4.9 (NaAc), xylanase (EC 3.2.1.32, Fluka) 0.4 mg/ml in NaAc, and a mixture of cellulase and xylanase (1:1) in NaAc, were used to treat the sections at 35°C for 2 h (Liners et al. 1994). After being washed with NaAc and TBS (3×5 min each), the sections were incubated with CCRC-M1 antibodies as described above.

Sections were stained with aqueous uranyl acetate and lead citrate at room temperature before examination in a Zeiss EM-10 transmission electron microscope operated at 60 kV.

For the quantitative analysis of labeling, gold particles over each variant were counted on 10 to 15 micrographs. Areas were measured with a Tomaya Digital Planimeter (Tomaya Technics Inc., Sokutama, Japan). The density of labeling was expressed as the number of gold particles per mm^2 . Statistical analyses of the differences between modified and normal cell wall were based on the *t* test (Moore and McCabe 1989).

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