A Putative Amino Acid Transporter Is Specifically Expressed in Haustoria of the Rust Fungus *Uromyces fabae*

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A cDNA library constructed from haustoria of the rust fungus *Uromyces fabae* was screened for clones that are differentially expressed in haustoria. One family of cDNAs (in planta-induced gene 2 [*PIG2*]) was isolated and found to encode a protein with high homologies to fungal amino acid transporters. A cDNA clone containing the complete coding region of *PIG2* and the corresponding genomic clone were isolated and sequenced, revealing the presence of 17 introns in the *PIG2* gene. Expression of *PIG2* mRNA appeared to be restricted to haustoria. With antibodies raised against synthetic peptides, the *PIG2*-encoded protein was found in membrane fractions of isolated haustoria but not of germinated rust spores. With immunofluorescence microscopy, the putative amino acid transporter was localized to plasma membranes of the haustorial bodies, but not detected in the haustorial neck, haustorial mother cells, or intercellular fungal hyphae growing within infected leaf tissue. These data present for the first time molecular evidence that the rust haustorium plays a special role in the uptake of nutrients from an infected host cell.

Additional keywords: permease, plant pathogen.

Fungal haustoria are formed within living cells of a host organism. Three taxonomically diverse groups of obligate plant pathogens, namely the rust fungi (Basidiomycetes), the powdery mildew fungi (Ascomycetes), and the downy mildew fungi (Oomycetes), share the ability to differentiate these specialized infection structures. Haustorial fungi have been extremely successful during the course of evolution. Most land plants suffer from at least one of these pathogens. Because of their apparent significance for biotrophic nutrition, and the problems encountered in purifying them for biochemical studies (Bushnell 1972), haustoria have been extensively studied by cytological techniques (Harder and Chong 1991). In the plant cell, they are surrounded by an invagination of the host plasma membrane, called the extrahaustorial membrane. The fungal plasma membrane is surrounded by the haustorial

wall and the extrahaustorial matrix. Thus, the fungal wall and a matrix separate the plasma membranes of the two organisms. Although it is a long-standing opinion that haustoria play a major role in the absorption of plant metabolites, this has been clearly demonstrated so far only for powdery mildews (Manners and Gay 1982; Aist and Bushnell 1991). In rust fungi, few experimental data are available that support the role of haustoria in nutrient uptake (Mendgen 1981).

Functional studies on haustoria are difficult because they exist within host cells. Nevertheless, isolated haustoria have recently been used for the identification of their molecular components. Monoclonal antibodies have been generated that recognize specific glycoprotein epitopes of powdery mildew haustorial complexes (Mackie et al. 1991; Callow et al. 1992). We have isolated haustoria of the rust fungus *Uromyces fabae* from infected leaves (Hahn and Mendgen 1992) and used them for the preparation of mRNA and the construction of a haustorium-specific cDNA library. Differential screening of this library resulted in the isolation of 31 different clones that are strongly expressed in the rust fungus during biotrophic growth but not in hyphae formed in the absence of the plant (Hahn and Mendgen 1997). Sequence analysis of some of these in planta-induced genes (*PIGs*) revealed similarities of their translation products to proteins involved in vitamin B1 biosynthesis, short chain dehydrogenases, cytochrome P-450s, metallothioneins, and peptidyl prolyl isomerase (Hahn and Mendgen 1997). In this paper, we report that one of these genes, *PIG2*, encodes a putative amino acid transporter that appears to be expressed only in plasma membranes of haustoria.

RESULTS

PIG2 encodes a putative amino acid permease.

Differential screening of a haustorium-specific cDNA library resulted in the isolation of 31 families of cDNAs representing *PIGs* (Hahn and Mendgen 1997). Clones of each of these families were sequenced and compared with the protein data bases. The translated sequence of a 1.6-kb cDNA (*PIG2*-c54) revealed high similarity to amino acid permeases from other fungi. The missing 5' terminal part of the *PIG2* cDNA was amplified by 5' RACE (rapid amplification of cDNA ends; Frohman et al. 1988), with mRNA from rust-infected leaves (5 days after inoculation) as a template. The amplified cDNA fragment (clone *PIG2*-c7) was used as a probe for the

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length *PIG2* cDNA (clone *PIG2*-c10), and of another cDNA (*PIG2*-c8) of only 0.8 kb that contained the longest stretch of 5' sequence (Fig. 1). From these data, it was apparent that *PIG2*-c7, which was obtained by 5' RACE, did not represent the 5' end of the *PIG2* mRNA. In none of the cDNA clones

Fig. 1. Sequence and translation of in planta-induced gene (*PIG*) *PIG2* cDNA. Arrows with numbers in brackets indicate the termini of cDNA clones. The 3' terminus of *PIG2*-c8 was not determined. Two large arrows indicate the positions of the two primers used for 5' RACE (rapid amplification of cDNA ends). The two bases flanking each intron in the genomic sequence are underlined. The translation of two open reading frames is shown, the second of which encodes the putative amino acid permease. Hydrophobic stretches of amino acids that could represent transmembrane domains are underlined.

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1                               51
FIG2 .....M ILIQKKDQKNDSPRIEDELEKHANQAYYESTEVNASSSPTGDGAVGE.NTVHRALEQRHI
INA1 .....MSKEESHVTPKEDGNVDVYQASTTV.LPSEGP...ERDANWFTRNGLNVDSFKK...KHYGPGMVE.....LERPMKARHL
PUTX .....MSPPSAKSMEEGRTPSVQYGYGDPKTLGEIEEHTATKRGLSSRQL
GAP1 MSNTSSYEKNNPDNLKHNGITIDSEFLTQEPITIPSNQSAVSDIDETGSGSKWQDFK.DSFKRVPKPIEVDPNLSAEKVAIITAQTP...LKHLKNRHL
CAN1 .....MPEDYEKMRGSSNESHQSKVQPISSSISKSNKTKKHQTDVFQDSDIEASSINDEFGEVKRDLKARHV
LYSP .....VSETKTTTEAPG...LRRELKARHL
+
CC ++ ***

101                          151
FIG2 SMIAIGGTIGTGLFLMGGEALNKGGLPLGLILGYMVMGLVYVYAMTIALGEMVTMFPVSG.SFTHYPCRFVDPALGFAVGWNYWYSLTIAVPSEVIAATIV.
INA1 HMIAIGGSIGAGFFVSGGALAKGGPGSLFVDFLIIGIMMNVVYALGELAIMYPVSG.SFYTYSARFIDPAWGFAMGWNYVLQWAAVLPLELTVCGIT.
PUTX QLLAIGGCIGTGLFVGTSTVLTQTGPAPLLMSYIVMASIVWFVNMVNLGEMTTYLPIRGVSVPYLIGRFTEPSIGFASGYNYWYSFAMLLACEVSTMALLS
GAP1 QMIAIGGAIGTGLLVGSGTALRTGGPASLLIGWGSGTGTMIYAMMALGELAVIFPISG.GFTTYATRFIDESFGYANNFNMYLQWLVLVPLEIVSASIT.
CAN1 SMIAIGGTIGTGLFISTGSLHTTGPMVMSLISFLFVTTICFSVTQSLGEMATIYIPISG.SFAQFVTRWVSKSCGAANGWLYWFSWAVTFGLELSVVGQV.
LYSP TMIAIGGSIGTGLFVASGATISQAGPGGALLSYMLIGLVMVYFLMTSLGELAAVMPVSG.SFATYQNYVEEGFGFALGWNYWYNWAVTIAVDLVAAQLV.
++++* **++*+ + * ** ++h h * h h h ***+ hh*h+* ++ + ++ *** ++++h ++h h +h

101                          251
FIG2 .VEYW.KAPVNV.VVWIITFIVLSCGINFFGVRWYGETEFAPAAVKVVAIVLILVIGIVIDIG...GGPOTHER...IGFRHFDPGPFNLHGIPGALG
INA1 .ISYWNSEIT.T.AAWISLPLGVIIINLFGALGYAEEFWASCFKLAATVIFMIIAFVLVLG...GGPKDGRYHEYWGARYWYDPGAFKN...G....
PUTX PLSCWNPDPNVGHCLGLIIEYWNPPVSVGLWIAIVLVESEFWFAGLKILAIIGLIILGVVLFPG...GGPNHER...LGFYRWQDPGAFNP.YLVPGDTG
GAP1 .VNFWGTDPKYR.DGFVALFWLAIVIINMFGVKGYGEAEFVSFIKVIITVVGFIILGIIILNCG...GGPTGG...YIGGKYWHPDGAFAAG...DTPGA
CAN1 .IQFW.TDAVPL.AAWISIFFVILTIFNFFPVKFGYGEVEFWIASIKIIAVFGWIIYAFIMVCGAGKTGP.....VGFRYWRNGYAWGDGILVNMNGK
LYSP .MNVWFPDTPGW.I.WSALFLGVIFLLNYISVRGFEAEYWSLKIIVTTVIVFIIIVGLVMIIGIFKGAQPAWG.....SNWTIGEAPFAG....G....
h * + ++ +h h h+h+ + *** +++ h*h + h h+h+h+h * +++ + +++ +++++

101                          351
FIG2 RFLAFWSVFTQASFSYM.GTEMVAITAGEAANPRKTPVKAIERVYRILVYFGLSTLVVGLLVYSSPQLLGS..TG..DASSPPFVIINRAGITV..L
INA1 .FKGFCVSVFTAASF.S.GTELVLGLAAESTNPTKNMPPGAIKQVFWRITIFYLGLFFVGLLINSDDPALLSS..AAYADSKASPFVLVKGKYAGLKG..F
PUTX KFLGFWTALIKSGFSFIFSPELITTAAGEVEAPRRNPKATKRFIYRVFTFYILGSLVIGVTVAYNPTLEAGVESGGSGAGASPFVVAIKTLVLEGSTM
GAP1 KFKGVCVSVFTAASF.S.GSELVLGLAAESVPRKSVKAAQVFWRITLFIYILSLLMIGLLVYNDKSLI.G..ASSVDAASPFVIAIKTHGIGK..L
CAN1 YVAAFVSGLINSIFTQ.GSELVAVTAGEA..SPRALRSARKVMFRILVYFVCLMFLMGLLVYNDPKLTQD..GGF..TRNSPFLIAMENSGTKV..L
LYSP .FAAMIGVAMIVGFSFQ.GTELIGIAAGESEDPKNI PRAVRQVFWRIILFYVFAILLIISLIIPYTDPSLLRN..DV.KDISVSPSTLVFQHAGLLS..A
+ + ++h *** + *** +*** + + h* * c ++h* h**h+ ++h+++++ ++ * + ***+h+ * h

101                          451
FIG2 PDMINVVILLSAISTASSKLYGSSRVLYGLSKDGMARPFSSKCNASGLPMWSLLATSSSTAALSFMCNLNSKAGVAFQWQNLAMTGILTWTVLVSVYL..
INA1 DHFMNLVILASVLSIGVSGVYGSRTLTALAQQGYAPKLFTYIDKSGRPLPSVIFLILFGFIAYVSLDATGPVVDWLLAISGLAALFTWGSVCLAH..
PUTX SSMLPSGSLPG..HPVTHGCYAGSEKLYSLAGEGQAPKIFTRTNRTGVPYVAVLATWTIGLLSFLNLSSSGQTVFYWFTNITTVGGFINWLVGIAYLVC
GAP1 PSVVNVVILIAVLVSGNSAIYACSRMTVALAEQRFLPEIFSVDKRGRPLVGIATVSAFGLIAFVAASKKEGEVFNWLLALSGLSSLTWGGICICHI..
CAN1 PHIFNAVIVTTIISAGNSNYSYSGRILYGLAQAGVAPKFFLTNTKGGVPFFAVFAAFAFGALGYLACSSQGNKAFWTLLNITATAGLISWGFISVSHI..
LYSP AAVMNAVILTAVLSAGNSGMYASTRMLYTLACDGKAPRIKAFKLSRGVPRNALYATTVIAGLCFLTSMFGNQTVYLVLLNTSGMTGFIWLGIAISHY..
hh+ +++ hh+ + + * +++ ++ *+ + +*ch* * * hhh+ + + +h ++ *+++ ++h * + h +h

101                          551
FIG2 .....FYQGLKVQGLSRDKLHYKAPFPQYASWIALAMLTLIMFMSGFETFLKGGWSIS....DFLANYLTLPIFLGFYLYWKI.....
INA1 .....RFRKAWKYHGHTLDEIPFKAAGVYGSYGLGFICIVILMAQPYTAAAPPSPGVGTAEDEFFKQYLAAPVVLGFWIVGWLW....
PUTX FPPSLHLNTPDQQRFRKALQFHGM.LDMLPFKTPLPQYGTYYVMPFIISLTLTNGYAVFFP....GRFTASDFLVSYIVFAIFLALYAGHKIYRT..
GAP1 .....RFRKALAAQGRGLDELSFKSPTGVWGSYGLFMVIMFIAQFYVAVFPVGDSPS...AEGFFEAYLSFPLVMVMYIGHKIY....
CAN1 .....RFMKTLQRRGISRDTLFPKAFMPFSAYYGMVVCFIVVLVIGFTVFD.....FNASDFFTAYISVILFVVLVWGFHFFFYGFG
LYSP .....RFRGRYVLQGHINDLPYRSGFFPLGPFIIFILCLITLQNGYEAFLKDTIDWGGVAAT....YIGIPLFLIIFWGYKLI....
+++++ + * +++++ +h+ +h hhh +h h + + +h ++ *h h+h++ h h+ h

101                          651
FIG2 ....STRSKFVSSKHVDVFTGTRELN...EMDAQEREKEIIPTTKWKGFIDKLL
INA1 ...KR..QPFLRTKNIDVDVTGLREFDWD...EINAERTRIAPLPARRRIIHHTF
PUTX .....PWLTKVSEVDIFTGKDEIDR.....LCENDMERQPRNWLRLVWVWIF
GAP1 ...KRNWKLFIAPAEKMDIDTGRREVLDLLKQEIABEKAIMATKPRWYRIWNFWC
CAN1 KDSFKMSNILVPLDECDIDSGVRDIN.....DAEFDIPPPKNAWDKFWANVA
LYSP ...KGT..HFVRYSEMFPQNDKK.....
+ +h ++ ++ + +

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Fig. 2. Alignment of the in planta-induced gene (*PIG*) *PIG2*-encoded protein with amino acid permeases. INA1: *Trichoderma harzianum* putative amino acid permease (P34054). PUTX: *Aspergillus nidulans* proline-specific permease (P18696). GAP1: *Saccharomyces cerevisiae* general amino acid permease (P19145). CAN1: *S. cerevisiae* arginine/lysine-specific permease (P43059). LYSP: *Escherichia coli* lysine-specific permease (P25737). *: completely conserved amino acids. +: in at least 4 sequences conserved amino acids. h: conserved hydrophobic amino acid (V, L, I, A, W, F, Y). The alignment was made with the Genetics Computer Group (Madison, WI) program MULTALIGN.

was a poly(A) tail observed, which was probably due to exonuclease activities during cDNA library construction (Hahn and Mendgen 1997). Therefore, the 3' terminus of the *PIG2* mRNA could not be determined. Inspection of the cDNA sequence covered by *PIG2*-c8 and *PIG2*-c10 revealed the presence of a very short 5' open reading frame encoding a peptide of 18 amino acids with unknown function. Downstream of this open reading frame was the start codon of another, long open reading frame encoding a putative protein of 561 amino acids, with a molecular mass of 61,712 Da. The *PIG2* protein showed extensive similarities to various amino acid permeases from other fungi and from *Escherichia coli* (Fig. 2), with 30 to 40% identical amino acids. Furthermore, its sequence indicated the presence of 12 highly hydrophobic, potentially membrane-spanning domains that are typical for a large family of

transport proteins from a variety of organisms (Fig. 1; Griffith et al. 1992).

A lambda EMBL3 genomic library of *U. fabae* was hybridized with a cDNA probe (*PIG2*-c54), and two hybridizing plaques were isolated. Restriction and hybridization analysis revealed that the two genomic clones, *PIG2*-g2 and *PIG2*-g4, were derived from the same genomic region. Both clones contained the complete *PIG2* gene in an overlapping region, confirming that they were authentic and not rearranged. From clone *PIG2*-g2, three *EcoRI* fragments of 4.9, 2.2, and 0.9 kb were subcloned. From these subclones, 6,325 bp covering *PIG2* were sequenced, consisting of 3,265 bp comprising the coding region including the introns, 1,817 bp of the 5' non-coding region, and 1,243 bp of the 3' noncoding region (Fig. 3; GenBank accession number U81794). Comparison with the cDNA revealed 17 introns, covering a total of 1,582 bp. The introns are in the size range from 68 to 153 bp, with an average size of 93 bp (Table 1). The 5' donor as well as the 3' acceptor splice sites were similar to the consensus sequences known from other fungi (Edelmann and Staben 1994). Less conservation of the position and sequence of the internal splice branch site was observed, similar to what has been described for introns in the saprophytic basidiomycete *Coprinus cinereus* (Table 1; Seitz et al. 1996).

To determine the copy number of *PIG2* in the rust genome, a *PIG2*-c54 probe was hybridized to various digests of *U. fabae* total DNA. The size of the *EcoRI* and *EcoRV* fragments that hybridized was identical to that of fragments found in two genomic *PIG2* clones (Fig. 4; data not shown), strongly indicating the presence of a single copy of *PIG2* in the genome of *U. fabae*.

Expression of *PIG2*.

Transcript levels of *PIG2* in different stages of rust development were determined by RNA blot analysis (Fig. 5). No hybridization signals were detected with RNA samples obtained from spores, germinated spores, or infection structures grown on inductive membranes, including germ tubes, appressoria, infection hyphae, and haustorial mother cells. A very strong hybridization signal was observed with haustorial RNA, and a weak signal with RNA from infected leaves. The

Table 1. Introns in the in planta-induced gene *PIG2*^a

No.	Size (bp)	Position	5' splice junction ^b	Branch site	3' splice junction ^b
1	153	209	G-GCAAGT	CCTAAT (-28)	TCAG-
2	112	288	T-GTGAGT	ATTGAT (-24)	ACAG-
3	86	322	G-GTTTGT	ACTAAC (-39)	ATAG-
4	87	407	G-GTATGT	ACTGAC (-39)	AAAG-
5	93	423	G-GTGGGT	GCTAAA (-17)	TCAG-
6	83	523	G-GTATGT	TCTAAT (-34)	CTAG-
7	92	577	G-GTGAGT	ACTAAC (-14)	ATAG-
8	87	593	G-GTAAGC	ACTAAC (-40)	TTAG-
9	76	653	G-GTGAGG	GTTAAC (-25)	CTAG-
10	101	687	G-GTGAGC	TCTGAC (-45)	ATAG-
11	68	762	G-GTAAGT	CCTGAG (-18)	ACAG-
12	80	882	G-GTCAGT	TCTGAT (-36)	CCAG-
13	76	1085	T-GTTAGC	TCTGAA (-24)	GCAG-
14	87	1309	G-GTAAGT	ACTCAG (-35)	ATAG-
15	77	1470	G-GTAAGT	GCTCAT (-19)	ACAG-
16	140	1536	G-GTTGGT	TTTGAA (-28)	ACAG-
17	84	1678	G-GTCAGT	ACTGAC (-19)	ACAG-
Consensus:			G-GTraGT	NCTRAy	aYAG-

^a The first base of each intron is numbered relative to the first base of the start codon of the large open reading frame. The positions (in parentheses) of the putative branch sites are counted from the 3' end of the introns to the conserved A residues.

^b Consensus sequences for *PIG2* are shown. Capital letters: 75 to 100% conservation. Lowercase letters: 50 to 75% conservation.

Fig. 3. Physical map of the in planta-induced gene (*PIG*) *PIG2*. Partial *EcoRI* restriction maps of two overlapping EMBL3 clones carrying *PIG2* are shown. Below is a restriction map of the sequenced DNA covering 6,325 bp. The transcribed region of *PIG2* is indicated by the large arrow. Sa: *SalI*. E: *EcoRI*. H: *HindIII*. V: *EcoRV*. S: *SspI*.

estimated size (2.3 kb) of the *PIG2* mRNA correlated well with the size of the *PIG2* cDNA (2,243 bp) composed of clones *PIG2*-c8 and *PIG2*-c10, confirming that the obtained cDNA sequence is near full-length. No cross-hybridization of the probe with leaf RNA was detected.

In order to study the expression of the *PIG2* protein, polyclonal antibodies raised against a mixture of three peptides of the deduced *PIG2* amino acid sequence were used for Western (immunoblot) analysis. The specificity of the antibodies was verified with yeast cells expressing the *PIG2* cDNA constitutively. In yeasts carrying the recombinant expression plasmid,

Fig. 4. Southern hybridization of genomic *Uromyces fabae* DNA with a digoxigenin-labeled in planta-induced gene (*PIG*) *PIG2* cDNA probe. Five micrograms of total DNA was loaded per lane, after digestion with the following enzymes. 1: *Kpn*I; 2: *Eco*RV; 3: *Hind*III; 4: *Eco*RI. Development of the nylon membrane was performed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Fig. 5. In planta-induced gene (*PIG*) *PIG2* mRNA levels during rust development. The top shows an ethidium bromide-stained agarose gel before transfer of the RNA, below is the autoradiograph with the hybridizing signals after chemiluminescent detection. Five micrograms of total RNA was loaded per lane. Lane 1: nongerminated urediospores. Lane 2: 4 h germinated spores. Lanes 3 to 6: in vitro differentiated rust infection structures. Lane 3: 6 h; lane 4: 12 h; lane 5: 18 h; lane 6: 24 h. Lane 7: haustoria. Lane 8: rust-infected *Vicia faba* leaves (5 days after inoculation). Lane 9: *V. faba* leaves.

but not in those containing only the vector, a protein of about 60 kDa, similar to the predicted size of the *PIG2* protein, was detected in microsomal membranes (Fig. 6A). In addition, microsomal membranes were isolated from 4-h-germinated, non-differentiated spores, and from isolated haustoria. Because the haustoria were contaminated with about twice the number of chloroplasts (Hahn and Mendgen 1997), chloroplast membranes were also analyzed as a control. An immunoreactive protein identical in size to that in yeasts expressing *PIG2* was detected in membranes from haustoria but not in membranes from chloroplasts or germinated rust spores (Fig. 6B).

Light microscopy of sections from rust-infected *Vicia faba* leaves with *PIG2*-specific antibodies and fluorescein-labeled secondary antibodies was performed. Significant peripheral fluorescence of haustorial bodies was observed. In most cases, the labeling was weaker or absent in the proximal parts of the haustorial bodies. No labeling of haustorial necks, haustorial mother cells, or intercellular hyphae was observed (Fig. 7). After incubation with preimmune serum, no specific fluorescence of fungal structures was observed. Preincubation of the *PIG2* antibodies with the three peptides used for their generation suppressed the labeling of the haustorial surfaces (data not shown).

DISCUSSION

Differential screening of a cDNA library from rust haustoria has allowed the identification of *PIGs* (Hahn and Mendgen 1997). Their patterns of regulation suggest that the *PIGs* fulfill special functions for parasitic growth of the rust fungus. By sequence analysis, *PIG2* and *PIG27* were preliminarily identified as genes encoding amino acid transporters. It seems that the capacity for amino acid uptake in the rust fungus is fully induced only during biotrophic growth. This finding is in line with the observation that artificial cultures of rust mycelium that cannot differentiate haustoria exhibit only delayed, slow growth and not the abundant sporulation observed in leaves (Williams 1984; Fasters et al. 1993). By application of radio-

Fig. 6. Identification of the in planta-induced gene (*PIG*) *PIG2*-encoded protein by Western blot (immunoblot) analysis. Five micrograms of microsomal membrane proteins was loaded per lane. **A**, *Saccharomyces cerevisiae* strain JT16 (Tanaka and Fink 1985), carrying a recombinant plasmid expressing *PIG2* (lane 1) and the vector pDR195 (lane 2), respectively. **B**, Lane 1: 4 h germinated rust spores. Lane 2: haustoria. Lane 3: chloroplasts. Lane 4: prestained molecular mass standards (BIORAD, Hercules, CA).

labeled amino acids to rust-infected wheat plants, it has been demonstrated that the amino acids in the rust mycelium are derived mainly from the host plant and only to a small extent by fungal biosynthesis (Jäger and Reisener 1969). To understand the regulation and mechanism of amino acid transport in rust fungi in more detail, we have started to analyze structure and expression of the *PIG2* gene.

The *PIG2* cDNA was found to contain two open reading frames starting with ATG. The role of the upstream open reading frame, which could encode 18 amino acids, is not clear, but the nucleotides surrounding this codon do not fit the rules that apply for efficient translation initiation by eukaryotic ribosomes (Kozak 1989). The second open reading frame, which follows 78 bp downstream, was found to encode a protein of 561 amino acids with high similarity to amino acid permeases from other fungi and *E. coli* (Sophianopoulou and Diallinas 1995). *PIG2* is present as a single copy gene in the genome of *U. fabae* and contains 17 introns. This is one of the highest numbers of introns reported for fungal genes. A very large gene (*rad9*), containing 26 introns and encoding a protein of 2,157 amino acids, has been described recently from *Coprinus cinereus* (Seitz et al. 1996).

The expression of *PIG2* appears to be strictly regulated during rust development. *PIG2* mRNA was not detectable in any of the rust growth stages that are formed by the fungus in the absence of the plant, such as appressoria, infection hyphae, and primary haustorial mother cells. In contrast, high levels of *PIG2* mRNA were detected in haustoria. The presence of comparatively low amounts of *PIG2* transcripts in rust-infected leaves is probably due to the dilution of the fungal transcripts by plant RNA. With anti-peptide antibodies, a 60-kDa membrane protein was detected both in transgenic yeasts expressing a *PIG2* cDNA from a plasmid and in haustoria, but not in germinated spores. In rust-infected leaf tissue, anti-*PIG2* antibodies labeled exclusively the periphery of haustorial bodies, indicating the location of the *PIG2* protein in haustorial plasma membranes. Interestingly, the border of membrane labeling was not identical to the neckband, which is located in the middle of the haustorial neck, but was in the proximal area of the haustorial body. No labeling was observed of the other fungal structures (haustorial neck, haustorial mother cell, intercellular hypha) growing in the plant. Al-

though we cannot exclude a very low expression in other parts of the mycelium, our data suggest that *PIG2* is expressed only in haustoria.

In yeast and *Aspergillus nidulans*, amino acid transporters are either constitutively expressed, or they are regulated in response to physiological conditions, e.g., by substrate induction or catabolite repression in the presence of nitrogen sources such as ammonia (Sophianopoulou and Diallinas 1995). Interestingly, in the mycoparasitic fungus *Trichoderma reesei*, a gene encoding a putative amino acid transporter was found to be induced by mycelial wall fragments of the host fungus *Rhizoctonia solani* (Vasseur et al. 1995). Similar to *U. fabae*, a signal from the host organism might induce morphological and physiological events related to parasitic functions. In rust fungi, the natural trigger leading to haustorium formation is unknown (Heath 1995).

A number of membrane transporters from a variety of organisms have been shown to be active and properly targeted when expressed in yeast (Frommer and Ninnemann 1995). The *PIG2* protein was expressed in yeasts, but no functional complementation of several amino acid uptake mutants (for His, Pro, Arg, Lys, Asp, Glu) was observed by plate tests (data not shown). Nevertheless, we think that the *PIG2* protein is an amino acid permease, since its similarity to fungal amino acid permeases (30 to 40%) is in the same range as the homologies found between the different yeast permeases. Possibly, the *PIG2* protein is specific for amino acid(s) that have not yet been tested for by complementation of appropriate mutants.

Evidence for the role of rust haustoria in nutrient uptake has been obtained previously only by cytological studies. Rapid translocation of radioactively labeled amino acids from rust-infected leaves into haustoria was shown by electron microscopic autoradiography (Mendgen 1981). With cytochemistry in combination with electron microscopy, vanadate-sensitive ATPase activity was shown to be present in the wall-lining plant plasma membrane but not in the extrahaustorial membrane. Variable results were obtained for the haustorial plasma membranes that were reported to contain or to lack ATPase activity, depending on the rust species (Baka et al. 1995).

Recently, a biochemical characterization of the rust plasma membrane ATPase was performed. The enzyme was found to be severalfold more active in haustoria of *U. fabae* than in spores or germ tubes (Struck et al. 1996), which emphasizes its importance for haustorial function. The fungal amino acid permeases to which *PIG2* is homologous are symport carriers that transport their substrates together with protons (Horak 1986). These carriers are using the transmembrane pH gradient generated by the activity of the plasma membrane ATPase. Because *PIG2* is haustorium-specifically expressed, the metabolite(s) it transports might not be taken up by other hyphae of the rust fungus. Taken together, our data provide molecular evidence for the hypothesis that the activity of the H⁺-ATPase cooperates with symport carriers in the haustorial plasma membrane to achieve an efficient uptake of plant metabolites (Fig. 8).

MATERIALS AND METHODS

Plant and fungal materials.

Broad bean plants (*Vicia faba* cv. Con Amore) were cultivated as described (Deising et al. 1991). Spray inoculation of

Fig. 7. Detection of the in planta-induced gene (*PIG*) *PIG2*-encoded protein in rust-infected leaves by immunofluorescence microscopy. **A**, Early stage of haustorium development. **B**, Fully developed haustorium. Only the surface of the haustorial bodies (h) is showing fluorescence labeling. Haustorial neck (arrow), haustorial mother cells (m), and intercellular hyphae (i) exhibit no fluorescence ($\times 2,200$).

the plants with *Uromyces fabae* race I₂ and isolation of haustoria were performed as described (Hahn and Mendgen 1992).

DNA and RNA manipulations.

Total DNA from *U. fabae* was isolated as described (Hahn and Mendgen 1997). Plasmid isolation, subcloning, transformation of *E. coli*, and polymerase chain reactions were performed according to standard protocols (Sambrook et al. 1989).

Sequencing of the *PIG2* cDNAs was done with the Δ Taq cycle sequencing kit (Amersham, Buckinghamshire, U.K.) with digoxigenin-labeled sequencing primers, and the direct blotting electrophoresis system (GATC, Konstanz, Germany; Pohl and Maier 1995). The genomic *PIG2* DNA was sequenced with the ABI PRISM dye terminator sequencing kit (Perkin Elmer, Foster City, CA) and an ABI 373 automated sequencer. Analysis of the DNA sequences was performed with the software package from the Genetics Computer Group (GCG; Madison, WI).

Isolation of RNA from rust and leaf tissue was done as described (Hahn and Mendgen 1997). For amplification of the 5' terminal *PIG2* cDNA end (5' RACE), an anchor oligonucleotide ligated to the cDNA by T4 RNA ligase was used (Edwards et al. 1991). The position of the *PIG2*-specific primers used for 5' RACE is shown in Figure 1. They have the following sequence: RACE 1: CTCTCCGTACCATCTCAC; RACE 2: CGTGATGATCCAAACGACC. Construction of a

λ gt10 cDNA library from rust-infected *V. faba* leaves (5 to 6 days after inoculation) was done as described for the haustorial cDNA library (Hahn and Mendgen 1997).

For expression of the *PIG2* protein, a 2.2-kb *NotI* fragment representing clone 10 was inserted into the plasmid pDR195 in an orientation to allow expression of the *PIG2* cDNA from the constitutive yeast *PMAI* promoter (Rentsch et al. 1995). Transformation of yeast was done as described (Dohmen et al. 1991).

Hybridization experiments with digoxigenin-labeled DNA probes were performed according to the standard protocols supplied by Boehringer Mannheim, except for Northern (RNA) hybridizations, which were done as described (Hahn and Mendgen 1997).

Generation of antibodies.

Antibodies against the *PIG2* protein were generated after synthesis of three peptides from the *PIG2* coding region—PEPPIG2a:(C)GLSRDKLHYK (449 to 458); PEPPIG2b:(C)TRSDVFSSKH (516 to 525); PEPPIG2c:(C)NEMDAQEREKEIPTTKWKGKFDKLL (536 to 561). The three peptides were coupled as a mixture via their N-terminal cysteines to keyhole limpet hemocyanine and the conjugate injected into rabbits (Baldwin 1994). The antiserum was affinity purified with a Sepharose 4B column to which the three peptides had been coupled (Pagano 1996).

Membrane preparation and immunological detection.

Microsomal membranes from germinated rust spores and from isolated haustoria were prepared as described (Struck et al. 1996). Membrane proteins were separated by sodium dodecyl sulfate polyacrylamide electrophoresis (Schägger and von Jagow 1987) and transferred to a polyvinylidene difluoride membrane (Kyhse-Andersen 1984). Immunological detection was performed with affinity-purified antibodies diluted 1:10,000, according to Blake et al. (1984).

Immuno-microscopy.

Rust-infected leaf pieces (8 days after inoculation) were vacuum-infiltrated with 8% (vol/vol) methanol in water and high pressure frozen with an HPM 010 high pressure instrument (Balzers, Liechtenstein) as described (Mendgen et al. 1991). Specimens were freeze substituted in acetone at -90°C and embedded in a mixture of 75% butylmethacrylate and 25% methylmethacrylate, 0.5% benzoinethylether and 10 mM dithiothreitol (Aldrich Chemical Corp., Milwaukee, WI) in mixtures with acetone (20, 50, 75, and 100%) for 1 day each at 4°C . Polymerization took place at 4°C during 4 days under UV light. Sections, 1 to 2 μm thick, were spread with water on microscope slides coated with Biobond (British Biocell, Cardiff, U.K.), and allowed to dry at 30°C . The slides were treated 3 times for 15 min with blocking buffer (1% [wt/vol] bovine serum albumin, 1% [wt/vol] autoclaved yeast cell walls, in TBS [10 mM Tris-HCl, 150 mM NaCl, pH 7.4]), and incubated with primary antibodies (preimmune serum or affinity-purified antibodies), diluted 1:50 with TBS, for 1 h. After three washes for 15 min with TBS, sections were incubated with the secondary antibody (fluorescein-conjugated goat anti-rabbit IgG, Dianova, Hamburg, Germany), diluted 1:50 with TBS, for 1 h at 20°C . After three rinses (3 min each) with TBS and a flush with water, specimens were

Fig. 8. Model for the secondary active transport of amino acids from an infected leaf cell into a rust haustorium (AA = amino acid).

mounted in citifluor (Citifluor Inc., London, U. K.). Samples were examined with a Zeiss Axioscop microscope equipped for epifluorescence (filters BP 490, FT 510, LP 565).

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