Possible Roles for Mannitol and Mannitol Dehydrogenase in the Biotrophic Plant Pathogen *Uromyces fabae*[1]

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Levels of the C6-polyol mannitol were observed to rise dramatically in the biotrophic interaction of the rust fungus *Uromyces fabae* and its host plant *Vicia faba*. Mannitol was found in millimolar concentrations in extracts and apoplastic fluids of infected leaves and also in extracts of spores. We suggest that this polyol might have at least a dual function: first, as a carbohydrate storage compound, and second, as a scavenger of reactive oxygen species. Mannitol accumulation is accompanied by high expression of a mannitol dehydrogenase (*MAD1*) in haustoria. While *MAD1* transcripts were detected in haustoria only, immunolocalization studies show that the gene product is also present in spores. Kinetic and thermodynamic analyses of the MAD1p catalyzed reactions indicate that the enzyme might be responsible for the production of mannitol in haustoria and for the utilization of mannitol in spores. Since *V. faba* is normally unable to synthesize or utilize polyols, the multipurpose usage of mannitol seems an ideal strategy for the fungal pathogen.

Acyclic polyhydroxy alcohols or polyols are secondary metabolites typically associated with the fungal kingdom (Lewis and Smith, 1967). Although polyols have also been found in a number of plant species (Stoop et al., 1996), fungi remain the major producers and accumulators of these substances (Lewis and Smith, 1967). A variety of different physiological functions have been attributed to these polyols, among them (1) carbohydrate storage; (2) translocation; (3) osmoprotection; and (4) coenzyme regulation and storage of reducing power (Jennings, 1984). Polyols are frequently found in fungal spores and are thought to act as storage compounds (Reisener et al., 1962; Maclean and Scott, 1976). Koide et al. (2000) also found a role for mannitol in the translocation of carbohydrates in mycorrhiza, and polyols have been shown to be involved in osmoprotection in fungi (Shen et al., 1999; Clark et al., 2003). It has been suggested that some fungi are able to switch between anabolic and catabolic pathways and to store reducing power by the interconversion of NADH and NADPH via the mannitol cycle (Hult et al., 1980). More recently, it has been shown that polyols can be powerful radical scavengers in vitro (Smirnoff and Cumbes, 1989) and in vivo (Shen et al., 1997a, 1997b). This has led to the demonstration that mannitol plays a role in pathogenicity of plant and animal pathogens. Chaturvedi et al. (1996a) showed that a mannitol low-producing mutant of the animal pathogen *Cryptococcus neoformans* was impaired in pathogenicity and stress tolerance. The addition of mannitol, on the other hand, increased survival of *C. neoformans* in vitro (Chaturvedi et al., 1996b). Jennings et al. (1998) observed that infection by the mannitol-secreting fungus *Alternaria alternata* induced expression of a mannitol dehydrogenase (MTD) in tobacco (*Nicotiana tabacum*), a mannitol-nonproducing plant. The hypothesis was raised that MTD induction served to counteract fungal suppression of reactive oxygen species (ROS)-mediated defense strategies. Support for this hypothesis was obtained by constitutive transgenic expression of a celery MTD in tobacco plants, which resulted in an increased resistance to the mannitol-secreting fungus *A. alternata* but not to the non-mannitol-secreting pathogen *Cercospora nicotianae* (Jennings et al., 2002).

At present, several different pathways are known for the synthesis of mannitol. In plants, mannitol is formed from Fru-6-P by the successive action of Man-6-P ketol-isomerase (EC 5.3.1.8), mannitol-1-P:$NADP^+$ 6-oxidoreductase (EC 1.1.1.224), and mannitol-1-P phosphohydrolase (EC 3.1.3.22; Stoop et al., 1996). In Fungi Imperfecti and possibly in Ascomycetes, mannitol may be produced via Fru-6-P, and mannitol-1-P in the mannitol cycle by the consecutive action of hexokinase (EC 2.7.1.1) and mannitol-1-P:NAD^+ 5-oxidoreductase (EC 1.1.1.17; Hult and Gatenbeck, 1979). However, in Basidiomycetes mannitol-1-P dehydrogenase seems to be absent (Hult et al., 1980). The plant MTD described by Jennings et al. (1998) is a mannitol:NAD^+ 1-oxidoreductase (EC 1.1.1.255). The reaction is NAD^+ coupled and thought to proceed

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primarily in the direction of Man formation (Stoop et al., 1996). By contrast, most of the bacterial and fungal MTDs are mannitol 2-oxidoreductases producing Fru in the forward reaction and using either NAD^+ (EC 1.1.1.67) or $NADP^+$ (EC 1.1.1.138) as a cofactor. Since mannitol-1-P dehydrogenase seems to be absent from Basidiomycetes (Hult et al., 1980), mannitol is most likely formed by direct reduction of Fru through a MTD (EC 1.1.1.67 or EC 1.1.1.138). Maclean (1990), for example, reported a NAD^+-dependent MTD activity in *Puccinia graminis* f. sp. *tritici* axenic cultures, and Clancy and Coffey (1980) have shown NAD^+- and $NADP^+$-dependent MTD activity in *Melampsora lini* axenic cultures and uredospores. The MTD of the Basidiomycete *Agaricus bisporus* has been studied biochemically in great detail (Morton et al., 1985; Stoop and Mooibroek, 1998; Hörer et al., 2001).

Biotrophic pathogens like the rust fungi are among the most devastating plant pathogens worldwide (Kawuki et al., 2003; Long, 2003). However, due to the intricate relationship of host and fungus, still very little is known about the molecular mechanisms underlying the biotrophic lifestyle. One aspect of our research deals with the uptake and metabolism of carbohydrates in the rust fungus *Uromyces fabae*. Recently, we were able to show that carbohydrate uptake in *U. fabae* is specific for Glc and Fru and proceeds exclusively via haustoria (Voegele et al., 2001). This study reveals that, during the course of infection, much of the carbohydrate is converted into the C6-polyol mannitol. Our results indicate at least a dual role for mannitol in the suppression of ROS-mediated plant defenses and as a carbohydrate storage compound deposited in spores. Mannitol in the pathosystem *U. fabae*/*Vicia faba* seems to be formed by a fungal mannitol:$NADP^+$ 2-oxidoreductase (EC 1.1.1.138), which also appears to be responsible for the utilization of mannitol upon spore germination.

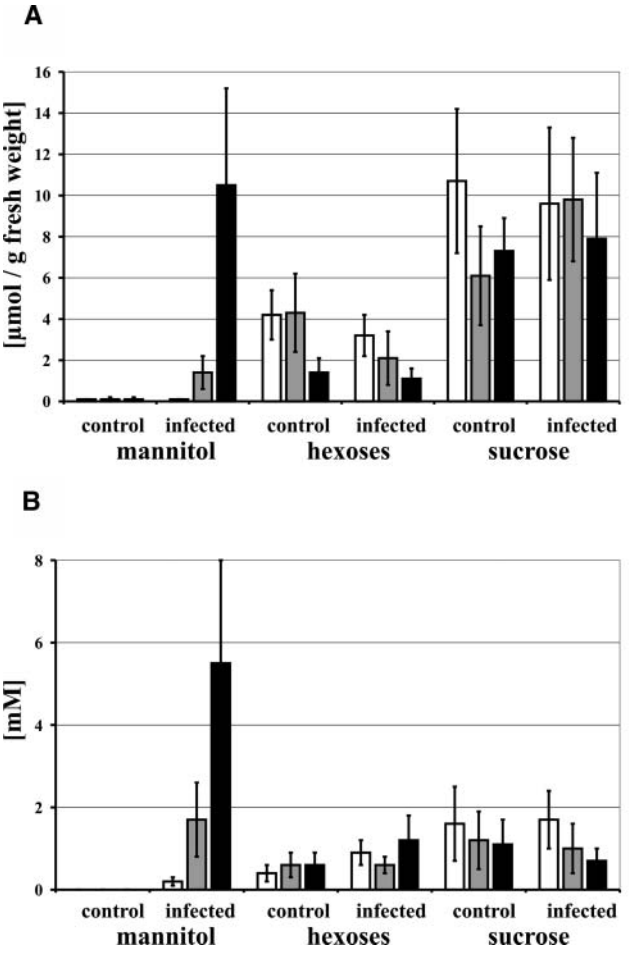

Figure 1. Sugar and sugar alcohol concentrations in infected and noninfected (control) *V. faba* leaf extracts (A) and AFs (B). Metabolites were analyzed by HPLC 3 (white bars), 5 (gray bars), and 12 d (black bars) postinfection. Concentrations are given as micromoles per gram FW for leaf extracts and mM for AFs. Bars represent the mean ($n = 6$ for leaf extracts, $n = 10$–15 for AF) ±SE. Hexoses represents the sum of Glc and Fru.

RESULTS

Mannitol Increases Dramatically in *V. faba* Leaves Infected with *U. fabae*

Extending our work on the hexose metabolism in the obligate biotrophic rust fungus *U. fabae* (Voegele et al., 2001; Voegele and Mendgen, 2003), we set out to analyze alterations in metabolite pools of *V. faba* leaves and apoplastic fluids (AFs) in response to attack by the pathogen 3, 5, and 12 d postinfection. Special emphasis was given to amino acids, sugars, and sugar alcohols, as well as inorganic ions. Of all the compounds analyzed, only two showed considerable changes following infection in a time dependent manner. One of these compounds was identified by NMR as arabitol (data not shown). The changes in arabitol content and the source for this sugar alcohol will be described separately (T. Link, G. Lohaus, I. Heiser, K. Mendgen, M. Hahn, and R.T. Voegele, unpublished data). The second metabolite was unequivocally identified as mannitol based on its retention time with respect to standard compounds in HPLC analysis. Figure 1 shows that levels of free hexoses (Glc and Fru) and Suc varied over time, but not in response to pathogen attack. Mannitol, however, rose from virtually nondetectable to concentrations of up to 10.5 μmol/g fresh weight (FW) in cell extracts (Fig. 1A) and up to 5.5 mM in AFs (Fig. 1B) of rust-infected leaves. By contrast, in control plants, the level of mannitol remained below the detection limit.

ROS Quenching by Mannitol

Of all the postulated roles for mannitol, its presence in the apoplast is most likely compatible with its quenching capabilities for ROS. We therefore tested whether the mannitol produced during the pathogenic interaction would be sufficient to quench ROS derived from H_2O_2. This was done in an in vitro system by coupling the Fenton reaction to the conversion of α-keto-β-methiol-butyric acid (KMB) to ethylene and

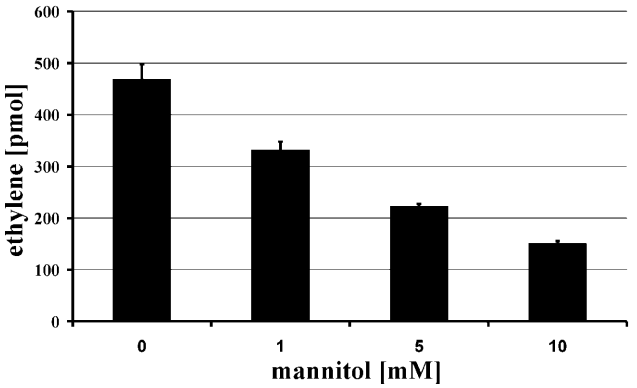

Figure 2. ROS quenching capacity of mannitol. Different concentrations of mannitol were added to the in vitro reaction containing KMB and the reduction in ethylene production was followed on a gas chromatograph. Bars represent the means of four independent experiments ±SE.

monitoring product formation via gas chromatography (GC). Figure 2 shows that quenching by concentrations of mannitol present in the apoplast suppresses ROS to about one-half the level found in the absence of mannitol.

Mannitol as a Storage Compound in Spores

We also analyzed uredospores and in vitro-grown infection structures (germlings, appressoria, infection hyphae, and haustorial mother cells; Deising et al., 1991) of *U. fabae* for the presence of mannitol and sugars. Suc, Fru, and Glc were below the detection limit of 10 μM in spores and all infection structures analyzed (data not shown). Mannitol, however, was found in large quantities in spores, but disappeared rapidly upon infection structure formation (Fig. 3). This strongly suggests a role for mannitol as a storage compound.

The Origin of Mannitol

During the characterization of plant-induced rust genes (*PIG*s), we had identified a gene, *PIG8*, encoding a protein that was classified as a short-chain alcohol dehydrogenase (Hahn and Mendgen, 1997). Subsequent analysis revealed strong homology (74% similarity, 60% identity) to a MTD from *A. bisporus* (O93868; Stoop and Mooibroek, 1998). The complete cDNA open reading frame of *PIG8* was subcloned into the yeast (*Saccharomyces cerevisiae*) vector pDR195, and the resulting plasmid pDR195::*MAD1* was transformed into yeast strain 23344c (Marini et al., 2000). Transformants were analyzed for alcohol dehydrogenase activity. No activity was detected with any of the substrates tested in yeast transformants carrying the vector alone and using either NAD(H) or NADP(H) as a cofactor (data not shown). Activity in transgenes was only found using NADP(H) as a cofactor (Fig. 4; data not shown). For the forward reaction (oxidation of alcohol), we found a strong preference for mannitol and a small side activity for sorbitol (Fig. 4A). The reverse reaction (reduction of sugar) proceeded only when Fru was provided as substrate (Fig. 4B). On the basis of its substrate and cofactor specificity, PIG8p can be unequivocally classified as a true mannitol:$NADP^+$ 2-oxidoreductase (EC 1.1.1.138). *PIG8* was therefore renamed *MAD1* (mannitol dehydrogenase 1).

Characterization of *U. fabae* MAD1p

Enzyme extraction procedures were adapted to yield high and stable MAD1p. MAD1p activity for the forward reaction was highest at pH values around 10.5, declining sharply at values above 11.0 and below 8.5. The reverse reaction exhibited a pH optimum around pH 6.0. The kinetic parameters for the forward and reverse reaction of MAD1p were determined in extracts of transgenic yeast, since our controls revealed the absence of a comparable activity from extracts of wild-type yeast. We found Michaelis-Menten constants of 78 mM for mannitol and 34 μM for $NADP^+$ for the forward reaction (Table I). V_{max} for both cofactor and substrate reactions in the heterologous system were 6.36 ± 0.2 μmol/min/mg protein. K_m values for Fru and NADPH were 804 and 38 μM, respectively (Table I). V_{max} for the reverse reaction was 19.4 ± 0.4 μmol/min/mg protein.

MAD1p Activity in Infected Leaves

Using the *U. fabae/V. faba* pathosystem, we set out to determine if MAD1p could be the source for the mannitol found in leaves and AFs as infection progressed. First we screened infected and noninfected plants for the MTD forward reaction under conditions optimal for MAD1p activity. Figure 5 shows that MTD activity in infected leaves went up more than 20-fold in the course of infection, whereas MTD activity

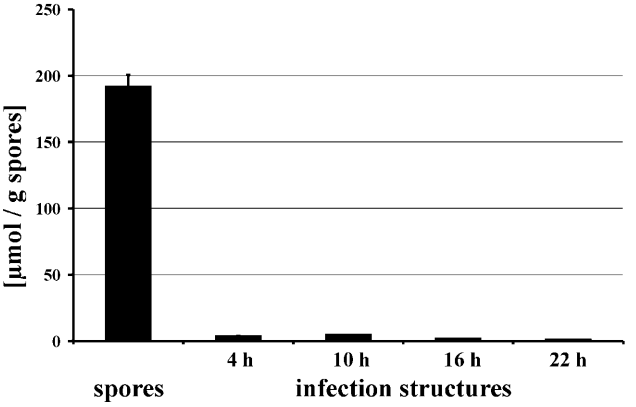

Figure 3. Mannitol as a storage compound. Mannitol present in spores and infection structures was quantified by HPLC of chloroform-methanol extracts. The amount of spores used for each experiment was used as a common denominator. Bars represent the means of duplicate experiments ±SE (n = 6 for spores, n = 2 for infection structures).

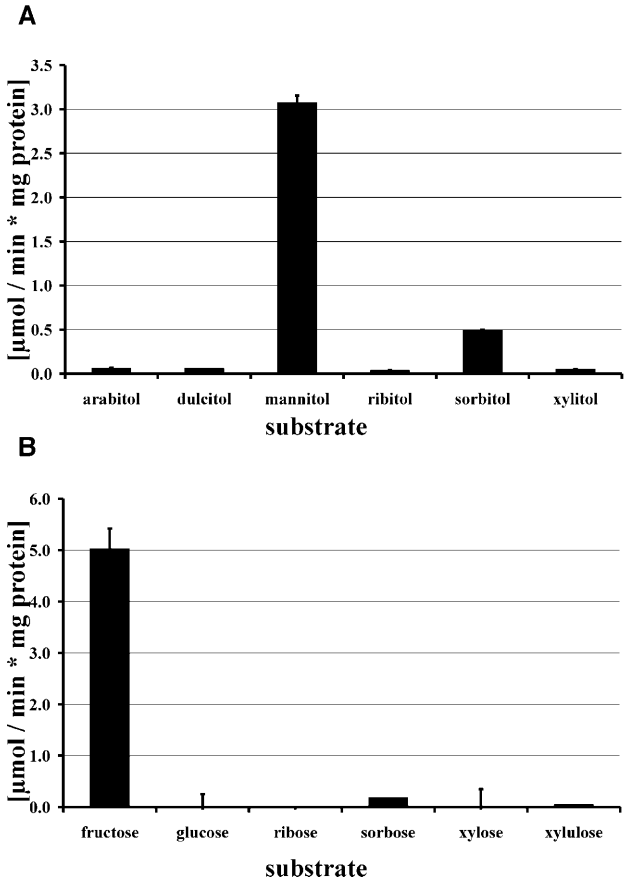

Figure 4. Substrate specificity of MAD1p. A, The forward reaction was assayed at a cofactor concentration of 0.4 mM $NADP^+$ and a substrate concentration of 500 mM, except for dulcitol, which was added to saturation. B, The reverse reaction was assayed at a cofactor concentration of 0.2 mM NADPH and a substrate concentration of 500 mM, except for xylulose, which was used at 100 mM. Bars represent the means of five experiments ±SE. Both reactions were followed by monitoring the absorbance change at 340 nm over time.

remained at the detection limit for noninfected leaves. To rule out the possibility that the activity determined would be due to a plant enzyme induced in the course of infection, we also assayed noninfected areas of infected leaves (Fig. 5). Like in control leaves, no significant changes in MTD activity could be found. To confirm that the activity determined in planta is indeed due to the action of MAD1p, we compared the kinetic fingerprints of the enzymatic reaction determined in transgenic yeast and infected and noninfected plants. To do this, we chose three different reaction conditions based on the kinetic determination shown in Table I. The ratio of these reaction velocities was then taken as an identifier for enzyme activity. Figure 6 shows the typical pattern obtained for MAD1p expressed in yeast. Only marginal MTD activity could be found in noninfected leaf samples for all reactions tested. Ratios resulting from these activities were always close to one. In the course of infection, the pattern obtained for infected leaves changed from one resembling noninfected leaves to a near-perfect match of the MAD1p pattern. This result strongly suggests that the MTD activity associated with infected *V. faba* leaves is largely due to the fungal enzyme MAD1p. However, the slight differences with respect to MAD1p alone also indicate the presence of other $NADP^+$-dependent enzymes involved in mannitol/Fru turnover.

Localization of MAD1p

To determine the localization of MAD1p more precisely, enzyme assays and immunofluorescence microscopy were used. In ungerminated uredospores, significant MTD activity was observed. V_{max} values for the forward and the reverse reaction were determined to be 0.4 μmol/min/mg protein and 1.0 μmol/min/mg protein, respectively. The kinetic fingerprint is consistent with MAD1p as the primary cause for this activity (Fig. 6). However, the differences to the pattern obtained for MAD1p expressed in yeast also indicate a possible contribution of other polyol dehydrogenases acting on mannitol. Further kinetic analyses confirmed K_m values and pH optima for the forward and the reverse reaction in uredospore extracts to be identical to the parameters determined for MAD1p in transgenic yeast (data not shown). The detection of MAD1p in spores was unexpected since *MAD1* (*PIG8*) was previously shown to be a typical in planta-induced gene, the expression of which appeared to be limited to haustoria (Hahn and Mendgen, 1997). To localize MAD1p in the biotrophic mycelium, we raised and purified polyclonal antibodies against MAD1p. Figure 7 shows that a strong signal could be detected in the lumen of haustoria. A weaker, but significant, signal was detected in spores, whereas no labeling was obtained for intercellular hyphae. No signal was obtained using preimmune serum (data not shown). This result indicates that MAD1p protein is present in haustoria and, despite the absence of *MAD1* transcripts, also in uredospores.

Table I. *Kinetics of MAD1p*

Substrate for the forward reaction was mannitol, with $NADP^+$ as a cofactor, whereas the reverse reaction was assayed with Fru and NADPH as a cofactor. Reactions were followed by monitoring the absorbance change at 340 nm.

pH		Forward Reaction		Reverse Reaction	
		V_{max}	K_m	V_{max}	K_m
		µmol/min/mg protein	*mM*	*µmol/min/mg protein*	*mM*
5.2	Substrate	0.37	695	19.7	804
	Cofactor	0.17	0.08	19.0	0.04
7.0	Substrate	1.60	346	7.6	461
	Cofactor	1.30	0.14	13.6	0.33
10.0	Substrate	6.30	78	n.d.	n.d.
	Cofactor	6.40	0.03	n.d.	n.d.

n.d., Not determined.

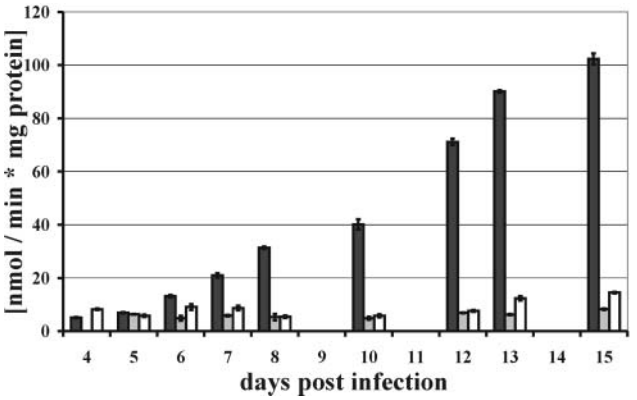

Figure 5. MTD activity in infected plants is due to MAD1p. MTD forward reaction was assayed under standard conditions using the following extracts of leaves: infected (black bars), noninfected (white bars), and noninfected areas of infected leaves (gray bars). Bars represent the means of triplicate experiments ±SE. The reaction was followed by monitoring the absorbance change at 340 nm over time.

A Dual Role for MAD1p

We have shown a correlation between the accumulation of mannitol in rust-infected leaves and the appearance of MAD1p, thus providing strong evidence that MAD1p is responsible for the formation of mannitol from Fru in haustoria. However, this does not explain the role of MAD1p in spores that are rich in mannitol but devoid of Fru (data not shown). To test the hypothesis that MAD1p is responsible for the rapid consumption of mannitol upon germination, we repeated the kinetic analyses using the same pH for both directions of the reaction and used the Haldane equation to calculate the equilibrium constant (Table I). For a neutral pH, we calculated an equilibrium constant of 6.2×10^{-9} M. Using the optimal assay pH of the reverse reaction for both enzyme directions, this value shifted to 2.7×10^{-10} M. This means that, at equilibrium, concentrations of mannitol and $NADP^+$ are much higher than the concentrations of Fru, NADPH, and protons. In haustoria, MAD1p will consequently form mannitol from Fru taken up through the action of HXT1p. In germinating spores, on the other hand, Fru levels are below the detection limit (data not shown). This might be due to the fact that it is immediately removed from the equilibrium through the action of glycolytic enzymes. Under these conditions, the reaction will proceed toward the formation of Fru. Given these thermodynamic considerations, it seems likely that MAD1p could be responsible for the formation of mannitol in haustoria and for the metabolism of mannitol deposited in spores.

DISCUSSION

We have analyzed the source and roles of the C6-polyol mannitol in the obligate biotrophic fungal plant pathogen *U. fabae*. Mannitol levels rose dramatically in the course of infection of the host *V. faba.* Whereas mannitol was virtually absent from noninfected leaves, it could be detected in large quantities in extracts as well as AFs of infected leaves. Mannitol was also present in spores but disappeared rapidly upon germination. The most likely source for this polyol is a MTD of fungal origin. The enzyme MAD1p was characterized biochemically and located in haustoria and spores using immunocytological techniques.

Mannitol and other acyclic polyhydroxy alcohols have been found to accumulate in a variety of fungi (Lewis and Smith, 1967). For example, axenic cultures of another rust fungus, *P. graminis*, were found to produce substantial quantities of glucitol (sorbitol) and also mannitol (Manners et al., 1982, 1984). Many other fungi analyzed showed accumulation of mannitol and arabitol (Maclean, 1982). Our own work indicates that *U. fabae* has a strong preference to accumulate mannitol and arabitol. Arabitol built up to similar concentrations as mannitol in leaf extracts and was also found in the apoplast at slightly lower concentrations than mannitol (T. Link, G. Lohaus, I. Heiser, K. Mendgen, M. Hahn, and R.T. Voegele, unpublished data). Preliminary data from a project to quantify *U. fabae* in planta indicate that the rise of both polyols approximately matches the increase in fungal biomass (R.T. Voegele, unpublished data). In contrast to the situation in *P. graminis*, we have no indication that sorbitol plays a role in *U. fabae.* Thus, there seem to be considerable differences in the polyol patterns even between closely related fungal species (Pfyffer et al., 1986).

As outlined by Lewis and Smith (1967), polyols might provide a means for fungi to store carbohydrate and reducing power in a form not accessible to the host. This view would be consistent with the observed accumulation of mannitol as infection of *V. faba* progressed. Conversion of carbohydrates taken up by the fungus into polyols would also maintain a gradient of metabolites toward the pathogen in times when nor-

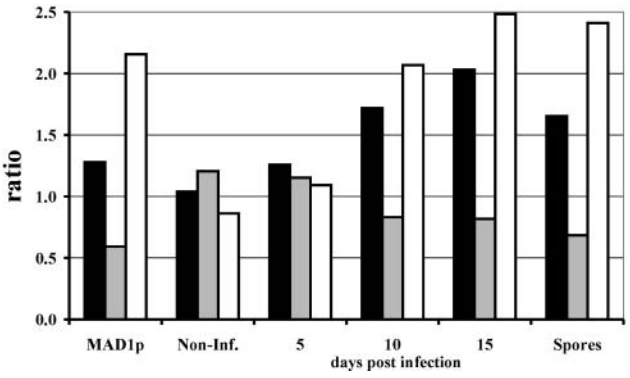

Figure 6. Kinetic fingerprint of MAD1p. MTD activity was assayed under three different conditions (Rev1, Rev2, and Fwd1; for details, see "Materials and Methods"). Bars represent the ratios of Rev1/Fwd1 (black bars), Rev2/Fwd1 (gray bars), and Rev1/Rev2 (white bars). Experiments were performed in triplicate.

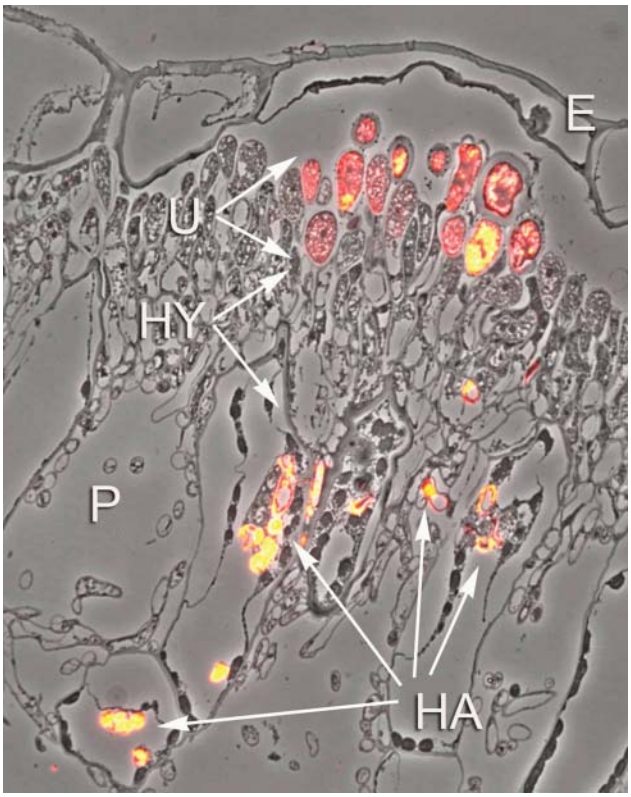

Figure 7. Localization of MAD1p. Superimposed phase contrast and immunofluorescence images depicting a cross-section through a young rust pustule within an infected leaf. The epidermis (E) still covers the pustule. Labeling of MAD1p with antibody S717p resulted in fluorescence signals in the lumen of haustoria (HA) and in the lumen of uredospores (U). Sporogenous and intercellular hyphae (HY) did not exhibit fluorescence. P, Parenchymatic tissue (magnification 650-fold).

mal metabolic pathways are working to full capacity. However, our results do not rule out a role of polyols in regulating osmotic pressure during times of water stress, as suggested by Chaturvedi et al. (1997). A role as translocatory compound as suggested for mannitol in some mycorrhiza interactions (Koide et al., 2000) also seems possible.

The fact that mannitol was found in large quantities in AFs hints at its role in suppression of ROS-related defense mechanisms. For the animal pathogen *C. neoformans*, for example, it was shown that mannitol serves to protect the fungus from oxidative damage caused by ROS (Chaturvedi et al., 1996a, 1996b). We were able to show that the amount of mannitol found in AFs is sufficient to suppress ROS to about one-half the level present in the absence of mannitol (Fig. 2). Since biotrophic interactions are characterized by a phase of prolonged coexistence of pathogen and host, such a suppression of host defense seems indispensable. Support for this hypothesis comes from work by Jennings et al. (1998, 2002), who have shown that the removal of mannitol from pathosystems by the action of a mannitol:NAD^+ 1-oxidoreductase (EC 1.1.1.255) in transgenic plants enhances resistance of the host against mannitol-secreting, but not against mannitol-nonsecreting pathogens. Our results are therefore consistent with a pivotal role of mannitol in the biotrophic interaction of *U. fabae* with its host *V. faba*.

In addition to the increase of mannitol during the parasitic growth phase, we also found mannitol in large quantities deposited in uredospores. Assuming a water content of spores of 20%, the concentration of mannitol found in spores is 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 (Reisener et al., 1962; Maclean and Scott, 1976). Such a mechanism might suggest a role as a carbohydrate storage compound and/or in stress protection. Ruijter et al. (2003) reported the requirement of mannitol for stress tolerance, but not as a storage compound in *Aspergillus niger* conidiospores. Maclean and Scott (1976) reported the presence of mannitol and arabitol both in ungerminated and germinated uredospores of *P. graminis* f. sp. *tritici*, a finding consistent with a role of both polyols in stress tolerance. Niederpruem and Hunt (1967), on the other hand, reported the disappearance of arabitol but not of mannitol during germination of basidiospores of *Schizophyllum commune*, indicating a role for arabitol in carbohydrate storage. 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 (Shu et al., 1954; Bago et al., 1999; Solomon et al., 2003); however, utilizing the pool of mannitol first would enable a quick start of glycolysis, since the conversion of mannitol to Fru is a single-enzyme step. At the same time, oxidation of mannitol to Fru would provide reducing power for anabolic processes.

We have found the mannitol in *U. fabae* predominantly to be produced from Fru by the action of a MTD encoded by *MAD1*. This enzyme was originally identified as an in planta-induced gene, *PIG8*, by Hahn and Mendgen (1997). They were able to show that gene expression was limited to haustoria and identified PIG8p as a short-chain alcohol dehydrogenase based on sequence homology. Further analysis revealed strong homology to a MTD from *A. bisporus* (Stoop and Mooibroek, 1998). *PIG8* was therefore renamed *MAD1*. The enzyme showed typical characteristics for a mannitol:$NADP^+$ 2-oxidoreductase (EC 1.1.1.138), both when expressed in yeast as well as in the native system. Kinetic parameters were very similar to the *A. bisporus* enzyme (Morton et al., 1985). With regard to other rust fungi, a NAD^+-dependent MTD activity was observed in axenic cultures of *P. graminis* f. sp. *tritici* (Maclean, 1990), whereas in spore extracts of *Melampsora lini*, a strong $NADP^+$-dependent but only a minor NAD^+-dependent MTD activity was found (Clancy and Coffey, 1980). The meaning of the differential cofactor specificity found in the different rust species remains to be elucidated.

Our results strongly indicate that the increasing levels of mannitol in *V. faba* plants infected with *U. fabae* can be correlated with the action of MAD1p (Fig. 6) and make an induction of a plant endogenous enzyme highly unlikely. Further evidence against a contribution of a plant enzyme stems from the fact that mannitol in plants is generated via a mannitol-1-P phosphohydrolase (EC 3.1.3.22) and oxidation of mannitol proceeds via a mannitol:NAD^+ 1-oxidoreductase (EC 1.1.1.255) producing Man instead of Fru (Stoop et al., 1996).

Immunolocalization of MAD1p is only partly consistent with the gene expression data reported by Hahn and Mendgen (1997). Besides a localization in haustoria, as expected from the expression data, substantial amounts of the enzyme are also found in spores (Fig. 7). However, the absence of any *MAD1* transcript in nongerminated and germinated spores (Hahn and Mendgen, 1997) indicates that MAD1p present in spores will consequently be diluted and possibly degraded during germination. The thermodynamic calculations reported in this article are in harmony with the numbers calculated for the *A. bisporus* enzyme (Morton et al., 1985) and indicate that the equilibrium favors the formation of mannitol. In haustoria, the level of Fru may be considered fairly high, since Fru is taken up by haustoria through the action of HXT1p (Voegele et al., 2001). Under these conditions, MAD1p would proceed in the reverse direction even if substantial amounts of mannitol are present. By contrast, we were not able to identify free Fru in uredospores of *U. fabae* presumably because it is rapidly fed into metabolism. Under high-mannitol/low-Fru conditions, MAD1p would proceed in the forward reaction. However, our thermodynamic calculations can only give a rough estimate of the direction of carbon flow. As an obligate biotrophic pathogen with no gene manipulation possible, definitive proof for the roles of any enzyme will be hard to come by. More detailed studies involving radiotracers as used by Manners et al. (1982) or in vivo NMR studies (Ceccaroli et al., 2003) might provide an alternative to address the question of carbon flow in *U. fabae* at a particular developmental stage.

Taken together, our results suggest a dual role for MAD1p: the formation of mannitol during the parasitic growth phase in haustoria and the mobilization of mannitol upon germination in uredospores. This scenario is consistent with the belief that biochemical materials are present in the spores ready to begin functioning (Gottlieb, 1976).

MATERIALS AND METHODS

Cultivation of Plants and Microorganisms

Cultivation of *Vicia faba* cv con Amore, inoculation with *Uromyces fabae* (Pers.) Schroet. uredospores, germination of spores, and growth of in vitro-grown infection structures were performed as described (Deising et al., 1991; Hahn and Mendgen, 1997). Yeast (*Saccharomyces cerevisiae*) strains were grown in SC medium with 2% Glc as a carbon source and dropout mix lacking uracil for selection of transformants (Sherman, 1991).

Nucleic Acid Manipulations

Molecular procedures were performed according to standard protocols (Sambrook et al., 1989). Yeast transformation was carried out according to Elble (1992). Sequencing was done using the Big-Dye Terminator Cycle Sequencing Ready Reaction mix (PE-Applied Biosystems, Foster City, CA) on an ABI 377 HT automated sequencer (GATC, Konstanz, Germany). Sequencing data were evaluated and analyzed using Chromas1.45 (Technelysium Pty., Helensvale, Australia) and DNA-STAR (DNASTAR, Madison, WI).

Plasmid Constructions

For expression of *MAD1* in yeast strain 23344c (Marini et al., 2000), the cDNA insert of the *PIG8* clone described by Hahn and Mendgen (1997) was excised via *Not*I digest and ligated into *Not*I digested and dephosphorylated vector pDR195 (Rentsch et al., 1995). Ampicillin-resistant transformants were screened for the correct orientation of the insert using colony PCR with vector- and gene-specific primers.

For the generation of anti-MAD1p antibodies, the complete open reading frame of *MAD1* without the native start codon was cloned into expression vector pET28a(+) (Novagene, Madison, WI) by introducing unique *Eco*RI and *Xho*I restriction sites via PCR using primers PIG8-5′-1, 5′-CCGAATTCA[82]-CATTTACCATTGACTTGAAGG-3′, and PIG8-3′-1, 5′-GTTCTCGAGG[905]-CAGTTTAACATGATTGGAGG-3′ (the introduced restriction sites are underlined and the numbers in brackets give the position of the preceding nucleotide in GenBank sequence U81790). The resulting plasmid pET28a(+)::*MAD1* encoded a His-tagged fusion protein consisting of 36 newly introduced amino acids at the N terminus (including a 6x-His cluster) and amino acids 2 to 256 of MAD1p. All plasmid constructs were verified by sequencing.

Expression of His-Tagged MAD1p Fusion Protein and Antibody Generation

Overexpression of the fusion protein encoded by plasmid pET28a(+)::*MAD1* was done using *Escherichia coli* strain BL21(DE3) and induction with isopropyl-β-D-thiogalactopyranoside (Studier and Moffat, 1986). Purification of the fusion proteins was performed using immobilized metal ion affinity chromatography under denaturing conditions according to the manufacturer's protocol (the QIAexpressionist; Qiagen, Hilden, Germany). Antibodies were obtained by repeated biweekly injection of a female New Zealand White rabbit with 500 μg purified fusion protein together with Freund's adjuvant. Serum S717 was purified in a two-step procedure as described (Voegele et al., 2001). The antigen used for negative adsorption was encoded by pET28a(+)::*MAD2*, another fusion protein identical to the MAD1 fusion protein with respect to the newly introduced amino acids, except for two.

Enzyme Extraction

Protein was prepared from infected and noninfected plants, in vitro infection structures, and uredospores by grinding the tissue in extraction buffer together with 1/10 of its wet weight of acid-washed sea sand. Extraction from transgenic yeast used extensive vortexing of cells in extraction buffer together with an equal volume of 1-mm glass beads after washing the cells once with water. This was followed by two centrifugation steps at 16,000*g* and 45,000*g*, respectively. Extraction buffer for maximum MAD1p activity and stability was 100 mM Tris-HCl, pH 9.0, 200 mM NaCl, 20% glycerol.

Enzyme Assays

MAD1p activity was assayed following the absorbance change of $NAD(P)^+$/NAD(P)H at 340 nm. A molar extinction coefficient for NAD(P)H of 6.22×10^6 cm^2 mol^{-1} was used for calculations. Reactions were monitored with a HITACHI U-2000 spectrophotometer or a TECAN Spectra Classic ELISA reader operated with TECAN Magellan2 software (TECAN, Grödig, Austria). Standard reaction buffer for the forward reaction was 50 mM glycin/NaOH, pH 10, 200 mM NaCl, 10% glycerol. For the reverse reaction, 100 mM citrate/disodium phosphate buffer, pH 5.2, was used. For kinetic experiments, substrate concentrations were varied between 6 mM and 1.82 M and cofactor concentrations were between 6 and 700 μM. For kinetic fingerprinting, the

following substrate and cofactor concentrations were used: Rev1 (465 mM Fru/100 μM NADPH), Rev2 (100 mM Fru/100 μM NADPH), and Fwd1 (500 mM mannitol/400 μM $NADP^+$). Enzyme activity is expressed as micromoles per minute per milligram total protein.

Protein concentrations were determined by the Bradford method (Bradford, 1976) using the Bio-Rad protein assay dye (Bio-Rad, Hercules, CA) following the microassay procedure with dilutions of bovine serum albumin as standard.

Extraction of Apoplastic Washing Fluids from Leaves

Leaves from 24-, 26-, and 33-d-old plants were cut 7 to 8 h after the onset of illumination and carefully washed with deionized water. Leaves were placed into a syringe filled with 40 mL deionized water and infiltrated by pulling the plunger, producing a reduced pressure of approximately 20 kPa. Thereafter, intact leaves were blotted dry, positioned with the xylem wound up in a 10-mL vessel, and centrifuged immediately at 75*g* for 4 min at 4°C. Due to the fact that infiltration of the apoplastic air space leads to a dilution of the AF, the solute concentrations in the apoplastic washing fluid (AWF) have to be corrected by the ratio volume of the infiltration solution (V_{inf}, which corresponds to the volume of the apoplastic air space V_{air}) to the volume of the apoplastic water space (V_{water}). Calculation of the ion concentration in the apoplastic water space was done by multiplying the ion concentration in the AWF by the dilution factor ($F_{dil} = V_{water} + V_{air}/V_{water}$). V_{water} was 111 μL g^{-1} FW and V_{air} was 885 μL g^{-1} FW (Lohaus et al., 2001). These data revealed that the concentrations of the AF were diluted 9-fold by the infiltration procedure. Cellular contamination of AWFs was quantified by comparing the activity of malate dehydrogenase in the AWF with leaf extracts (Lohaus et al., 2001). The cellular contamination was always below 0.1%.

Extraction of Metabolites from Leaves, Infection Structures, and Uredospores

Metabolites were extracted from infected and noninfected plants, in vitro-grown infection structures, and uredospores by grinding the tissue in liquid nitrogen. After addition of 5 mL chloroform:methanol (1.5:3.5, v/v), the sample was homogenized further with mortar and pestle until completely thawed and kept on ice for 30 min. The homogenate was then extracted twice with 3 mL water. The aqueous phases were combined and evaporated in a rotary evaporator. The dried residue was dissolved in 1.5 mL water and stored at −80°C until analysis.

Sugar and Sugar Alcohol Analysis

Sugars in tissue extracts and AWFs were assayed by HPLC. An ion-exchange column (CarboMA1; Dionex, Sunnyvale, CA) was eluted isocratically with 500 mM NaOH (flow rate of 0.4 mL min^{-1}). Sugars and sugar alcohols were detected by a Pulse Amperometric detector with gold electrode (ESA, model 5200; Coulochem II, Bedford, MA). Pulse setting was at 50, 700, and −800 mV for 500, 540, and 540 ms, respectively.

ROS Quenching Assay

The ability of mannitol to quench ROS distal to H_2O_2 was assayed by coupling the Fenton reaction to the cleavage of KMB. KMB is fragmented by the attack of ROS yielding ethylene. The amount of ethylene directly correlates with the amount of ROS formed (von Kruedener et al., 1995) and was determined by GC. The reaction mixture contained 0.1 M phosphate buffer, pH 7.4, 1 mM KMB, 10 μM H_2O_2, 10 μM $FeSO_4$, 50 μM EDTA, and mannitol in different concentrations. Each vessel was sealed gas tight and placed in a waterbath at 30°C. For quantification, 1 mL of gas was withdrawn after 30 min. Samples were analyzed using a Varian GC (Varian Aerograph 3300) equipped with a one-eighth-in × 100-cm aluminum oxide column and flame ionization detector. The GC was set up as follows: column temperature 60°C, injector temperature 80°C, and flame ionization detector temperature 225°C. Retention time of ethylene is 0.9 min under these conditions.

Immunocytochemistry

Microscopic analyses were performed as described (Voegele et al., 2001).

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