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Nutrient uptake in rust fungi: how sweet is parasitic life?

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Abstract A better understanding of the fundamental principles of host-pathogen interactions should enable us to develop new strategies to control disease and to eliminate or at least manage their causative agents. This is especially true for obligate biotrophic parasites like the rust fungi. One vital aspect in the field of obligate biotrophic host-pathogen interactions is the mobilization, acquisition and metabolism of nutrients by the pathogen. This includes transporters necessary for the uptake of nutrients as well as enzymes necessary for their mobilization and metabolism. In a broader sense effector molecules reprogramming the host or triggering the infected cell into metabolic shifts favorable for the pathogen also play an important role in pathogen alimentation.

Keywords Nutrient acquisition · Nutrient utilization · Nutrient uptake · Obligate biotrophs

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Introduction

On a global scale, some of the most serious fungal plant pathogens are *obligate biotrophic* parasites (Brown and Hovmøller 2002). The term obligate biotroph characterizes a specific lifestyle in which the host suffers only minor damage. The pathogen in turn is dependent on the living host plant to complete its life cycle (Staples 2000). This form of parasitism stands in contrast to necrotrophic parasites, which quickly kill their hosts (Staples 2001). Hemibiotrophic fungi, like *Colletotrichum* spp., are characterized by a more or less extended biotrophic phase, before switching to necrotrophic growth (Perfect and Green 2001).

In order to distinguish true obligate biotrophic fungi from hemibiotrophs or necrotrophs the following six criteria were suggested: 1. Obligate biotrophs are not culturable in vitro (at least not to a point representing the parasitic phase), 2. They form highly differentiated infection structures (variations of the normally tubular cell shape, which are necessary for pathogenesis), 3. They have limited secretory activity, 4. They establish a narrow contact zone separating fungal and plant plasma membranes, 5. They engage in a long-term suppression of host defense responses, 6. They form haustoria (specialized hyphae that penetrate host cells) (Voegele et al. 2009). According to these criteria the range of organisms designated as true obligate biotrophs comprises the downy mildews (Oomycota), the powdery mildews (Ascomycota) and

the rusts (Basidiomycota). Looking at this broad phylogenetic spectrum it seems more than likely that the obligate biotrophic lifestyle has arisen more than once in the course of evolution (Hahn et al. 1997a). Therefore, it does not seem appropriate to restrict the analysis of the molecular basis of this type of host-parasite interaction on members of a single phylum. While some progress has been made especially in the analysis of powdery mildew–plant interactions (Zhang et al. 2005), the analysis of other obligate biotrophic systems is lagging behind.

The haustorium

The haustorium represents one of the hallmarks of obligate biotrophic parasites. These structures have generated the interest of plant pathologists ever since their first description about 160 years ago (von Mohl 1853). Already in naming these structures [fr. L. haurire (haurio, hausi, haustum): to drink, to draw] de Bary (1863) proposed a possible function for haustoria—the uptake of nutrients.

The dikaryotic rust haustorium develops from the haustorial mother cell with a slender neck and a haustorial body (Heath and Skalamera 1997). During formation of the haustorium the cell wall of the host cell is breached. The expanding haustorium invaginates the host plasma membrane and new membrane is synthesized. Therefore, the haustorium is not truly intracellular (Fig. 1).

With the development of the haustorial body a zone of separation between the plasma membranes of parasite and host is formed. It is composed of the fungal cell wall and the extrahaustorial matrix (Hahn et al. 1997a). The extrahaustorial matrix resembles an amorphous mixture of mainly carbohydrates and proteins, partly of fungal but primarily of plant origin (Harder and Chong 1991). It seems likely that this zone of separation plays an important role in maintaining the biotrophic lifestyle. This hypothesis is supported by the cytological analysis of hemibiotrophic parasites. The initial biotrophic phase of hemibiotrophs is also characterized by the presence of a narrow contact zone between host and parasite (Mendgen and Hahn 2002; Perfect and Green 2001). Upon the switch to necrotrophic growth the host plasma membrane surrounding the hyphae disintegrates and parasitic growth continues with narrower unsheathed hyphae. Undoubtedly the extrahaustorial matrix represents a formidable trading place for the exchange of nutrients and information (Heath and

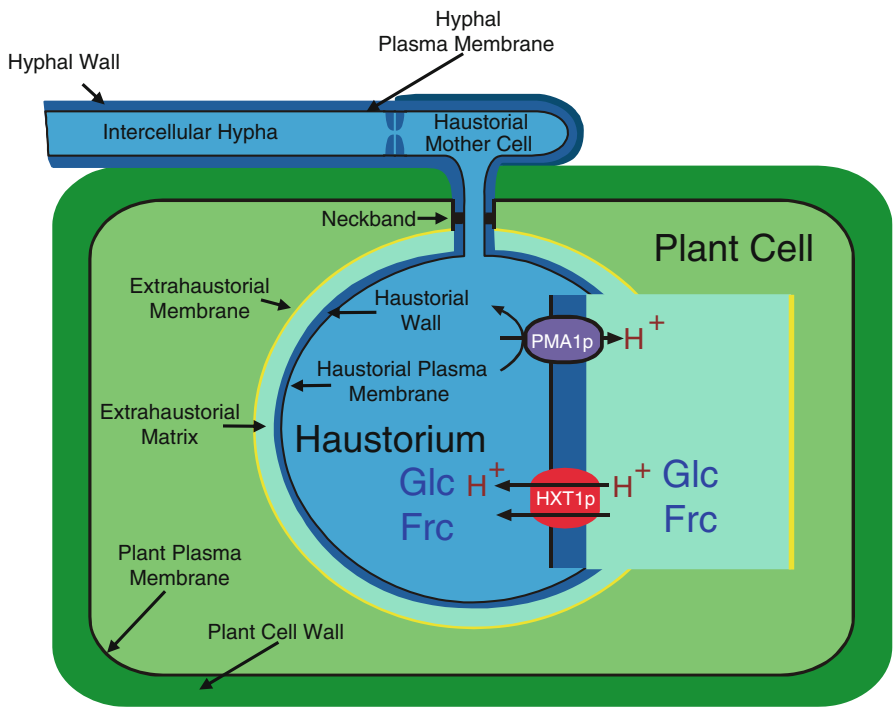

Fig. 1 Schematic representation of a dikaryotic rust haustorium. Structures derived from the fungus are depicted in *blue*, structures contributed by the plant are shown in *green*. The extrahaustorial matrix is shown in *light blue* and the extrahaustorial membrane in *yellow*. Drawing from Voegele (2006), with modifications

Skalamera 1997). In a study on *Puccinia hemerocallidis* Mims et al. (2002) showed long tubular extensions contiguous with the extrahaustorial matrix. Similar structures were already described by Stark-Urnau and Mendgen (1995) for monokaryotic haustoria of *Uromyces vignae*. These structures reach far into the host cytoplasm and exhibit vesicle-like bodies at their tip. However, it still remains to be shown if there is any kind of trafficking linked to these structures.

There is 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 and Chong (1991) summarized results obtained by freeze fracture electron microscopy with bean rust and oat crown rust. In both interactions the extrahaustorial membrane lacks intramembranous particles, and exhibits a dramatic reduction of sterols (Harder and Mendgen 1982). Cytochemical studies on powdery mildew haustoria (Gay et al. 1987; Manners 1989) and later work by 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. Further support for a modification of the extrahaustorial membrane comes from recent results obtained with GFP-tagged membrane proteins in the pathosystem *Erysiphe cichoracearum/Arabidopsis thaliana* (Koh et al. 2005). In this study eight different plasma membrane markers were excluded from the extrahaustorial membrane and accumulated in a collar-like formation around the haustorial neck. This neck region is characterized by electron-dense material, apparently joining the two plasma membranes (Harder and Chong 1984). This "neckband" seals the extrahaustorial matrix against the bulk apoplast (Heath 1976). Based on the sealing by the neckband and the presence of the plant plasma membrane surrounding the whole structure it was suggested that the extrahaustorial matrix should be considered a symplastic compartment (Heath and Skalamera 1997). However, it might also be regarded as a highly specialized portion of the apoplast, providing conditions different from those present in the bulk apoplast.

Analyses of the potential role(s) of rust haustoria were hampered by the fact that haustoria are exclusively formed *in planta* and that their isolation encountered numerous problems (Bushnell 1972). As a result, haustoria were mostly studied using cytological techniques (Harder and Chong 1991). The introduction of molecular biology into the field of phytopathology opened a new dimension to investigate the role(s) of haustoria. A picture is beginning to emerge indicating that haustoria do not only serve in nutrient uptake. In fact, they seem to perform biosynthetic duties and are thought to be engaged in the suppression of host defense responses and in redirecting and/or reprogramming the host's metabolic flow.

The dawn of a new era

Because haustoria are only formed *in planta* and cannot be produced in axenic culture, there have been a number of attempts to establish protocols for the isolation of haustoria. A procedure developed to isolate haustoria of the powdery mildew fungus *Erysiphe pisi* involved sucrose density gradient centrifugation (Gil and Gay 1977). However, this method proved too laborious and inefficient when applied to rust fungi (Cantrill and Deverall 1993; Tiburzy et al. 1992). A milestone in the research involving rust haustoria was the introduction of a chromatographic method to isolate haustoria by Hahn and Mendgen (1992). The method is based on a selective binding of oligosaccharides present in the haustorial wall to immobilized ConcanavalinA. Repeated cycling of cell extracts of infected broad bean leaves yielded considerable quantities of highly pure haustoria.

Of rusts, beans, and *PIG*s

One of the first publications arising from molecular work on rust haustoria based on this technique was the paper by Hahn and Mendgen (1997) on the characterization of 31 *in planta*-induced rust genes isolated from a haustorium-specific cDNA library. These *in Planta*-Induced Genes, or *PIG*s, show exclusive or at least preferential expression in rust haustoria compared to their expression in other, earlier infection structures. This in turn was taken as evidence for a role of the corresponding gene products in structure and/or function of haustoria.

An interesting finding was that homologs for only about one third of the genes could be found. Among the sequences for which a function could tentatively be assigned were two genes encoding amino acid permeases. This result seemed to corroborate the long standing hypothesis that rust haustoria are responsible for nutrient acquisition. However, the two most highly expressed genes in haustoria were found to encode components of vitamin B1 biosynthesis. It can therefore be concluded that besides their role in nutrient acquisition, haustoria also fulfill vital biosynthetic functions. Another interesting finding was the fact that the remaining two thirds of the *PIG*s did not exhibit homology to known genes. This was taken as evidence that at least some of these genes might be linked to virulence or pathogenicity of rust fungi in general or *U. fabae* in particular. The initial screen for *PIG*s by Hahn and Mendgen (1997) was recently followed up by Jakupovic et al. (2006). Using an EST sequencing project and microarray hybridization the authors concluded that in rust fungi a strong shift in gene expression takes place between germination and the biotrophic stage characterized by haustoria.

Rusty power plants

As shown by Hahn and Mendgen (1997) in the rust fungus *U. fabae* the switch from early stages of host plant invasion to parasitic growth is accompanied by the activation of a number of *PIG*s. Two of them, *THI1* (former designation *PIG1*) and *THI2* (*PIG4*), were highly expressed in haustoria (Sohn et al. 2000). We showed that transcripts of both genes together make up more than 5% of the total haustorial mRNA (Sohn et al. 2000). The genes exhibit homology to genes involved in thiamin biosynthesis. Based on these homologies, THI1p is likely to be involved in the synthesis of the pyrimidine moiety, whereas THI2p seems to participate in the synthesis of the thiazol moiety of thiamin. Their functional identities were confirmed by complementation of *Schizosaccharomyces pombe* thiamin auxotrophic mutants. In contrast to thiamin biosynthesis genes of other fungi that are completely suppressed by thiamin, *THI1* and *THI2* expression was not affected by the addition of thiamin to rust hyphae either in vitro or *in planta*. Western blot analysis revealed decreasing amounts of THI1p in extracts of spores, germlings, and in vitro grown infection structures. Immunofluorescence microscopy of rust-infected leaves detected high concentrations of THI1p in haustoria, whereas only low amounts were found in intercellular hyphae. In sporulating mycelium, THI1p was found in the basal hyphae of the uredinia, but not in the pedicels and only at very low levels in urediniospores. These results indicate that the haustorium is an essential structure of the biotrophic rust mycelium for the biosynthesis of metabolites such as thiamin. Therefore, haustoria can be considered power plants providing essential nutrients through de novo synthesis.

A function for the name

Already in naming these structures de Bary (1863) suggested the uptake of nutrients from the host as a possible function of haustoria. Earlier attempts to elucidate a potential role of haustoria in nutrient acquisition for the parasite mainly involved feeding experiments. Martin and Ellingboe (1978) employed ^{32}P-labeled substances and Manners and Gay (1982) used $^{14}CO_2$ to analyze substrate translocation in members of the *Erysiphales*, while Mendgen (1979, 1981) applied ^{3}H-labeled amino acids using *Uromyces* spp. These experiments gave indirect evidence for a role of haustoria in nutrient uptake without providing conclusive proof.

Employing the haustoria isolation protocol developed by Hahn and Mendgen (1992), Struck et al. (1996) showed a strong increase in the activity of a plasma membrane H^+-ATPase [EC 3.6.1.35] in haustoria compared to other fungal structures. A detailed characterization of the *PMA1* gene and the corresponding gene product PMA1p followed 2 years later (Struck et al. 1998). In contrast to the increased PMA1p activity in microsomal vesicles derived from haustoria, only reduced amounts of the corresponding transcripts were found in haustorial preparations. Analysis of wild type enzyme and a C-terminal deletion mutant in a heterologous expression system indicated a role of the C-terminus in auto-regulation of the enzyme. The observed net activity increase could be explained on the basis of a strong biochemical auto-activation paired with the decrease in mRNA level. These results suggested that the electrochemical gradient generated by the H^+-ATPase of haustoria plays an important role in their function,

possibly by promoting nutrient uptake from host cells.

Among the *PIG*s, putative secondary transporters for amino acids were identified (Hahn and Mendgen 1997; Hahn et al. 1997b). These findings supported the potential role of rust haustoria in nutrient uptake (Hahn et al. 1997b). However, while an exclusive localization of AAT2p (PIG2p) in haustoria could be shown, no transport activity could be detected (Mendgen et al. 2000). AAT1p (PIG27p) was characterized as a broad specificity amino acid transporter with a main specificity for L-histidine and L-lysine (Struck et al. 2002). However, a localization of the transporter is still to come. AAT3p, another amino acid transporter identified, exhibits a substrate preference for L-leucine and the sulphur containing amino acids L-methionine and L-cysteine (Struck et al. 2004). However, there have been no localization studies, so far, and RT-PCR analyses indicate that expression of *AAT3* is not restricted to haustoria. Taken together it seems that amino acid uptake in *U. fabae* is not limited to haustoria, but the transporters characterized are clearly energized by the proton-motive force, and show a preference for amino acids present in low abundance in infected leaves (Struck et al. 2004).

Sugar uptake on the other hand seems to proceed exclusively via haustoria (Voegele et al. 2001). HXT1p was localized preferentially at the tip of monokaryotic haustoria (Voegele and Mendgen 2003), and in the periphery of the body of dikaryotic haustoria (Voegele et al. 2001; Fig. 2b). No specific antibody labeling could be found in intercellular hyphae. Neither nested PCR, nor genomic Southern blot analyses provided evidence for additional hexose transporters in any of the developmental stages tested (Voegele et al. 2001). This stands in contrast to the pronounced redundancy of sugar transporters in many other organisms, for example *Saccharomyces cerevisiae* (Boles and Hollenberg 1997). Based on its primary sequence, HXT1p can be placed into the sugar porter subfamily of the MFS [TC 2.A.1.1.]. HXT1p was characterized biochemically by heterologous expression. The data revealed that HXT1p is a proton-motive force driven monosaccharide transporter. Specificity was found for D-glucose and D-fructose with similar K_M-, and V_{max}-values (Voegele et al. 2001). This means that in contrast

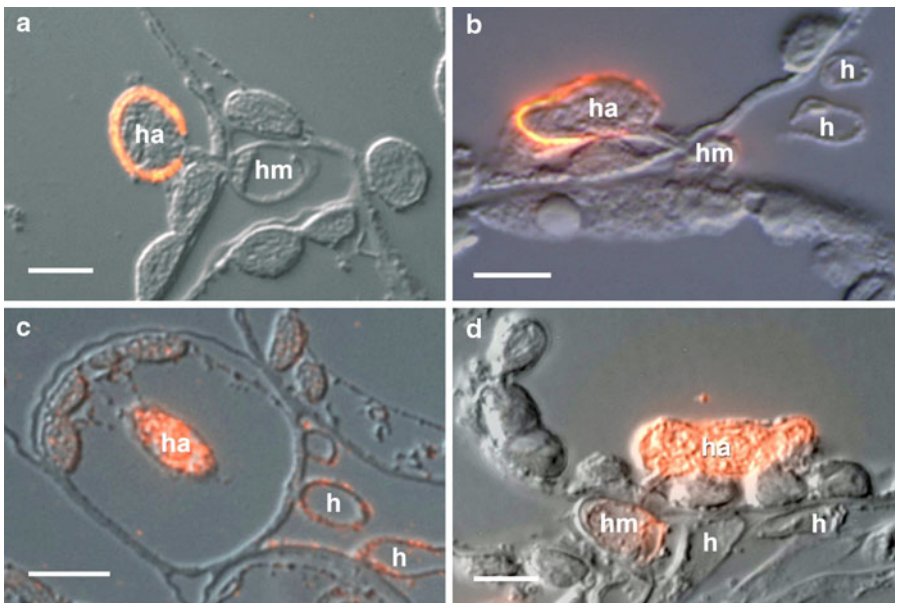

Fig. 2 Infected plant tissue containing haustoria (ha) within the plant cells and haustorial mother cells (hm) together with hyphae (h) in the extracellular space. Differential interference micrographs are shown with the fluorescence signal of Cy3-coupled antibodies against the different proteins superimposed. *Bar* 5 μm. **a** INV1p localizes exclusively in the periphery of the haustorium, most probably the extrahaustorial matrix; **b** HXT1p is restricted to the periphery of the haustorium and originates from the haustorial plasma membrane; **c** GLK1p is detected in the cytoplasm of the haustorium only; **d** MAD1p is also found in the cytoplasm of the haustorium. Neither hyphae nor the haustorial mother cell exhibit a distinct signal

to the situation with *Am*-MST1p, the sole hexose transporter in the ecto-mycorrhizal fungus *Amanita muscaria* and a close homolog of HXT1p, HXT1p seems to transport both hexoses with similar efficiencies. Our work on *HXT1*/HXT1p provided the first conclusive proof that rust haustoria are indeed nutrient uptake devices (Voegele et al. 2001).

Overall a picture is beginning to emerge which indicates that *U. fabae* makes use of several strategies to cover its nutritional demands. 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.

Where do the sweets come from?

Elucidating the mechanism and specificity of carbohydrate uptake in *U. fabae* provided an important advance for understanding the obligate biotrophic relationship, but at the same time put forward a series of new questions (Szabo and Bushnell 2001). One of the most important questions to address was the source of the substrates of HXT1p (Voegele et al. 2001).

Earlier research had shown that the level of free hexoses is fairly low in *V. faba* leaves (Lohaus et al. 2001). However, a carbohydrate that is present in abundance in virtually every plant is the disaccharide sucrose [1-α-D-Glucopyranosyl-2-ß-D-fructofuranoside] (Weber and Roitsch 2000). Source tissue designates plant organs which act as net exporters of carbohydrates (Williams et al. 2000). Heterotrophic tissue such as roots, or reproductive structures are net importers of sugars and hence referred to as sink tissues. Source tissue infected by a plant pathogen is thought to be converted into sink tissue (Ayres et al. 1996; Wright et al. 1995). With the major plant carbohydrate transport form, sucrose, being diverted directly to the plant pathogen, it seems obvious that the pathogen should try to use this nutrient source and Manners (1989) suggested that sucrose is indeed the major metabolite absorbed by powdery mildews. However, Mendgen and Nass (1988), Aked and Hall (1993), and later Sutton et al. (1999) were able to show that D-glucose is a more likely candidate carbohydrate for uptake by powdery mildew fungi. These data together with the substrate specificity determined for HXT1p in *U. fabae* indicate that it is not sucrose itself which seems to be utilized by the parasite. However, considering the building blocks of sucrose, D-glucose and D-fructose, it appears likely that sucrose may be the source of the carbohydrates consumed by the pathogen, but an enzymatic cleavage of the disaccharide has to precede the uptake.

Carbon partitioning in higher plants, the re-distribution of sucrose, seems to be largely determined by the activity of sucrose cleaving enzymes, such as invertases (Sturm 1999; Sturm and Tang 1999). Invertases (ß-D-fructofuranoside fructohydrolase [EC 3.2.1.26]) catalyze the hydrolysis of terminal non-reducing ß-D-fructofuranoside residues in ß-D-fructofuranosides, with sucrose being the major substrate (Myrbäck 1960). Plants contain different isoforms of invertases, which can be distinguished by their subcellular localization, pH optimum, and isoelectric point (Godt and Roitsch 1997). The invertase(s) responsible for sucrose partitioning are insoluble acid invertases located in the apoplastic space (Eschrich 1989; Tymowska-Lalanne and Kreis 1998). Hence, this invertase isoform may also be responsible for phloem unloading at the site of pathogen infection. There have been a number of reports of increased invertase activity in plants upon wounding or infection (Benhamou et al. 1991; Heisterüber et al. 1994; Sturm and Chrispeels 1990; Tang et al. 1996; Wright et al. 1995), and this might be explained on the basis of an increased demand for nutrients, for example, for defense reactions. However, for most of the systems analyzed, it has not been possible to distinguish the contribution of plant or fungus to the observed invertase activity increase (Billett et al. 1977; Callow et al. 1980; Krishnan and Pueppke 1988; Tang et al. 1996; Williams et al. 1984). Using reverse-transcriptase-PCR Voegele et al. (2006) showed that whereas transcript for cell-wall bound invertase 2 of *Vicia faba* (CWINV2p) was present in roots, but absent from leaf tissue, infection with *U. fabae* induced expression of *CWINV2* in leaves and elevated expression of the gene in root tissue. Although not resolved to the single cell level, these results provided strong support for the hypothesis that infection with a pathogen establishes a new sink, which stands in competition with already existing sinks.

However, it seems highly unlikely that a pathogen would solely rely on a host enzymatic system. Nevertheless, so far it has only been possible to demonstrate a contribution of the fungus to the increased invertase activity during infection in the pathosystem *Botrytis cinerea/Vitis vinifera* (Ruffner et al. 1992; Ruiz and Ruffner 2002). In the course of our research involving carbohydrate metabolism in *U. fabae* we identified a gene with homology to invertases, cloned the gene, and characterized the gene product using heterologous expression. The gene is expressed during parasitic growth and the gene product catalyses the irreversible breakdown of sucrose into D-glucose and D-fructose (Voegele et al. 2006; Fig. 2a). INV1p was the first invertase described for an obligate biotrophic pathogen. Our results indicate that a rise in host invertase expression as well as a fungal sucrolytic activity contribute to the overall increase in invertase activity in this pathosystem (Voegele et al. 2006).

Another source at least for the HXT1p substrate D-glucose could be BGL1p, a ß-glucosidase (ß-D-glucoside glucohydrolase [EC 3.2.1.21]) (Haerter and Voegele 2004). ß-glucosidases are a subgroup of O-glycosyl hydrolases, and occur widely in prokaryotes and eukaryotes (Bhatia et al. 2002). In fungi and bacteria, for example, ß-glucosidases are involved in cellulose and cellobiose catabolism as part of the cellulase complex and thus play a role in the process of biomass conversion (Leah et al. 1995). BGL1p has the capacity to use cellobiose, a breakdown product of cellulose, as a substrate, and consequently could make degradation products of the plant cell wall available for fungal nutrition (Haerter and Voegele 2004). However, *BGL1* shows a different expression pattern than *HXT1*. Therefore, there might be alternative or additional roles for this enzyme (Haerter and Voegele 2004).

Where do the sweets go to?

Another important question to address was the fate of the monosaccharides D-glucose and D-fructose once they are taken up by haustoria.

The level of free hexoses such as D-glucose and D-fructose has to be tightly regulated since especially D-glucose is also a powerful regulator of gene expression (Leon and Sheen 2003; Sturm and Tang 1999). There was evidence from an EST sequencing project that both glycolysis and the pentose phosphate pathway (PPP) are operational in haustoria of *U. fabae* (Hahn unpublished results; Jakupovic et al. 2006). In any case, the first enzyme to act on the monosaccharides translocated by HXT1p should be a hexokinase (ATP:D-hexose 6-phosphotransferase [EC 2.7.1.1]), if both D-fructose and D-glucose are funneled into these two pathways, or a glucokinase (ATP:D-glucose 6-phosphotransferase [EC 2.7.1.2]), if only D-glucose is metabolized this way. We cloned a *U. fabae* glucokinase (*GLK1*) using degenerate primers derived from *S. cerevisiae* hexokinases (Seibel and Voegele unpublished results). The sequence of *GLK1* was determined at both cDNA and genomic DNA level. Analysis of the amino acid sequence displayed considerable sequence homology with other fungal glucokinases. A detailed biochemical analysis of GLK1p was performed using heterologous expression of *GLK1* in *Escherichia coli*. Biochemical assays revealed substrate discrimination between aldoses and ketoses defining GLK1p as a glucokinase. Both real-time PCR assays and immunolocalization data using two GLK1p-specific antibodies revealed expression of *GLK1* exclusively/preferentially in haustoria (Fig. 2c). The presence of a glucokinase in haustoria hints at a preferred if not exclusive usage of D-glucose as substrate in the pathways mentioned above.

But what about the second substrate of HXT1p, D-fructose? During the initial characterization of *PIG*s, one gene, *PIG8*, was identified, that exhibited substantial homology to short-chain alcohol dehydrogenases (Hahn and Mendgen 1997). Subsequent analysis revealed strong homology to a mannitol dehydrogenase from *Agaricus bisporus* (Stoop and Mooibroek 1998). Functional characterization of the gene product in the heterologous expression system *S. cerevisiae* revealed that PIG8p constitutes a $NADP^+$-dependent mannitol dehydrogenase (D-mannitol:$NADP^+$ 2-oxidoreductase [EC 1.1.1.138]). As a result the designation of *PIG8* was altered to mannitol dehydrogenase 1, *MAD1* (Voegele et al. 2005). Thermodynamic evaluation of our kinetics data suggested that although termed mannitol dehydrogenase, the equilibrium of the reaction lies far on the side of the reaction educts, which in turn means that the enzyme is more likely to act as a D-fructose reductase (reverse reaction). To illustrate this scenario, we plotted the net

reaction velocity as a function of mannitol and D-fructose concentration (assumptions for this calculations were: neutral pH and equimolar concentrations of $NADP^+$ and NADPH).

As shown in Fig. 3 the reaction proceeds in the direction of mannitol formation under almost all conditions. Only under extremely high mannitol concentrations and only negligible amounts of D-fructose does the reaction proceed in the direction of D-fructose formation. MAD1p could therefore be responsible for utilization of the HXT1p substrate, D-fructose, in haustoria of *U. fabae*. Acyclic polyhydroxy alcohols or polyols are secondary metabolites typically associated with the fungal kingdom (Lewis and Smith 1967). A variety of different physiological functions were attributed to these polyols, including a role in carbohydrate translocation and storage (Jennings 1984). This role becomes especially important given that while some plants are able to synthesize mannitol from D-mannose (mannitol:NAD^+ 1-oxidoreductase [EC 1.1.1.255]) (Jennings et al. 1998), most plants are not able to utilize it. The production of mannitol would therefore be an ideal strategy for a pathogen to store carbohydrate in a soluble form that is freely diffusible in the mycelium, but cannot be accessed by the host.

Our metabolite analyses indeed indicated a dramatic increase in mannitol in infected leaves and large amounts of mannitol in urediniospores (Voegele et al. 2005). Assuming a water content of spores of 20%, the concentration of mannitol found in spores would be around 1 M, which is close to the solubility level of this polyol. Deposition of sugar alcohols in spores has been described for a number of fungi, including closely related rust species (Maclean and Scott 1976; Reisener 1969). Such a mechanism might suggest a role as a carbohydrate storage compound and/or in stress protection. Our results indicate a role for mannitol as a carbohydrate storage compound because of its rapid disappearance upon germination without ruling out a role in stress protection. There is no doubt that lipids and proteins constitute the major substrates during spore germination (Bago et al. 1999; Shu et al. 1954; Solomon et al. 2003). However, utilizing the pool of mannitol first would enable a quick start of glycolysis, since the conversion of mannitol to D-fructose is a single enzyme step. At the same time, oxidation of mannitol to D-fructose provides reducing power for anabolic processes. In this contest it is interesting to highlight the fact that while *MAD1* transcript was only detected in haustoria, MAD1p could be found in the lumen of haustoria (Fig. 2d) and in urediniospores (Voegele et al. 2005). Our enzymatic characterization clearly identified the mannitol dehydrogenase activity associated with spores as being due to MAD1p. The high level of mannitol in spores in combination with low D-fructose concentrations (Lohaus unpublished results) provides the ideal ground for the forward reaction of MAD1p (Fig. 3). Therefore, MAD1p seems to be responsible for the formation of mannitol from D-fructose in haustoria and for the mobilization of mannitol for metabolism in germinating urediniospores (Voegele et al. 2005).

This scenario requires the presence of a hexokinase [EC 2.7.1.1] in spores of *U. fabae*. The glucokinase,

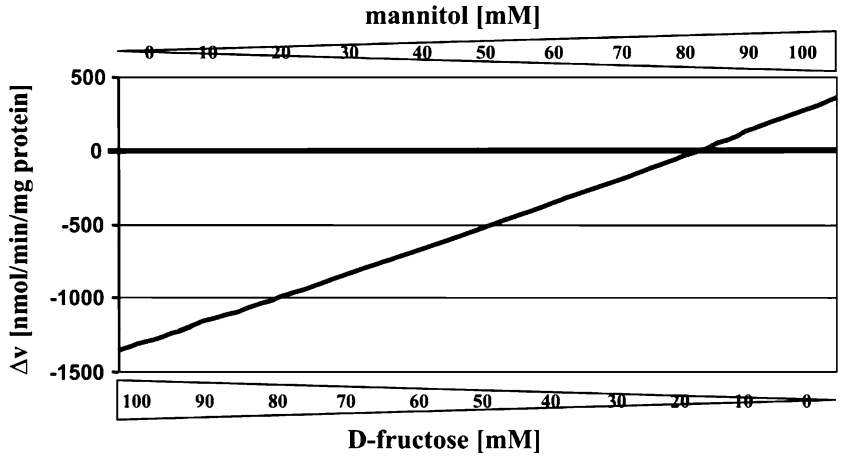

Fig. 3 Net-reaction velocities of the MAD1p-catalyzed reaction. Reaction velocities were calculated as a function of D-fructose and mannitol concentration. The following assumptions were made: Velocities were calculated for neutral pH and an assumption of equimolar concentrations of $NADP^+$ and NADPH

GLK1p, identified by us (Seibel and Voegele unpublished results) would not be suited to catalyze this reaction. Furthermore, GLK1p could not be localized in spores. This hints at the presence of an additional yet to be identified hexose phosphorylating activity in *U. fabae*.

Another important aspect of our work on hexose metabolism in *U. fabae* was the identification of a novel enzyme (Link et al. 2005). D-arabitol dehydrogenase 1 (D-arabinitol:$NADP^+$ oxidoreductase [EC 1.1.1.287]), ARD1p, acts on D-ribulose, D-xylulose, and D-arabitol using $NADP^+$/H as a cofactor. The enzyme could be localized in the lumen of haustoria. High levels of D-arabitol were found in infected leaves and ungerminated urediniospores. Again, upon germination D-arabitol diminished rapidly, also suggesting a role of this polyol in carbohydrate storage. However, since no ARD1p was detected in spores, utilization of D-arabitol in germinating spores has to proceed via a different enzymatic pathway. Mannitol and other acyclic polyhydroxy alcohols accumulate in a variety of fungi (Lewis and Smith 1967). For example, axenic cultures of *P. graminis* produce substantial quantities of both D-sorbitol and mannitol (Manners et al. 1982, 1984). Many other fungi showed accumulation of mannitol and D-arabitol (Maclean 1982). Our own work indicates that *U. fabae* has a strong preference to accumulate both mannitol and D-arabitol. In contrast to the situation in *P. graminis*, there is no indication that D-sorbitol plays a role in *U. fabae*. Thus, there might be considerable differences in the polyol patterns even between closely related fungal species (Pfyffer et al. 1986).

Our work regarding hexose mobilization, uptake and utilization clearly identified a number of important genes/gene products in *U. fabae* (Fig. 4). Haustoria are clearly uptake devices for some of the most important nutrients, but at the same time they are also the place where major parts of the ensuing metabolic pathways are carried out. In addition, haustoria also seem to be responsible for the production of polyols used as storage compounds in spores. However, it remains to be elucidated how the polyols are translocated from haustoria, through the mycelium and deposited in the urediniospores. Additional functions for these polyols are highly likely (see below).

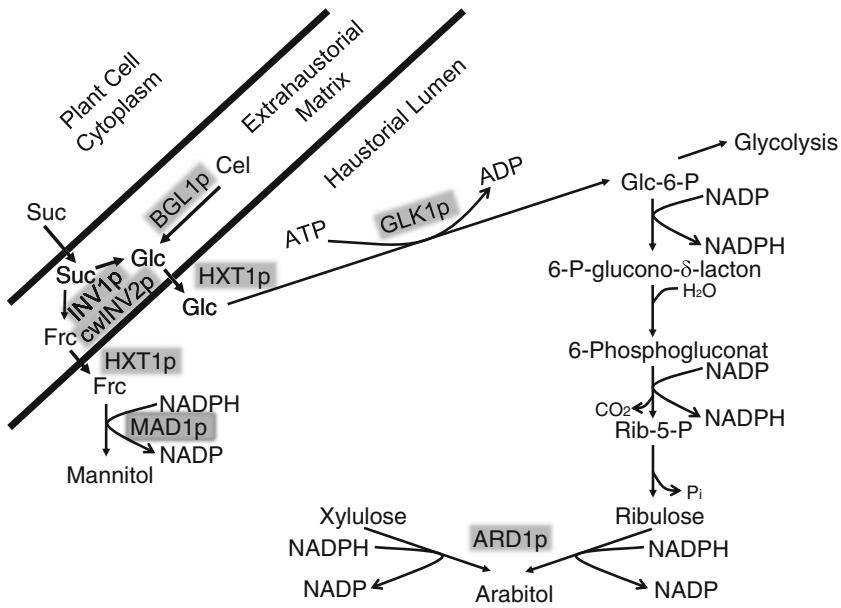

Fig. 4 Mobilization, uptake, and utilization of hexoses in haustoria of *U. fabae*. Sucrose (Suc) is released from the infected plant cell either actively or passively. The disaccharide is cleaved by the fungal invertase INV1p and possibly the action of plant enzymes. The resulting monosaccharides, D-glucose (Glc) and D-fructose (Frc), are taken up via the hexose transporter HXT1p. Glc might also be provided through the breakdown of cellobiose (Cel) by the action of BGL1p. Glc is funneled into glycolysis and the pentose phosphate pathway (PPP) by phosphorylation through GLK1p. D-fructose on the other hand is converted into mannitol by the action of MAD1p. At the bottom of the PPP, the novel enzyme ARD1p is generating D-arabitol

A link from nutrients to the obligate biotrophic lifestyle

In order to establish the obligate biotrophic relationship, the pathogen needs to evade or suppress host defense reactions. Rust fungi seem to have evolved a number of mechanisms to avoid recognition by host surveillance systems.

Analyses of the structural components of early rust infection structures indicated the most obvious differences between infection structures produced on the outside of the leaf, and those produced once the fungus has entered the tissue (Freytag and Mendgen 1991b; Kapooria and Mendgen 1985). One explanation for these differences would be the protection of chitin containing rust infection structures within the leaf against the action of glucanases and chitinases (Freytag and Mendgen 1991a). Another explanation would be the conversion of chitin to chitosan by the action of a chitin deacetylase (El Gueddari et al. 2002). General recognition of patterns common to a whole group of pathogens, so called Pathogen Associated Molecular Patterns, or PAMPs, by the plant innate immune system is thought to be one of the basic defense responses of a plant (Nürnberger and Brunner 2002; Parker 2003). Therefore, masking of fungal infection structures by obscuring or modifying for example chitin, might be one possibility for the pathogen to avoid recognition by the host.

The ß-glucosidase BGL1p (see above) might also play a role in the suppression of host defenses (Haerter and Voegele 2004). The protein shows high homology to other fungal ß-glucosidases involved in the detoxification of saponins. It is therefore quite possible that BGL1p has additional or alternative functions than providing substrate for HXT1p. This hypothesis, however, awaits verification.

Since some of the carbohydrates under investigation are not only superb nutrients, but also powerful regulators of gene expression (Leon and Sheen 2003; Sturm and Tang 1999), it is also conceivable that alterations in the concentration of one or more of these compounds could result in altered gene regulation in either host or parasite, or both. There is evidence from the pathosystem *Albugo candida*/*Arabidopsis thaliana* that the level of all three carbohydrates rises as a result of infection (Chou et al. 2000; Tang et al. 1996). However, there is still no conclusive proof that alterations in gene expression are the direct result of altered carbohydrate levels. In the pathosystem *U. fabae*/*V. faba* there seem to be no significant changes in the level of free hexoses or sucrose (Link et al. 2005; Voegele et al. 2005). The balancing of the levels of the different carbohydrates may actually be another way for the pathogen to evade detection by host surveillance systems. For example, by keeping the level of D-glucose within certain limits, it might be possible for the pathogen to prevent the induction of host signaling cascades involved in mounting defense responses.

Infection with *U. fabae* has far reaching effects on host metabolism exceeding the boundary of the infected cell. This has been shown by expression analysis of *V. faba* genes in response to attack by the pathogen (Wirsel et al. 2001). Several of the genes analyzed showed altered expression patterns in the infected organ as expected. However, some of the analyzed genes also showed alterations in expression in far remote organs, such as stems and roots. Our work therefore clearly shows that the influence on host metabolism by a leaf pathogen is not limited to the infected organ alone. Our results regarding the expression of host and pathogen invertases also show far reaching effects on host metabolism caused by infection with *U. fabae* (Voegele et al. 2006). Alterations in the expression level of plant invertases indicate systemic effects of infection. An attractive explanation for the observed expression of the fungal invertase INV1p in early infection structures stems from the role insoluble acid plant invertases have in the determination of the sink strength of a plant organ. Apoplastic hydrolysis of sucrose would limit export of carbohydrates from the infected tissue via the phloem and therefore would condition the tissue for a conversion from a source to a sink, which then stands in competition with naturally occurring sinks (Voegele et al. 2006).

There is also evidence that mannitol and D-arabitol are released from the fungal mycelium into the apoplast (Link et al. 2005; Voegele et al. 2005). Results from mammalian (Chaturvedi et al. 1996) and from plant (Jennings et al. 2002) pathosystems indicate that at least mannitol can effectively be used to suppress host defense responses involving reactive oxygen species. The concentrations of mannitol and D-arabitol in infected *V. faba* tissue were shown to be

sufficient to effectively quench reactive oxygen species (Link et al. 2005; Voegele et al. 2005). In essence, there might be a direct link between nutrient acquisition and at least parts of the pathogen's system to prevent activation of host defense responses. But surely this cannot be the only strategy of the parasite to establish a long lasting obligate biotrophic relationship with its respective host.

There is more to establish and maintain an obligate biotrophic lifestyle

As already mentioned, the establishment and maintenance of biotrophy requires the evasion or suppression of host defense reactions. Besides masking fungal structures, the usage of sugar alcohols to quench reactive oxygen species, the use of detoxifying ß-glucosidases, and the potential control of the level of regulatory carbohydrates, rust fungi seem to have evolved further strategies to avoid recognition through host surveillance systems.

Analysis of the morphology of extrahaustorial membranes produced by *P. graminis* or *P. coronata* on oat for example revealed several differences. This in turn suggests that formation of the fine structure of the haustorial host-parasite interface is under the control of species-specific signals from the fungus (Harder and Chong 1991). Such signals may include suppressors, which have been implicated in maintaining basic compatibility between the parasite and its host plants (Bushnell and Rowell 1981). Evidence for such suppressors comes from a phenomenon called induced susceptibility. French bean tissue already infected by *U. vignae* supported additional infections by several non-host pathogens (Fernandez and Heath 1991). Suppressors for plant defense responses have been described, but they are either poorly characterized or non-proteinaceous (Basse et al. 1992; Knogge 1997; Moerschbacher et al. 1999). Nevertheless, it is reasonable to assume that fungi, like their bacterial counterparts, have evolved mechanisms to deliver proteins as effectors to take control of the host's metabolism.

Papers from Australian researchers working with the pathosystem *Melampsora lini/Linum usitatissimum* indicate that there are a number of haustorium-specific secreted proteins that interact directly with corresponding host resistance gene products (Catanzariti et al. 2006; Dodds et al. 2004, 2006). This work was done using heterologous expression systems and biochemical assays and confirms at the molecular level the gene for gene hypothesis put forward by Flor (1955, 1956) more than 60 years ago. However, it has to be kept in mind that the interaction of avirulence gene products and resistance gene products results in an incompatible interaction; that is, a failure of the pathogen to establish infection fully. While this is certainly an interesting aspect with respect to the basic understanding of resistance reactions and the identification of new avirulence gene—resistance gene combinations is also advantageous for breeders, this situation does not reflect the true obligate biotrophic lifestyle, which is based on a long lasting interaction of host and parasite.

Recently, Kemen et al. (2005) showed that one of the *PIG*s identified by Hahn and Mendgen (1997) is actually not only secreted into the extrahaustorial matrix as expected from its targeting sequences, it is further transferred to the host cell cytoplasm and nucleus. It remains to be shown if Rust Transferred Protein 1 (RTP1p) acts as a suppressor or has other functions. However, RTP1p distribution seems to be limited to the infected host cell. Since RTP1p does not have any homologs in publicly accessible databases, it is not possible to deduce potential functions from sequence homologies. In silico analysis of RTP1p revealed the presence of potential targeting signals and domains. We also identified *RTP1* homologs in the closely related rust fungus *U. striatus* (Kemen et al. 2005). Both proteins share an overall identity of 71%. However, if only the C-terminal halves of the proteins are compared the level of identity increases to more than 91%. This C-terminal portion also contains one *N*-glycosylation site common to both proteins which seems to be essential for proper folding and secretion of the protein (Kemen 2006a). The C-terminal half of the proteins also contains a ß-aggregation domain often associated with prion like proteins. Indeed, our attempts to overexpress, purify, and crystallize RTP1p have been hampered by the tendency of the protein to aggregate. Using the method developed by Lee and Eisenberg (2003), we showed that amorphous aggregates of RTP1p can be converted into fibrils (Kemen 2006b). The stability of these filaments may be linked to a potential function of RTP1p: the prevention of a collapse of the host cell and the protection of the haustorium against plant

defenses. However, this hypothesis still awaits proof. At the same time it is still unclear how RTP1p is transferred from the extrahaustorial matrix into the infected host cell, and how it reaches the host cytoplasm, once it has entered the cell.

While our initial hypothesis was that *RTP1* distribution might be limited to a few species, i.e. *U. fabae* and some closely related rust fungi, it now appears that *RTP1* homologs are present in a number of rust fungi. So far, we identified 30 *RTP1* homologs in a broad spectrum of rust fungi (Pretsch and Voegele unpublished results). It now seems that RTP1p might be a protein specific for rust fungi in general, since to date no homologs were found outside the Uredinales.

Outlook

Our analyses of the roles played by haustoria in establishing and maintaining the obligate biotrophic relationship have come a long way. We have shown that haustoria are indeed nutrient uptake devices that additionally seem to function as small power plants for the pathogen. There seems to be a tight link between standard metabolic pathways and potential suppression of host defense reactions. Our research has also shown that, like their bacterial counterparts, rust fungi are able to deliver effector proteins into the infected host cell, although the mechanism of this transfer is still elusive. Establishing a system for the stable transformation of *U. fabae* will enable us to lift our research to a higher level. There is certainly much more to the obligate biotrophic lifestyle awaiting to be elucidated.

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