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Review

Research review

Rust haustoria: nutrient uptake and beyond

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Summary

Haustoria are morphological features of an extremely successful class of plant parasites, the obligate biotrophs. The broad phylogenetic spectrum of organisms producing haustoria suggests that these structures have arisen many times in the course of evolution and represent specific adaptations of these organisms to the close interaction with their respective host plants. This close interaction and the fact that these structures cannot be produced *in vitro* have hampered an analysis of the roles of haustoria in biotrophy for many decades. Only recently has it become possible to analyse haustorial function at a molecular level. A picture is beginning to emerge indicating that haustoria do not only serve in nutrient uptake – a task postulated for these elements ever since their discovery. Moreover, they seem to perform enormous biosynthetic duties. They also seem to be engaged in the suppression of host defense responses and in redirecting or reprogramming the host's metabolic flow. This review intends to summarize current knowledge about the structure and function especially of rust haustoria.

Introduction

Many representatives of the fungal kingdom form partners in relationships with plants. The spectrum of associations is broad and extends from mutualistic symbiosis to parasitism. There are first of all the arbuscular- and the ecto-mycorrhizal fungi associated with almost all terrestrial plants beneficial for both partners (Harrison, 1999; Wiemken & Boller, 2002). This group is followed by endosymbionts, which coexist with their respective host plants virtually without apparent symptoms or benefits (Saikkonen *et al.*, 1998). Last but not least there is a vast number of plant parasitic fungi, many of which can cause devastating disease on wild and crop plants (Oerke & Dehne, 1997).

Some of the most serious fungal plant pathogens worldwide are obligate biotrophic parasites (Brown & Hovmoller, 2002). This term characterizes a specific lifestyle in which the host as a whole suffers only minor damage over a longer period of time, since the pathogen is dependent on the living host plant to complete its life cycle (Staples, 2000). This form of parasitism stands in sharp contrast to necrotrophic parasites, which kill their hosts quickly after infection and subsequently thrive on the dead plant material (Staples, 2001). Hemibiotrophic fungi, like *Colletotrichum* spp., are typified by a more or less extended biotrophic phase, before switching to necrotrophic growth and killing of their host. In order to mark off the true obligate biotrophic fungi from hemibiotrophs or necrotrophs we have suggested the following criteria:

(a) highly differentiated infection structures; (b) limited secretory activity; (c) a narrow contact zones separating fungal and plant plasma membranes; (d) long-term suppression of host defense responses; (e) the formation of haustoria (Mendgen & Hahn, 2002).

These haustoria especially have generated the interest of plant pathologists ever since their discovery by Zanardini about 150 years ago (von Mohl, 1853). However, knowledge about this key element of the obligate biotrophic lifestyle is still fairly scarce. The main reasons for this are the preserving lack of functional transformation systems for haustoria forming fungi and the fact that these organs are not formed in culture. This excludes them from the application of many molecular techniques successfully in use for other systems. *Claviceps purpurea*, *Ustilago maydis* and several hemibiotrophs on the other hand are well-studied and amenable to modern molecular tools. However, molecular studies of these organisms can only give limited evidence about the processes and types of interactions involved in a true obligate biotrophic relationship, since haustoria are not formed in these systems. More detailed studies regarding the highly specialized obligate biotrophic pathogens are therefore urgently needed. This review aims to give an overview of the current knowledge about one of the key elements of biotrophy, the haustorium, with special emphasis on current research involving rust haustoria.

Haustoria

Haustoria are one of the hallmarks of true obligate biotrophic fungi. Yet, looking at the broad phylogenetic spectrum of haustoria-forming organisms – they comprise the downey mildews (Oomycota), powdery mildew (Ascomycota) and the rust fungi (Basidiomycota) – it seems more than likely that these structures have arisen more than once in the course of evolution (Hahn *et al.*, 1997a). Extending this idea to the structurally similar arbuscules produced by arbuscular mycorrhizas, such an apparatus seems to represent a particularly successful adaptation of these organisms to their interactions with their respective host plants.

Whereas initial infection structures of rust fungi may be produced *in vitro*, haustoria are only formed *in planta* (Deising *et al.*, 1991). This makes it extremely difficult to analyse processes involving these structures at a molecular level. Although axenic cultures have been established for some biotrophs (Mendgen & Hahn, 2002), most of the economically important biotrophic parasites remain nonculturable, at least not to a point equivalent to the biotrophic phase. Studies by Heath (1990) have indicated that it is not a mere lack of specific nutrients that prevent haustoria being formed *in vitro*. Rather one or more signals from the host are missing in culture either to induce, or to complete the differentiation of haustoria *in vitro*.

Haustoria may be produced by mono- or dikaryotic mycelium of rust fungi. Monokaryotic haustoria (M-haustoria) merely appear as intracellular extensions of intercellular hyphae with no significant morphological specialization (Gold & Mendgen, 1991). Dikaryotic haustoria develop from external haustorial mother cells (HMC) with a slender neck that penetrates into the host cell and a haustorial body that forms distally to the neck (Heath & Skalamera, 1997). The HMC therefore functionally resembles an appressorium. However, it remains to be elucidated if the functional similarity extends to the molecular level.

The expanding haustorium invaginates the host plasma membrane and new membrane is probably synthesized. Therefore, haustoria are not truly intracellular. They remain outside the physiological barrier of the host cell. Recent research concentrated on the dikaryotic stage of rust haustoria and for that reason, if not stated otherwise, we will concentrate on this more differentiated stage of these structures.

With the formation of the haustorial body a zone of separation between the plasma membranes of host and parasite is established. It consists of the fungal cell wall and the extrahaustorial matrix (Hahn *et al.*, 1997a). The extrahaustorial matrix resembles an amorphous mixture of components, mainly carbohydrates and proteins, partly of fungal but primarily of plant origin (Harder & Chong, 1991). The initial biotrophic phase of hemibiotrophs like *Colletotrichum lindemuthianum* is also characterized by the presence of an interfacial matrix separating host and parasite plasma membranes (Perfect & Green, 2001). Upon the switch to necrotrophic growth the host plasma membrane surrounding the hyphae disintegrates and parasitic growth continues with narrower unsheathed hyphae. It therefore seems likely that this zone of separation plays an important role in the maintenance of the biotrophic lifestyle. Undoubtedly the extrahaustorial matrix represents a formidable trading place for the exchange of nutrients and information between the host and the fungus (Heath & Skalamera, 1997). Recent studies by Mims and coworkers (Mims *et al.*, 2002) on *Puccinia hemerocallidinis* have shown long tubular extensions contiguous with the extrahaustorial matrix. These structures reach far into the host cytoplasm and exhibit vesicle-like bodies unbinding at their tip. However, it remains to be shown if there is any kind of trafficking linked to these structures.

There is some evidence that the cytoplasmic membrane of the host enclosing the haustorial body, the so-called extrahaustorial membrane, is modified and therefore no longer resembles a conventional plant plasma membrane. Harder & Chong (1991) summarized results obtained by freeze fracture electron microscopy and other cytological methods showing that the extrahaustorial membrane lacks intramembranous particles. Cytochemical studies by Gay (Gay *et al.*, 1987) and Manners (1989) with powdery mildew haustoria and later work by Baka (Baka *et al.*, 1995) on rust haustoria suggested that the extrahaustorial membrane lacks ATPase activity. This implies that there would be no control over solute fluxes from the host cell. Yet the technique used in these studies has

been under considerable debate (Novikoff, 1970; Katz *et al.*, 1988). Immunolocalization studies are therefore still urgently needed to clarify the energization state of the extrahaustorial membrane.

The neck region of the haustorium is characterized by some electron-dense material, seemingly joining the two plasma membranes of host and parasite (Harder & Chong, 1984). It is assumed that this 'neckband' seals the extrahaustorial matrix against the bulk apoplast, not unlike the Casparian strip in the endodermis (Heath, 1976). Based on the sealing by the neckband and the presence of the plant plasma membrane surrounding the whole structure, Heath & Skalamera (1997) suggested that the extrahaustorial matrix might be considered a symplastic compartment. However, it might also be regarded as a highly specialized portion of the apoplast, providing conditions different from those present in the bulk apoplast.

Very little is known about the composition and structure of the fungal cell wall in haustoria. There is recent work on the modification of the cell wall in plant parasitic fungi after the invasion of the host (El Gueddari *et al.*, 2002). The authors suggest the alterations of cell wall composition as a means of the fungus to protect itself from enzymatic hydrolysis in the course of plant defense responses. However, this work does not extend to the analysis of haustoria.

Nutrient uptake by rust haustoria

Already in naming these structures [fr. L. haustor: pail] de Bary (1863) proposed one of the possible functions for haustoria – the uptake of nutrients from the host. However, until recently there was evidence for an involvement of haustoria in nutrient uptake for powdery mildew fungi only (Hall & Williams, 2000). Yet, for rust fungi, which grow inter- and intracellularly, the situation might be quite different from ectoparasites like the powdery mildew fungi.

Analysis of the potential role of rust haustoria in nutrient uptake has been hampered by the fact that haustoria are exclusively formed *in planta* and that their isolation encountered numerous problems (Bushnell, 1972). As a result, haustoria have mostly been studied by cytological techniques (Harder & Chong, 1991). Aside from these microscopic methods there were a number of attempts to use feeding experiments to elucidate a potential role of haustoria in nutrient uptake. Martin & Ellingboe (1978) used ^{32}P-labelled substances and Manners & Gay (1982) employed $^{14}CO_2$ to analyse substrate translocation in *Erysiphales*, while our group applied ^{3}H-labelled amino acids in *Uredinales* (Mendgen, 1979; Mendgen, 1981). Most experiments gave indirect evidence for a role of haustoria in nutrient uptake without providing a conclusive proof.

The introduction of molecular biology into the field of phytopathology, however, opened a new dimension to investigate the role(s) of haustoria. Results from our group revealed an increased plasma membrane H^+-ATPase activity for haustorial membranes compared with membranes from other fungal structures (Struck *et al.*, 1996; Struck *et al.*, 1998). The proton gradient generated by this ATPase was suggested to drive secondary active transport systems engaged in nutrient uptake by the parasite (Hahn *et al.*, 1997a). Later work identified a number of *in planta i*nduced *g*enes, termed *PIG*s, that were preferentially expressed in haustoria (Hahn & Mendgen, 1997). Among these genes were putative secondary transporters for amino acids (Hahn & Mendgen, 1997; Hahn *et al.*, 1997b). These findings strengthened the potential role of rust haustoria in nutrient uptake (Hahn *et al.*, 1997a). However, while an exclusive localization of AAT2p in haustoria could be shown, no transport activity could be assigned to this protein (Mendgen *et al.*, 2000). AAT1p, a close homologue of AAT2p, however, was recently characterized (Struck *et al.*, 2002). AAT1p represents a broad specificity amino acid transporter with a main specifi-city for L-histidine and L-lysine. Yet a defined localization of the carrier was not successful so far. However, Northern Blot data indicate that expression of *AAT1* is not limited to haustoria. The data for these two transporters taken together might be taken as evidence that bulk amino acid uptake at least in *Uromyces fabae* proceeds via haustoria but also to an unknown extend via intercellular hyphae (Fig. 1). Again, further immunological studies are needed to solve this question.

The situation for the uptake of carbohydrates on the other hand seems to be clear cut. Sugar uptake in *U. fabae* seems to proceed solely via haustoria (Voegele *et al.*, 2001b). Using immunofluorescence microscopy the hexose transporter HXT1p was localized preferentially at the tip of the differentiating monokaryotic haustorium (Fig. 2a,b). No specific labelling was found in intercellular hyphae. In the case of dikaryotic haustoria the haustorial body was labelled (Fig. 2c). Labelling usually decreased towards the neck region and no labelling was detected in intercellular hyphae. Results obtained with two independent sets of purified polyclonal HXT1p-specific antibodies were very similar (this work and Voegele *et al.*, 2001b). In addition, immunocytological analysis of *Medicago truncatula* leaves infected with *Uromyces striatus* using the *U. fabae*-HXT1p-specific antibodies gave a picture similar to dikaryotic haustoria of *U. fabae* (Fig. 2d). Therefore, these antibodies may be used to analyse the localization of hexose transporters in related systems. While HXT1p homologues seem to exist in closely related species, there does not seem to be a redundancy of monosaccharide transporters in *U. fabae* itself. Neither nested PCR using three different independent sets of degenerated primers, nor genomic Southern Blot analysis under low stringency conditions yielded evidence for additional hexose transporters present in *U. fabae* in any of the developmental stages tested (Voegele *et al.*, 2001b).

HXT1p was completely characterized on a biochemical level by heterologous expression of *HXT1* in *Saccharomyces*

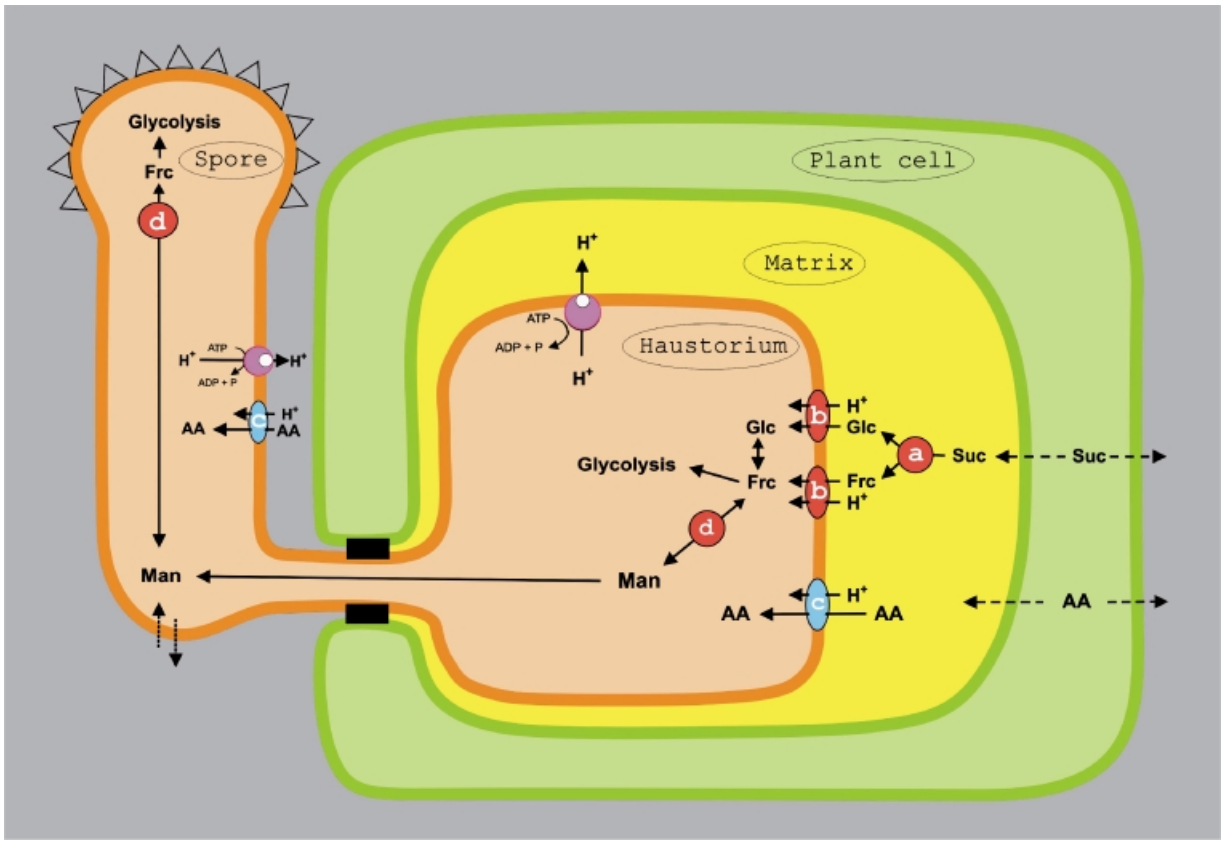

Fig. 1 Model for amino acid and hexose uptake and redistribution in rust fungi. Depicted is a schematic representation of a fungal spore, an intercellular hypha, and an haustorium (orange), an infected plant cell (green), and the interphase, the extrahaustorial matrix (yellow). The neckband is indicated by two black rectangles. (a) invertase INV1p; (b) hexose transporter HXT1p; (c) amino acid transporters AAT1p and AAT2p; (d) major alcohol dehydrogenase MAD1p; Glc: D-glucose; Frc: D-fructose; Man: D-mannitol; Suc, sucrose; AA: amino acids. Solid arrows specify confirmed enzymatic conversions or transport processes, dotted arrows indicate postulated solute fluxes.

cerevisiae and oocytes of *Xenopus laevis*. The data revealed that HXT1p is a proton-motive force driven monosaccharide transport system. Specificity was found for D-glucose, 2-deoxy-D-glucose, D-fructose and D-mannose, with increasing K_M-values in this order (Voegele *et al.*, 2001b). The substrate spectrum closely resembles the one determined for the closest characterized homologue of HXT1p, the *Amanita muscaria* monosaccharide transporter AmMst1p (Wiese *et al.*, 2000). However, there is a striking difference with respect to the K_M-values for D-fructose. AmMst1p exhibits a K_M for D-fructose of 4.2 mM, about a factor of 10 higher than the D-glucose K_M (0.46 mM) (Wiese *et al.*, 2000). As shown in Fig. 3 the K_M values of HXT1p for D-glucose and D-fructose are positioned much closer together (0.36 mM, and 1.0 mM, respectively) (Voegele *et al.*, 2001b). This difference might reflect specific adaptations of the fungi to their immediate environment. Whereas *U. fabae* is a haustoria-forming plant parasite, *A. muscaria* is an ectomycorrhizal fungus. The preferential use of D-glucose by AmMST1p would rapidly deplete the Hartig net of this metabolite. However, the preferential use of D-glucose would at the same time cause the fructose concentration to rise above the inductive threshold value. This would create slightly offset gradients for both hexoses. The different K_M values of AmMST1p for the two hexoses would therefore be an important factor for the induction of *AmMST1* and hence the effective utilization of both hexoses along the path from the Hartig net to the inner layers of the sheath (Nehls *et al.*, 2001). With the extrahaustorial matrix being sealed off against the bulk apoplast no such continuous spatial gradients are possible in haustoria-forming fungi. In order to prevent accumulation of D-fructose in the matrix it would have to be taken up with almost equal efficiency as D-glucose. We conclude that the two highly similar systems nevertheless show specific adaptation to their special needs.

Overall a picture is starting to emerge that indicates that rust fungi make use of several strategies to cover their nutritional demands (Fig. 1). Uptake of amino acids seems to occur via haustoria and also via intercellular hyphae. Uptake of carbohydrates on the other hand seems to be limited to haustoria. Substrate translocation is executed by secondary active transport systems which allow direct coupling of transport to the proton gradient established by the H^+-ATPase.

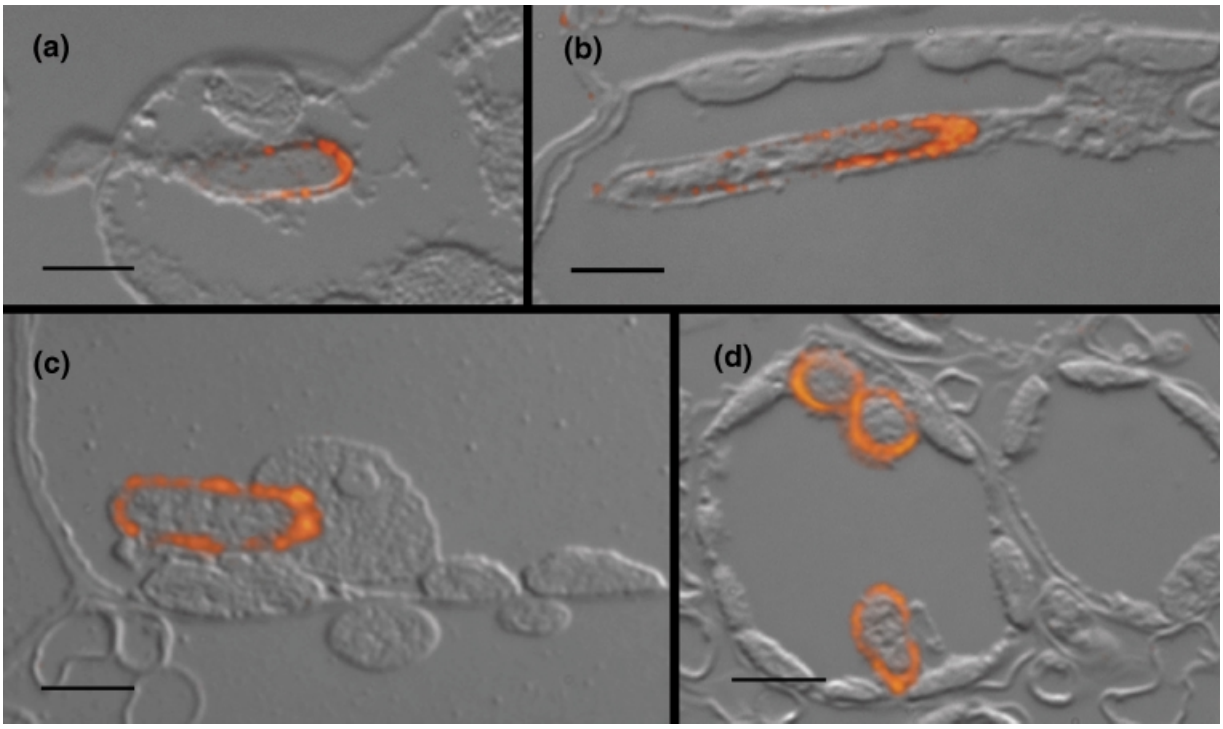

Fig. 2 Early (a) and late (b) stages of differentiation of a monokaryotic *U. fabae* haustorium. HXT1p shows accumulation at the tip of the developing haustorium and remains concentrated in the distal part of the fully differentiated haustorium. In dikaryotic haustoria of *U. fabae*, the haustorial body is labelled with anti-HXT1p-specific antibodies (c). A similar picture is observed with dikaryotic haustoria of *U. striatus* (d). Images obtained with Nomarski differential interference contrast and Cy3-fluorescence using the purified HXT1p-specific antibody S650p are superimposed. Bar: 5 μm.

Such energization is particularly useful in haustoria if the extrahaustorial membrane is indeed de-energized. However, such systems would also work rather efficiently in the bulk apoplast. In any case, accumulation factors and low K_M values of these transporters would ensure a good position of the parasite in the competition with the host for scarce nutrients.

Where do the hexoses come from and where do they go to?

Elucidating the mechanism and specificity of carbohydrate uptake in *U. fabae* provided an important advance in understanding the biotrophic relationship between host and parasite (Szabo & Bushnell, 2001). However, the findings regarding substrate specificity and energization of hexose uptake also posed some new challenging questions.

One of the most important questions to address is: 'Where does the hexose transporter obtain its substrates from?'. It has been shown that the levels of free hexoses (mainly D-glucose and D-fructose) in *Vicia faba* are rather low (Lohaus *et al.*, 2001). A carbohydrate that is present in abundance in most plants, however, is the disaccharide sucrose (Farrar, 1985). Invertase, the enzyme responsible for the cleavage of sucrose into D-glucose and D-fructose, is also present in all plants analysed (Tymowska-Lalanne & Kreis, 1998). Invertases come in

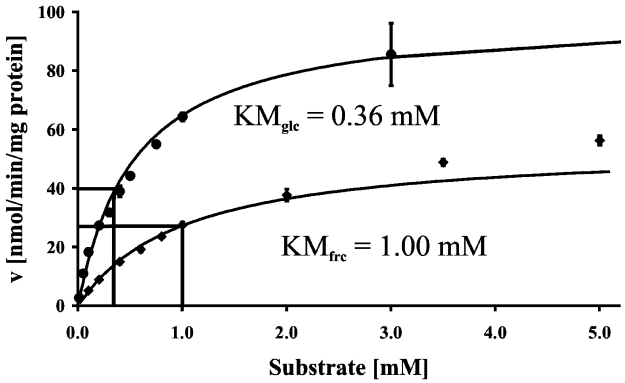

Fig. 3 Michaelis-Menten graphs for HXT1p with D-glucose (solid circle) and D-fructose (solid diamond) as substrate. The respective KM values extrapolated from linear transformations of the Michaelis-Menten plot are indicated. For details of the experiment see Voegele *et al*. (2001b).

different isoforms with different physiological and biochemical properties and different subcellular localizations. It has been suggested that these enzymes serve as key steps in carbon partitioning in higher plants, their activity basically determining the sink strength of a plant organ (Sturm, 1999). It is also accepted that wounding or pathogen attack generally convert

source tissue into sink tissue and this can be correlated with an increased invertase activity (Long *et al.*, 1975). However, it seems unlikely that a pathogen would rely solely on the action of a host enzyme to obtain its nutrients. Indeed, there have been a number of reports on invertases in fungal plant pathogens in general (Ruffner *et al.*, 1992; Ruiz & Ruffner, 2002), and on increased invertase activity in the case of biotrophic interactions in particular (Long *et al.*, 1975; Heisterüber *et al.*, 1994). However, in the latter case there was no conclusive proof for the activity increase being due to a fungal contribution (Farrar, 1985). We have now isolated a gene from *U. fabae* with homology to fungal and plant invertases and are currently investigating its biochemical characteristics and subcellular localization (Voegele *et al.*, 2001a). Our goal is to dissect the contribution of plant and fungal invertases to nutrient uptake by the fungus during the biotrophic stage.

Another aspect of interest is the fate of carbohydrates once they are taken up by the fungus. From an EST sequencing project involving a haustorial cDNA library we have evidence that major components of glycolysis and the pentose phosphate cycle are present in haustoria (M. Hahn *et al.* manuscript in preparation). It is therefore likely that dissimilation of the hexoses in haustoria proceeds via these normal pathways. However, substrate specificity and energization of hexose transporter HXT1p might pose a problem for the fungus. Concentrative uptake of free hexoses by secondary active transport systems may well exceed the capacity of subsequent enzymatic reactions. This could result in *trans*-inhibition of the uptake process, and/or the accumulation of these substances to toxic levels.

We have identified a *M*ajor *A*lcohol *D*ehydrogenase, MAD1p, in *Uromyces fabae* with a specificity for mannitol (R. T. Voegele *et al.*, manuscript in preparation). Mannitol is a C6-polyol derived from D-fructose that has been found as a major metabolite in a variety of fungi (Jennings, 1984). A role as a mobile carbon storage compound, in osmoprotection, in interconversion of reduction equivalents, and lately as radical scavenger has been proposed (Jennings, 1984; Jennings *et al.*, 1998). MAD1p was localized in the lumen of haustoria and to a lesser extend in the lumen of uredospores. The enzymatic characteristics indicate that the enzyme acts as a D-fructose reductase under the physiological conditions within a haustorium (high D-fructose level, high NADPH level, ambient pH). The enzyme might therefore act as a bypass valve and thus could be a key regulatory component of carbon flow. Under conditions of low D-fructose – and high mannitol concentrations, as determined for example in spores – the enzyme is able to re-convert mannitol to D-fructose to make it available for instant metabolism (Fig. 1) (R. T. Voegele *et al.*, manuscript in preparation). Mannitol in *U. fabae* therefore has to be considered a major mobile carbohydrate storage compound. However, we also have evidence that mannitol is released from the fungal mycelium into the apoplast (R. T. Voegele *et al.*, manuscript in preparation). There is growing evidence from mammalian – (Chaturvedi *et al.*, 1996) and lately from plant pathosystems (Jennings *et al.*, 2002) that mannitol can be effectively used by a pathogen to suppress host defense responses involving reactive oxygen species. MAD1p would therefore directly link nutrient acquisition and suppression of host defense responses in rust fungi.

Suppression of host defenses and influence on host metabolism

The use of mannitol as a radical scavenger to combat reactive oxygen species generated or released by the plant is one possible method of suppressing host defense responses. However, there are also other means of the fungus to deal with its host. Haustoria from related rust fungi revealed unique structural modifications for each species (Berndt & Oberwinckler, 1997). This specificity might be transduced to the host cell: Oat plants infected with *Puccinia graminis* develop extrahaustorial membranes with short tubular extensions, whereas after infection with *Puccinia coronata* they form long and narrow extensions (Harder & Chong, 1991). These observations suggest that formation of the fine structure of the haustorial host–parasite interface is under the control of species-specific signals from the fungus. Such signals may include suppressors, which have been implicated in maintaining basic compatibility between the parasite and its host plants (Bushnell & Rowell, 1981). Evidence comes from a phenomenon called induced susceptibility. French bean tissue already infected by *Uromyces vignae* supported additional infections by several nonhost pathogens (Fernandez & Heath, 1991). In the same way, haustoria of *Blumeria graminis* can induce such susceptibility (Lyngkjaer & Carver, 1999). However, experimental evidence for suppressors from these fungi is still weak and it is also still unclear if these effects can be linked to haustoria.

The fungal pathogen may also directly or indirectly influence host metabolism and nutrient flow. A recent study by Ayliffe and coworkers (Ayliffe *et al.*, 2002) has shown that a Δ1-pyrroline-5-carboxylate (P5C) dehydrogenase activity in flax is highly induced by the compatible rust fungus *Melampsora lini*. Induction was limited to infected and immediately surrounding leaf mesophyll cells and induction was not seen in incompatible interactions. The authors indicate a possible role in detoxification of the toxic metabolite P5C. Expression analyses of *Vicia faba* genes in response to attack by *U. fabae* have been performed by Wirsel *et al.* (2001). Several of the analysed genes showed altered expression pattern in the infected organ as expected. However, some of the analysed genes also showed alterations in expression in far remote organs, such as stem and roots. This work clearly shows that influence on host metabolism by a leaf pathogen is not limited to the infected organ alone. But again, it remains to be shown that at least some of these effects can be linked to the function of haustoria.

Biosynthesis

Not very much is known about the spectrum of biosynthetic reactions occurring in haustoria. However, two of the most abundant genes isolated from a haustorial cDNA library code for enzymes directly involved in the synthesis of Vitamin B1 (Hahn & Mendgen, 1997). *THI1* and *THI2* (formerly *PIG1* and *PIG4*, respectively) together make up about 5% of the total transcripts in haustoria. Thiamine diphosphate (Vitamin B1) is a cofactor required for the activity of several enzymes of the central carbon metabolism, such as pyruvate dehydrogenase, pyruvate decarboxylase, α-ketoglutarate decarboxylase and transketolase (Sohn *et al.*, 2000). Therefore haustoria can not only be viewed as nutrient uptake devices, but also have to be considered as power plants providing essential nutrients though *de novo* synthesis.

Conclusion

Recent research has proven that haustoria are indeed nutrient uptake devices, as suspected ever since their discovery (Voegele *et al.*, 2001b). However, we are also starting to see new facets of haustoria. There is increasing evidence that haustoria are involved in biosynthetic pathways, the suppression of host defenses and in redirecting the metabolic flow of the host. More molecular work on these immensely intricate structures is urgently needed to fully understand their contribution to the obligate biotrophic lifestyle. This will not only further our basic understanding of the mechanism involved in biotrophy. Utilizing plant promoters specifically induced in infected cells as suggested by Ayliffe *et al.* (2002) might be one direct application for basic research on biotrophic fungi. Another line was taken by Jennings and coworkers (Jennings *et al.*, 2002). The authors used expression of a mannitol dehydrogenase in transgenic tobacco to reduce the level of this polyol produced by the pathogen *Alternaria alternata*. Understanding the influence of a pathogen on host metabolism might be another means of aiming at cultivars exhibiting a higher degree of resistance to pathogens. Generation of transgenic plants could for example exchange components subject to pathogen influence with homologues that do not exhibit such sensitivity.

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