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Review article
Evidence for Molecular Differentiation in Powdery Mildew Haustoria Through the Use of Monoclonal Antibodies

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Abstract

The haustorium of biotrophic powdery mildew fungi is a specialised structure involved in the transfer of host nutrients to the fungus and in recognition and signalling between the plant and fungal partners. In order to study this interface, monoclonal antibodies (MAbs) have been raised to isolated haustorial complexes and to pea plasma membranes. Use of these MAbs in immunolocalisation and biochemical studies has revealed: (1) a major, 62 kDa N-linked glycoprotein of fungal walls and plasma membranes found in haustoria and mycelia (identified by MAb UB7); (b) specific glycoproteins uniquely located in the haustorial plasma membrane (MAbs UB8 and UB10) and on the extrahaustorial membrane (MAb UB11); (c) all these glycoproteins are incorporated into the haustorium at early stages of development and are present in healthy haustoria of susceptible and resistant plants; (d) a glycoprotein antigen (MAb UB9), which is present in uninfected pea cells, occurs on the outer face of the *ehm* of a sub-population of haustoria, appears late in development and may play some role in plant defence responses. These results provide for the first time, clear evidence for molecular differentiation at this haustorial interface. We tentatively introduce the term *'haustorins'* to describe those proteins and glycoproteins of either host or pathogen origin that are specifically expressed in the haustorial complex.

1. Introduction

The biotrophic powdery mildew fungi interact with host cells through the formation of sac-like *haustoria* within living epidermal cells. The haustorium is surrounded by an invaginated host plasma membrane, *the extrahaustorial membrane (ehm)*. Between the *ehm* and the fungal haustorial cell wall there is a polysaccharide-rich *extrahaustorial matrix*. The composite structure of host pathogen components is termed the *haustorial complex* (HC, Fig. 1). The
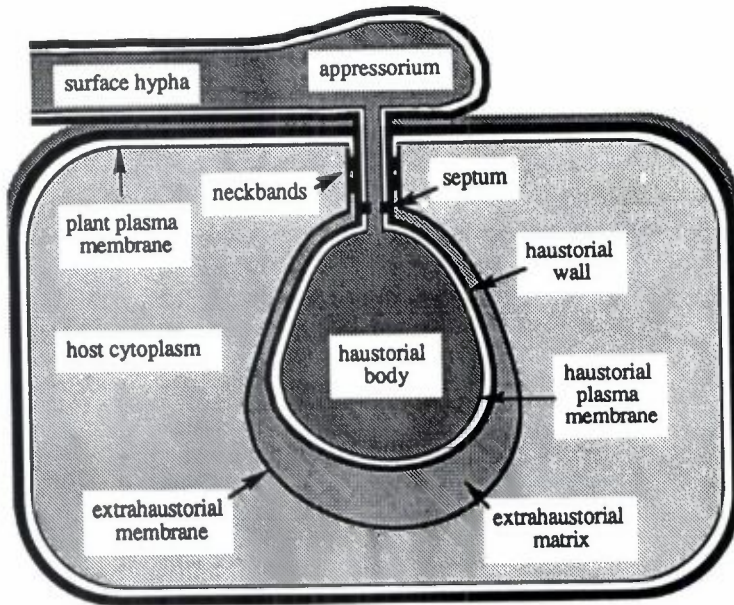

Figure 1. Diagram of a haustorial complex of *E. pisi* in a pea epidermal cell, showing the structural components referred to in this paper.

haustorium in powdery mildews is the only interface with the host cell and must therefore play a fundamental role in processes such as nutrient transfer, recognition and disease resistance. These specialised roles imply molecular differentiation of the various interface components of the haustorium compared with the equivalent structures in uninfected host cells and the fungal surface mycelium. Various studies (summarised in Gay, 1984, Manners and Gay, 1983) have shown that the *ehm*, in particular, has certain properties (e.g. membrane thickness, absence of intramembrane particles, presence of certain glycan moieties, absence of ATPase activity) that distinguishes it from the normal plant plasma membrane.

However, there is little or no information about the HC at the molecular level and in consequence our understanding of this interface is very poor. For example, we need to know whether there are proteins or glycoproteins specifically expressed at this interface in either host or pathogen structures. Alternatively, are there components of normal plant plasma membranes, for example, that are not expressed at the haustorial interface? If one can isolate and characterise these differentially expressed macromolecules and their associated gene sequences this may allow us to determine their functional roles. Finally, by comparing haustoria from susceptible and resistant hosts it may prove possible to identify those surface molecules that play some role in mutual plant-pathogen recognition.

One approach to the molecular analysis of structure and function at the interfaces of symbionts, that may provide at least partial answers to these questions, is through monoclonal antibodies (MAbs). The advantage of this approach is that from small amounts of immunogen, which does not need to be pure, antibodies can be obtained with a range of specificities, and they can be made in virtually unlimited quantities. The aim then, is to develop a set of specific affinity probes for components of the haustorial interface, with particular emphasis on proteins and glycoproteins that are differentially expressed. This approach has been very successfully applied to analyses of bacteroid components involved in the *Rhizobium*/legume symbiosis (Brewin et al., 1986), but the work described in this paper is the first to use such an approach for haustorial interfaces. Details of this work are contained in Mackie (1991); Mackie et al. (1991), Green et al. (1992).

2. Isolation of Haustoria and Raising of MAbs

Peas (*Pisum sativum* cv. Onward) were grown in a glasshouse under supplementary illumination and a 18 hr photoperiod. Four-week-old plants were inoculated with *Erysiphe pisi* by flicking infected leaves and stems above plants to be infected. Intact haustorial complexes (HCs, Figs. 2A,B,C) were isolated from infected leaves 7 days after infection by the method of Gil and Gay (1977) as modified and described by Mackie et al. (1991). HCs were then used as an immunogen as described by Mackie et al. (1991). Antibody-secreting hybridomas were screened by indirect immunofluorescence (IIF) on isolated HCs dried down onto microscope slide. The contaminating chloroplasts in the immunogen do not constitute a problem since antibodies to chloroplast components are easily screened out. MAbs were also raised to plasma membrane-enriched fractions from uninfected pea leaves (Roberts et al., in preparation) and screened for binding to plasma membranes by ELISA, and to HCs by IIF. The details
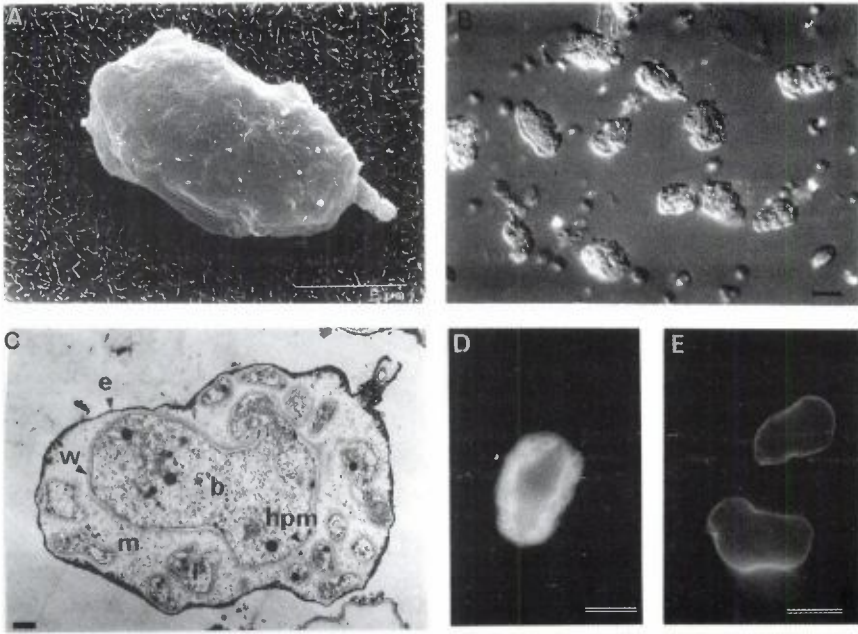
Figure 2. A, B, C, intact, isolated haustorial complexes of *E. pisi* in SEM (A), under inter-
ference contrast (B), and in section in the TEM (B). The smaller bodies in B are
contaminating chloroplasts. The TEM image shows the haustorial body (b) and
lobes (l) delimited by the haustorial plasma membrane (hpm) and haustorial wall
(w) and surrounded by the extrahaustorial matrix (m) and an intact, thickened
extrahaustorial membrane (e). D and E, indirect immunofluorescence of hausto-
rial complexes *in situ*, showing labelling with an internal binder (UB7, D) and a
peripheral binder (UB11, E). Scale bars are 5 μm (A), 10 μm (B), 0.5 μm (C),
5 μm (D and E).

of the methods used to raise,screen and characterise the antibodies are all
described in Mackie (1991) and Mackie et al. (1991).

The first fusions led to the selection of a panel of 5 MAbs displaying a range
of specificities (Table 1). Briefly, the MAbs can be divided into two groups
with respect to binding to HCs (Figs. 2D,E): those that bind to peripheral
components of the *ehm*, and those that recognise internal antigens (the isolated
HCs are dried down onto slides for IIF which increases their permeability to
MAbs). The characteristics of these two groups of antibodies is described
below.

Table 1. Characteristics of the panel of MAb probes raised to isolated haustorial complexes or plant membranes

MAb	Immunogen	Immuno-globulin subclass	Molecular nature of antigen	Probable identity of epitope	Location of antigen in haustorial complexes	Expression	Species-specificity[1]
UB7	Isolated haustorial complexes	IgG2a	62 kDa N-linked glycoprotein, probably polymeric	CHO	Internal, fungal plasma membrane and cell wall	Surface hyphae and haustoria	Present in many chitin-containing fungi
UB8	Isolated haustorial complexes	IgG1	62 kDa N-linked glycoprotein	protein	Internal, haustorial plasma membrane	Haustorium-specific, all stages of development	*E. pisi* only
UB9	Plasma membrane-enriched preparations from uninfected pea leaves	IgG1	200 kDa glycoprotein	CHO	Peripheral, cytoplasmic face of some *ehm*	Plasma membranes of host. *Ehm* of ~ 20% of older haustoria	*E. pisi* and pea downy mildew[2]
UB10	Isolated haustorial complexes	IgG1	45 kDa glycoprotein	CHO	Internal, fungal plasma membrane	Haustorium-specific	*E. pisi* only
UB11	Isolated haustorial complexes	IgG1	250 kDa glycoprotein	CHO	Peripheral, *ehm*	Haustorium-specific, all stages of development	*E. pisi* only

[1] Range of species tested includes, *Erysiphe pisi* on pea, *E. graminis* on wheat, *E. cichoracearum* on Antirrhinum, *Colletotrichum lindemuthianum* on bean, *Peronospora pisi* on pea (Machkie et al., in preparation).

[2] The species-specificity of UB9 has only been studied to a limited extent. It binds to ~ 20% of pea powdery mildew haustoria after 4 days infection, but not to haustoria of *Erysiphe cichoracearum* on Antirrhinum, or *E. graminis* on wheat, although the possibility cannot be excluded that certain UB9+ developmental stages might have been missed. However, UB9 also binds to haustoria of the pea downy mildew *Peronospora pisi* (Beale and Spencer-Phillips, pers. commun.).

3. MAbs Labelling Internal Antigens

MAb UB7 can be shown by immunogold labelling and ELISA on isolated cell fractions to bind to an antigen which is present on the fungal plasma membrane and in the cell walls of both haustoria and surface hyphae. It is, therefore, not differentially expressed in the haustorium. In Western blots the antigen has an M_r of 62 kDa on reducing gels but it may be polymeric in the native state. The antigen is a glycoprotein with some N-linked side-chains and the epitope is probably carbohydrate since it is labile to periodate oxidation but not pronase digestion. This antibody is not species-specific since it binds to hyphal walls and membranes of other chitin-containing fungi.

In contrast, UB8 recognises a glycoprotein antigen that is specifically expressed in the haustorial plasma membrane being absent from both fungal surface hyphae and uninfected plant cells. The antigen also has an M_r of 62 kDa, and is also an N-linked glycoprotein. However, the pattern of expression, differences in mobility following treatment with endo-F which cleaves N-linked glycans, and the identical mobility of the antigen on reducing and non-reducing gels, all show that the UB8 antigen is different to that recognised by UB7. Binding is sensitive to protease and insensitive to periodate suggesting a protein epitope. The expression of the antigen in the HC ceases at a point between the A and B neckbands. UB8 is specific for *E. pisi* (Table 1). The antigens recognised by UB7 and UB8 are expressed at very early stages in haustorial development, within a few hours of infection.

Another haustorium-specific antigen is recognised by UB10 but this has been less intensively studied than UB8. UB10 binds to a 45 kDa glycoprotein specifically expressed in the haustorial plasma membrane.

4. MAbs labelling Peripheral Antigens

UB9 was raised against plasma membrane-enriched preparations from uninfected pea leaves and confocal laser scanning microscopy of permeabilised epidermal strips, clearly shows binding to the pea plasma membrane (Roberts et al., in preparation). The antigen is a glycoprotein and elutes on HPLC with an M_r of 200 kDa. Binding is sensitive to periodate, suggesting that the antibody recognises a carbohydrate epitope. The UB9 antigen is not expressed in the modified host plasma membrane that forms the *ehm* around the invaginating haustorium for up to 7 days after infection, which suggests that this antigen plays no part in the functional properties of the active haustorium. However, it does bind to a sub-population of haustoria after 7 days, labelling 20% of HCs. Immunogold probing reveals that this antigen is deposited on the cytoplasmic

face of the *ehm*. Furthermore, in double-labelling immunofluorescence experiments, those haustoria that bind UB9 are not labelled by Texas Red labelled tomato lectin (TRTL). Since tomato lectin binds to oligo- and polysaccharides containing *N*-acetyl-glucosamine residues, we would therefore anticipate that TRTL would label the chitin-containing fungal cell wall of the haustorium. Approximately 80% of haustoria from a susceptible interaction were TRTL+/UB9–, but those which were TRTL– were UB9+ and *vice versa*. Our interpretation of these results is that deposition of the UB9 antigen may be a late response on the part of the host, possibly associated with defence, and that coating of some haustoria by the antigen thereby renders them impermeable to TRTL. Whilst we do not yet know the molecular identity of the UB9 antigen, the possibility that it might be callose has been excluded.

In contrast, UB11 binds to the periphery of all haustoria, labelling the *ehm* at very early stages of haustorial development (Fig. 2E). This antibody does not label the plant plasma membrane in uninfected epidermal cells, nor that region of the host plasma membrane lining the plant cell wall of an infected cell. The antigen is a large glycoprotein (M_r 250 kDa) and the epitope recognised by UB11 appears to be carbohydrate in nature. Whilst it is tempting to conclude that this antigen is a novel host component, specifically inserted into the modified host plasma membrane that forms the *ehm*, the possibility cannot be excluded as yet, that it may represent a fungal glycoprotein which is exported from the haustorium and inserted into the host membrane.

5. Discussion

The establishment of a successful infection by a biotrophic pathogen such as a powdery mildew, requires the development of a compatible, dynamic relationship with the living host cell, through the formation of a specific infection structure, the haustorium. This is a complex process requiring developmental, physiological and molecular changes in both the fungus and the host cell and one might consider that specific genes involved in this process constitute 'biotrophy genes'. On the pathogen side one might consider such genes to be a sub-set of the more familiar 'pathogenicity genes' since such genes are functioning to promote pathogenic success. However, the idea that the expression of specific host genes is required in the formation of a functional haustorium is perhaps a new concept that deserves further consideration. It is not difficult to accept the existence of host 'symbiosis genes' such as those that code for nodulins in the *Rhizobium*/legume symbiosis (Verma et al., 1986), where the expression of such genes is clearly to the benefit of the host in promoting a functional mutualistic relationship. It is more difficult to postulate the

existence of genes in the host whose expression results in the formation of a compatible relationship with a pathogen since we are normally accustomed to thinking of the expression of host genes that promote defence rather than compatibility.

Yet on *a priori* grounds the existence of such genes must be anticipated since the formation of the haustorium presumably involves the synthesis of plasma membrane components to form the *ehm* that invests the invaginating haustorium and we know that the *ehm* is structurally and functionally quite different to that of the host plasma membrane in uninfected cells. The solution to this apparent contradiction presumably is that either the pathogen influences host gene expression in such a way as to promote biotrophy and repress defence or that no new host genes are actually expressed, rather the pathogen inserts its own gene products into host membranes in order to create the appropriate properties, say with respect to the transport of metabolites.

The purpose of the research programme described in this paper is to obtain basic molecular information that might enable fundamental issues like these to be addressed and the strategy chosen was to raise MAbs that could then be used as molecular probes to investigate the expression of specific molecules at the level of the individual cell. The results illustrated in this paper and elsewhere (Mackie et al., 1991; Green et al, 1992), clearly show that this is a viable approach. HCs can be isolated in sufficient quantities both to raise and screen MAbs and the selection criteria and methods employed have enabled the selection of probes to both internal and peripheral antigens and to antigens which are differentially expressed in the various structures of the interface.

The most important general finding from this initial phase of the programme is that for the first time there is now clear evidence for molecular differentiation at an haustorial interface of a biotrophic pathogen since UB8,10 and 11 all recognise antigens that are specifically expressed in haustoria. In other systems, cytochemical changes in polysaccharide/glycoprotein composition of haustorial walls during their maturation in wheat stem rust have been reported (Chong et al., 1986) and novel polypeptide synthesis has been demonstrated in ectomycorrhizas (Hilbert and Martin, 1988) although these new products were not localised to any interface. In the case of the mutualistic *Rhizobium*-legume symbiosis, new gene products encoded by both host and microbe genomes have long been shown to be essential for nodule function and are referred to as 'nodulins' (e.g. Verma et al., 1986). By analogy, new genes specifically expressed in haustoria, might be termed 'haustorins'. UB8, which recognises a protein epitope, is probably detecting a novel gene product, and the UB8 antigen is therefore the first, tentative candidate for an 'haustorin'. However, we cannot, at this stage, claim that the novel antigens detected by UB10 and

UB11 represent new gene products since it is possible that these MAbs are detecting new carbohydrate epitopes of pre-existing antigens introduced through post-translational modifications in glycosylation.

At this stage we do not know the function of any of the novel antigens expressed in the HCs. The current programme is therefore directed towards this aspect. MAbs against protein epitopes (e.g. UB8) will be used for immunoscreening of a PCR-based cDNA expression library prepared from infected epidermal strips, providing direct access to the gene sequence. In other cases, where the antibody is against a carbohydrate epitope, a more conventional biochemical and immunochemical approach to antigen isolation will be adopted followed by microsequencing and construction of DNA probes that can then be used to screen the expression library. The identity of the gene products and their temporal and cellular expression will be studied.

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