Minireview / Minisynthèse

Cellular interactions between biotrophic fungal pathogens and host or nonhost plants

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Abstract: Biotrophic fungi, such as the rust fungi and the powdery mildew fungi, represent a distinct and economically important group of plant pathogens. Studies of nonhost interactions with *Uromyces vignae* (cowpea rust fungus, monokaryotic stage) or *Erysiphe cichoracearum* (plantain powdery mildew fungus) suggest that for both types of biotrophs, penetration through the epidermal wall commonly fails in association with wall-associated defense responses, in part elicited by breakdown products of the plant cell wall. Data suggest that extracellular hydrogen peroxide plays a primary role in this penetration failure, acting either as a signaling agent or as a necessary adjunct to some other defense response within the wall. Protoplast involvement, but not necessarily transcription or translation, is necessary for these wall-associated responses, and they can be reduced by reducing the adhesion between the plant cell wall and the plasma membrane. The latter observation suggests that this adhesion is important for at least some types of defensive signaling between the wall and the cell contents. In host or nonhost plants, *Erysiphe* species increase this adhesion and wall-associated responses occur even in susceptible plants. However, in its host species, *U. vignae* locally reduces this adhesion around the penetration site and, as an apparent consequence, no wall-associated responses can be detected. Thus, this latter system is ideal for studying postpenetration cellular and molecular changes in resistant or susceptible host cells without the confounding effect of responses to the trauma of cell wall penetration. Such studies indicate that not only is penetration failure in nonhost plants determined while the fungus is growing through the epidermal cell wall, but that it is at this stage of infection in host species that cellular responses are initiated that determine whether the plant cell will become susceptible to infection or exhibit the hypersensitive response.

Key words: adhesion, biotroph, cell wall, fungi, H_2O_2, hypersensitive response, powdery mildew, rust.

Résumé : Les champignons biotrophes tels que les champignons de rouille et les champignons de blanc forment un groupe d'agents phytopathogènes distinct et économiquement important. L'étude d'interactions incompatibles avec l'*Uromyces vignae* (le champignon de la rouille de la dolique, stade monocaryotique) ou l'*Erysiphe cichoracearum* (champignon du blanc du plantain) montre que pour les deux types de biotrophes, la pénétration au travers l'épiderme échoue habituellement à la suite de réactions de défense associées à la paroi et en partie provoquées par la dégradation de produits de la paroi cellulaire de la plante. Les données montrent que le peroxyde d'hydrogène extracellulaire joue un rôle primordial dans l'échec de cette pénétration en agissant comme un agent avertisseur ou comme un complément essentiel à quelque autre réaction de défense au sein de la paroi. L'implication du protoplasme, mais pas nécessairement par la transcription ou la traduction, est nécessaire pour ces réactions associées à la paroi qui peuvent être réduites en diminuant l'adhérence entre la paroi cellulaire de la plante et la membrane plasmique. Cette dernière observation montre que cette adhérence est importante pour au moins certains types de signaux défensifs entre la paroi et le contenu cellulaire. Dans les plantes, hôtes ou non, les *Erysiphe* spp. augmentent cette adhérence et les réactions associées à la paroi ont lieu même dans les plantes sensibles. Cependant, dans ses espèces hôtes, l'*U. vignae* réduit localement cette adhérence autour du point de pénétration et, comme conséquence apparente, aucune réaction associée à la paroi ne peut être détectée. Ainsi, ce dernier système est idéal pour l'étude des modifications cellulaires et moléculaires postpénétration dans les cellules d'hôtes résistants ou sensibles débarrassées du facteur de confusion que représentent les réactions au trauma de pénétration de la paroi cellulaire. De telles études indiquent que non seulement l'échec de la pénétration dans des hôtes incompatibles est déterminée lorsque le champignon croît au travers la paroi des cellules épidermiques, mais que c'est à ce stade de l'infection dans les espèces hôtes que les réponses cellulaires sont déclenchées pour déterminer si la cellule végétale deviendra sensible à l'infection ou aura une réponse d'hypersensibilité.

Mots clés : adhérence, biotrophe, paroi cellulaire, champignon, H_2O_2, réponse d'hypersensibilité, blanc, rouille.

Accepted 20 June 2002.

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Introduction

Biotrophic fungal plant pathogens obtain their nutrients from living cells and thus represent a distinct group of plant pathogens. Although there are some biotrophs that remain entirely in the plant's intercellular spaces, most form structures within the plant cell such as intracellular hyphae or haustoria. As only the plant wall, but not the plant protoplast, is breached by these structures, there must be some value to biotrophic pathogens being in intimate contact with the plant plasma membrane. Most probably this is the ability to form a compartment, delimited by the fungal plasma membrane and the adjacent surrounding extension of the plant plasma membrane, through which the exchange of signals and nutrients can be controlled (Heath and Škalamera 1997).

Although the biotrophic interaction between some fungi and the invaded cell may only last a day or two, for powdery mildew and rust fungi, the "compatible" relationship between host cell and parasite may last for weeks. Such long-lived biotrophy is usually associated with changes in translocation patterns within the plant such that the infection site becomes a nutrient sink, essentially allowing the fungus to access all of the plant's resources (Lewis 1973). This form of nutrient acquisition seems more efficient than that of necrotrophic pathogens, which have to constantly kill and grow into new dead cells to continue obtaining nutrients from their hosts. Perhaps this efficiency of parasitic biotrophy explains why it has evolved independently in several taxonomically distinct groups of fungi and oomycetes; however, the limited number of such taxa suggests that the biotrophic niche may be a difficult one to occupy. The responsiveness of plants to their environment, and the numerous defensive responses that can be induced by stress at a cellular level, suggest that overcoming the ability of a living cell to defend itself may be the most significant problem associated with the evolution of biotrophy (Heath and Škalamera 1997).

Biotrophic fungal pathogens include many species that are economically important in agriculture and forestry, and it seems reasonable to assume that the more we understand the details of the intimacy of the plant–fungal interaction, the more likely we are to find novel ways to combat the diseases that these fungi cause. This article reviews some of the work in my laboratory aimed at elucidating the factors that determine cellular resistance or susceptibility to rust and powdery mildew fungi. Our "model" rust fungus is *Uromyces vignae*, an autoecious species that forms both monokaryotic and dikaryotic parasitic stages in the same host, cowpea. The studies discussed here concentrate on those involving basidiospore-derived infections (the monokaryotic stage) because the initial direct penetration of the fungus into an epidermal cell is comparable with the formation of the first intracellular haustorium by an appressorium of a powdery mildew fungus. Two powdery mildew species have been studied, *Erysiphe cichoracearum* isolated from plantain and *Erysiphe polygoni* isolated from cowpea. *Erysiphe cichoracearum* differs from many *Erysiphe* species in that its first penetration attempt rarely succeeds even in a susceptible host, and up to three attempts may be made before a haustorium is formed (Fig. 1a). Nevertheless, in host species, penetration into the underlying epidermal cell by the two powdery mildew fungi, or by *U. vignae*, is almost always eventually successful. In contrast, on nonhost species, as is typical for many direct-penetrating fungal pathogens, penetration usually fails while the fungus is still within the epidermal wall or embedded in a callose-containing papilla between the wall and the plant plasma membrane. On those, often rare, occasions in which the fungus successfully breaches the nonhost wall and starts to develop within the cell lumen, the epidermal cell rapidly dies (the hypersensitive response (HR)) and fungal growth ceases. This HR is the more typical response seen in resistant host cultivars to *U. vignae* and penetration failure is rare.

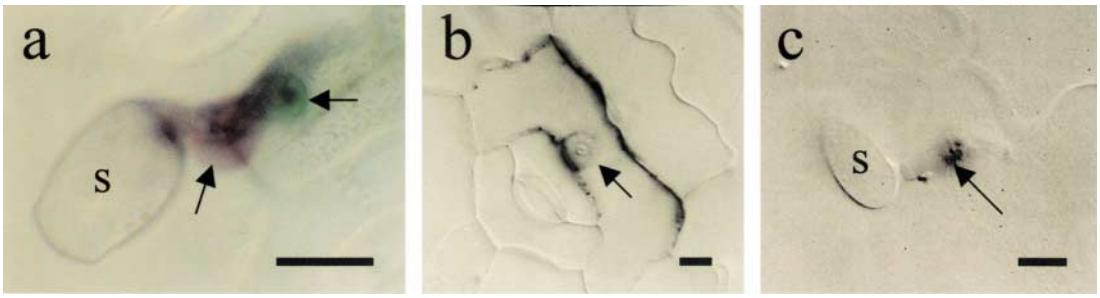
Defensive responses on and in the epidermal cell wall are the first line of defense against direct-penetrating fungi

Direct-penetrating fungi commonly fail to penetrate nonhost plants, suggesting that the epidermal cell wall is the first line of defense against such fungi, including biotrophic parasites, and that this defense is most successful when the fungus is on a plant to which it is not adapted. Numerous fungal or fungal product induced wall-associated responses have been reported that could hinder fungal ingress into a plant cell. These include the cross-linking of wall proteins (Brisson et al. 1994), the impregation of the wall with phenolics or silica (Fig. 1a) (Heath and Stumpf 1986), the deposition of callose between the cell wall and the plasma membrane (Figs. 1a and 1b) (Aist and Bushnell 1991; Perumalla and Heath 1989), and the extracellular generation of reactive oxygen species (Figs. 1b and 1c) (Thordal-Christensen et al. 1997). Many of these occur at the same site of attempted penetration, and there have been only a few attempts at proving which component is the most effective in inhibiting fungal growth (Aist and Bushnell 1991; Heath and Stumpf 1986; McLusky et al. 1999; Perumalla and Heath 1989, 1991). These have demonstrated that some responses are ineffective in particular plant–fungal interactions (Perumalla and Heath 1989), some potentially toxic compounds are not toxic (McLusky et al. 1999), and the relative importance of a particular component can increase if the intensity of another is reduced (Heath and Stumpf 1986).

Wall-associated responses during fungal penetration are primarily, but not completely, the response to localized wall degradation

To see what wall-associated responses are the inevitable outcome of the invasion process itself, we have tried to mimic the fungal infection process by lightly scratching the cuticles of cowpea leaves and treating them with a hemicellulase enzyme mixture (Heath et al. 1997). Cytochemical studies (Mellersh et al. 2002) show that localized extracellular generation of H_2O_2 occurs at the scratch sites followed by the accumulation of phenolic compounds within the wall and the eventual cross-linking of proteins. Deeper scratches cause the localized deposition of callose between the plasma membrane and the plant cell wall. It would be expected, therefore, that any fungus trying to breach these epidermal walls would trigger similar re-

Fig. 1. Light micrographs of the wall-associated responses to cowpea epidermal wall penetration by *Erysiphe cichoracearum*. (a) A spore (s) has produced an appressorium that has made two penetration attempts (arrows). One has triggered a callose-containing papilla (seen as a circle around the penetration point) and the deposition of phenolic compounds (stained greenish-blue with toluidine blue at low pH). The other has triggered the deposition of material staining purple with toluidine blue, which often represents silica deposition. (b) A failed penetration site stained with nitroblue tetrazolium. A blue colouration, indicative of superoxide generation, can be seen associated with parts of the anticlinal walls of the epidermal cell but not in the callose papilla (arrow) that surrounds the fungal penetration point. (c) A failed penetration site stained with diaminobenzidine. The brown colour in the papilla (arrow) around the penetration site indicates the generation of H_2O_2. Scale bars = 10 μm. (Photographs courtesy of D.G. Mellersh.)



sponses. As expected, such responses were observed when *U. vignae* attempted to penetrate nonhost pea plants or when either of the two *Erysiphe* species attempted to penetrate host or nonhost pea or cowpea plants. However, the results also suggested that specific activities of a fungus may additionally influence the plant response. For example, *E. cichoraceaum* elicited a transient burst of extracellular superoxide under the appressorium prior to penetration (similar to the unusually late burst shown in Fig. 1b), perhaps in response to the cutinase that some powdery mildew fungi are known to release (Pascholati et al. 1992). Interestingly, the later generation of extracellular H_2O_2 triggered by this fungus is more localized to the immediate penetration site (Fig. 1c), and the spatial separation of these two reactive oxygen species suggest that they may be generated by different mechanisms (Mellersh et al. 2002).

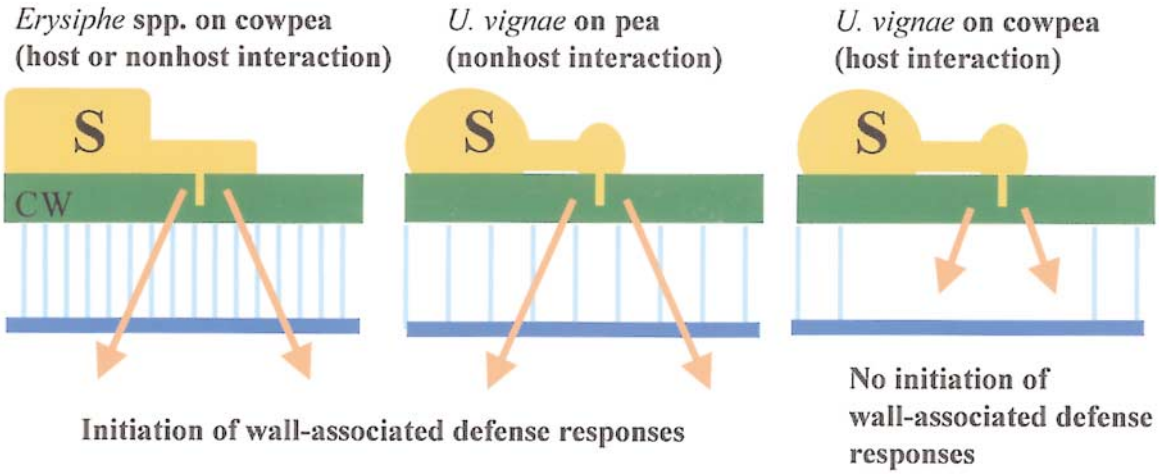
Hydrogen peroxide plays an important role in inhibiting fungal penetration in nonhost plants

More detailed time course studies of the nonhost interactions involving *U. vignae* or *E. cichoracearum* (Mellersh et al. 2002) have revealed that the only detected wall-associated response common to both interactions at the time of inhibition of fungal penetration is H_2O_2 generation. Specifically scavenging this extracellular H_2O_2 with exogenous, apoplastic catalase results in a significant increase in penetration frequency in both nonhost interactions. Interestingly, inhibitors of transcription or translation do not affect H_2O_2 generation, indicating that the generation process does not require new proteins to be synthesized in the cell. Nevertheless, interference with the actin cytoskeleton by cytochalasin E does reduce H_2O_2 generation, demonstrating that this response requires some involvement of the plant protoplast. Although transcription and translation inhibitors have no effect on H_2O_2 generation, they, like cytochalasin E, do cause an increase in penetration frequency. These data suggest that either the primary role of H_2O_2 is to act as a signal for some unknown wall-associated defense that requires gene activation or H_2O_2 and some other defense are both needed for the inhibition of fungal growth in the plant cell wall. Data tend to support the latter possibility for the *E. cichoracearum* – cowpea interaction, as penetration failure also correlates with the presence of phenolic compounds in the cell wall, and H_2O_2 could be involved in oxidative cross-linking of these compounds to wall components (Mellersh et al. 2002). Interestingly, a comparable study of *Colletotrichum coccodes* on young tomato leaves showed no evidence of the involvement of the protoplast in either the generation of H_2O_2 or the inhibition of fungal growth and supported the hypothesis that H_2O_2 alone was sufficient to cause penetration failure (Mellersh et al. 2002). The differences between these results and those involving *U. vignae* or *E. cichoracearum* emphasize the fact that superficially similar plant responses to pathogen invasion may differ in the details of the interactions taking place between the pathogen and the plant cell. Therefore, one has to be cautious when extrapolating from one system to another.

Rust and powdery mildew fungi cope with wall-associated responses in their host species in different ways

It is well reported in the literature that powdery mildew fungi typically elicit wall-associated responses in both resistant or susceptible host plants (Aist and Bushnell 1991). Similarly, in our studies, there was no difference in the types or frequencies of wall-associated responses in host or nonhost interactions with the *Erysiphe* species, although their intensity may have been somewhat less in host species. These observations suggest that *Erysiphe* species cope with these defenses in subtle ways, with the relative speed of penetration in relation to the speed of the defense responses perhaps being a significant factor in penetration success (Aist and Bushnell 1991). However, in stark contrast, no wall-associated responses were observed when the rust fungus penetrated resistant or susceptible cowpea cultivars, indicating that this fungus must have a cowpea-

Fig. 2. Diagrammatic illustration of the differences between penetration sites of the *Erysiphe* species and *Uromyces vignae* in the degree of adhesion (indicated by vertical lines) between the cell wall (CW; green horizontal lines) and the plasma membrane (blue horizontal lines). The localized reduction in adhesion induced by *U. vignae* in its host species appears to prevent signals (red arrows) from being transduced from the wall to the protoplast to induce wall-associated defense responses. S, spore. (Adapted from Mellersh and Heath 2001.)



specific means of suppressing nonspecific defense responses as it grows through the epidermal wall.

Adhesion between the plasma membrane and the plant wall is needed for the expression of wall-associated responses

The increase in penetration success, and the reduction in wall-associated defense responses, seen in nonhost interactions involving *U. vignae* or *E. cichoracearum* after interference with the actin cytoskeleton indicate that these responses require communication between the plant cell wall and the protoplast. In mammalian cells, communication between the extracellular matrix and the intracellular actin cytoskeleton is mediated by transmembrane proteins known as integrins. Binding of integrins to the extracellular matrix can be inhibited by peptides containing the RGD (arginine – glycine – aspartic acid) motif (Giancotti and Ruoslahti 1999), and although homologues of integrins have not been unequivocally demonstrated in plants, RGD peptides reduce the adhesion between the plant plasma membrane and the cell wall (Mellersh and Heath 2001). Significantly, RGD peptides also mimic the effect of the antiactin agent cytochalasin E and increase fungal penetration and reduce wall-associated responses in nonhost interactions involving *U. vignae* or *E. cichoracearum* (Mellersh and Heath 2001). These results suggest that the communication between the plant cell wall and the plant protoplast that results in wall-associated defenses requires adhesion between the cell wall and the plasma membrane.

Powdery mildew fungi increase plasma membrane – plant wall adhesion in host or nonhost plants whereas the rust fungus transiently decreases it in its host species

The degree of adhesion between the plasma membrane and the plant cell wall can be monitored using plasmolysis-inducing solutions that cause the plasma membrane to contract away from the cell wall. Normal adhesion points are indicated by thin "Hectian strands" of cytoplasm linking the plasmolysed protoplast to the cell wall, and these can be disrupted by the application of RGD peptides (Mellersh and Heath 2001). Increased degrees of adhesion are detectable as larger areas of plasma membrane – cell wall contact resulting in a "concave" plasmolysis morphology. Plasmolysis studies show that the powdery mildew fungi cause an increase in plasma membrane – cell wall adhesion in host and nonhost plants that is not disrupted by RGD peptides and that spreads to surrounding uninfected cells. In contrast, the rust fungus has no effect on adhesion in nonhost pea but causes a transient and highly localized decrease in adhesion as it penetrates resistant or susceptible host cowpea plants (Mellersh and Heath 2001). Since plasma membrane – cell wall adhesion is necessary for wall-associated responses to be elicited, it appears that the reason why these responses are absent during host penetration by the rust fungus is that it disrupts this adhesion and, therefore, the signaling system between the cell wall and the cell protoplast (Fig. 2). The fungal molecule(s) that is responsible for this disruption is under investigation.

Cellular resistance or susceptibility of host cultivars to the rust fungus is determined while the fungus is still penetrating the plant wall

In resistant cowpea cultivars, because of the absence of nonspecific, wall-associated defense responses, *U. vignae* normally successfully penetrates epidermal cells and it is some hours before the HR is manifested by the death of the invaded cell (Chen and Heath 1991). The requirement for cell metabolism, as well as some shared features with programmed cell death in animals, strongly suggest that the HR in all plant–pathogen interactions is a programmed cell death (Heath 2000a). In the cowpea cultivar that we have

studied, *U. vignae* induced cell death is controlled by two resistance genes (Heath 1994), requires protein synthesis (Chen and Heath 1994) as well as an influx of extracellular calcium (Xu and Heath 1998), and appears to be triggered by short fungal peptides that possibly are the products of avirulence genes (D'Silva and Heath 1997). The actual dismantling of the cell is a protracted process lasting several hours, and a summary of the temporal cellular changes that accompany the death process is illustrated in Fig. 4 in Heath (2000b)

Although the invaded cell does not die until some time after the fungus has entered the cell lumen, the peptide death elicitors are produced by the fungus just after the fungus forms an appressorium and before it starts to enter the epidermal cell wall (Chen and Heath 1990). Careful stereological analysis of polyribosome density on the endoplasmic reticulum (indicative of the degree of transcription) and nuclear pore density (indicative of the degree of trafficking between the nucleus and the cytoplasm) has shown that the resistance or susceptibility of the underlying cowpea epidermal cell can be predicted from its cytoplasmic response as the fungus starts to grow through the plant wall. Results suggest that transcription and translation increase at this time in resistant cells (Mould and Heath 1999), as does the level of cytosolic calcium (Xu and Heath 1998), while calcium levels remain normal and translation locally decreases in susceptible cells. Therefore, the first stages of resistance or susceptibility are initiated before the fungus reaches the plant plasma membrane and several hours before the invaded resistant cell manifests any visible sign of the HR.

The absence of nonspecific defense responses normally associated with fungal wall penetration makes the cowpea – *U. vignae* monokaryon system a unique pathosystem for investigating the changes within the plant cell that prepare it for the expression of susceptibility or resistance gene controlled resistance to rust infection. This pathosystem also is unique in that the stage at which the fate of the plant cell is determined, i.e., when the fungus has just entered the plant cell wall, can be identified in living cells because of the transient association of the plant nucleus with the penetration site at this time (Heath et al. 1997). To exploit the features of this pathosystem, we have extracted mRNA from individual resistant or susceptible cells during wall penetration to form an EST library with the aim of identifying genes that are up- or down-regulated at this critical time in the infection process (M.J.R. Mould, T. Xu, and M.C. Heath, unpublished data). Interestingly, the rather few genes that have been tentatively identified as being upregulated as the cell prepares for the HR are not those that have been suggested to be markers for the HR (Heath 2000a), nor is the profile similar to those reported from studies that sample tissue, rather than individual cells, during the HR. This last observation perhaps reflects the fact that tissue samples provide an averaged view of gene expression in both dying cells and those responding to their dying neighbours rather than a picture of how an individual cell prepares to die.

Conclusions

The data discussed here suggest that nonhost resistance to biotrophic rust (monokaryon) or powdery mildew fungi closely resembles that described for nonbiotrophic, directly penetrating fungi in the primary involvement of plant responses that prevent successful penetration of the epidermal wall. Similarly, these responses seem to almost universally involve the plant protoplast and, in particular, the actin cytoskeleton (Kobayashi et al. 1997) even in the absence of the need for transcription or translation. However, in their host species where penetration is successful, there are differences in the way that rust and powdery mildew fungi cope with these nonspecific, penetration-induced defenses, just as there are differences in the interface that develops between intracellular structures and the invaded plant cell (Heath and Škalamera 1997). These differences perhaps reflect the independent evolution of these intimate plant–fungal interactions. Because the cowpea rust fungus inhibits wall-associated defense responses in its host species, apparently by preventing the signaling between the plant cell wall and the protoplast, this represents an ideal system for investigating the specific cellular and molecular changes that accompany the expression of susceptibility, or resistance gene controlled resistance, without the confounding effects of nonspecific responses to the penetration of the cell wall.

Acknowledgments

The research described in this paper was funded by the Natural Sciences and Engineering Research Council of Canada. This paper is based on the presentation and abstract presented at the Australasian Plant Pathology Society meeting in September 2001.

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