

Characterization of In Planta–Induced Rust Genes Isolated from a Haustorium-Specific cDNA Library

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Rust fungi are plant parasites that depend on living host tissue for growth. For invasion of leaves, dikaryotic urediospores differentiate germ tubes and infection structures that penetrate through stomata. Biotrophic growth occurs by intercellular mycelia that form haustoria within host cells. A cDNA library was constructed from haustoria isolated from broad bean leaves infected by *Uromyces fabae*. Differential screening revealed that a high proportion (19%) of the haustorial cDNAs are specifically expressed in planta but are not expressed, or are much weaker, in germlings or infection structures produced in vitro. A total of 31 different in planta–induced genes (PIGs) were identified. Some of the PIGs are highly expressed in haustoria. The PIGs are single or low copy number genes in the rust genome. A variety of developmentally regulated expression patterns of PIG mRNAs were observed. Sequence analysis of PIG cDNAs revealed similarities to genes encoding proteins involved in amino acid transport, thiamine biosynthesis, short-chain dehydrogenases, metallothioneins, cytochrome P-450 monooxygenases, and peptidyl-prolyl isomerases.

Additional keywords: Uredinales, *U. viciae-fabae*.

The rust fungi (order: Uredinales; phylum: Basidiomycetes) are a large group of biotrophic obligate plant pathogens with a complex life cycle, including up to five different spore types (Littlefield 1981). During infection, dikaryotic rust hyphae show a high degree of morphogenetic and physiological differentiation. After landing on the epidermis, urediospores of *Uromyces fabae* firmly attach to the cuticle by forming an adhesion pad containing esterases, including cutinase (Deising et al. 1992). Germ tube growth occurs in close contact with the leaf surface. Topographical features of the stomatal guard cells and possibly also chemical signals induce the formation of an appressorium and additional infection structures, which invade the plant through the stomatal opening (Staples and Hoch 1987). In the leaf parenchyma, the rust fungus spreads with a mycelium consisting of intercellular hyphae, including haustorial mother cells, and haustoria that are formed within host cells. During infection structure development, cell-wall-

degrading enzymes are released by the rust fungus in a sequential and differentiation-dependent fashion (Deising et al. 1995; Mendgen et al. 1996). Haustorium formation occurs only within living host cells and probably requires other, yet unknown, signals from the plant (Heath 1995).

Very little is known about the physiology of rust fungi growing within host tissue. An intriguing phenomenon is the apparent absence of plant defense responses in a compatible interaction. There is some evidence for the release of suppressors by rust hyphae growing in the host (Heath 1995). Only a few facts are known about the mechanism of biotrophic nutrient uptake. Recent data favor an important function of the rust plasma membrane H⁺-ATPase and a haustorium-specific amino acid permease that seem to cooperate in the energy-dependent metabolite transport from the infected host cell into the haustorium (Struck et al. 1996; Hahn et al. 1997).

In order to gain further insight into haustorium-related functions, we have followed a molecular genetic approach. The availability of a method for the preparative isolation of rust haustoria from infected leaves (Hahn and Mendgen 1992) facilitated the construction of a haustorium-specific cDNA library. By differential screening, a large number of cDNAs were isolated that are specifically expressed in planta. Sequence analysis of these genes revealed various functions related to biotrophic growth, such as nutrient uptake and vitamin biosynthesis.

RESULTS

Construction of a haustorium-specific cDNA library.

Rust haustoria were isolated from infected *Vicia faba* leaves by ConA affinity chromatography (Hahn and Mendgen 1992). Due to a substantial proportion of chloroplasts in the haustorial preparations, the extent of contamination of the haustorial RNA with chloroplast RNA was analyzed. RNA was obtained from haustorial preparations (which also contained chloroplasts) in high yields, whereas only low amounts of RNA were extracted from a similar number of pure chloroplasts. From a sample containing 1.4×10^9 haustoria and 2.9×10^9 chloroplasts, 1.3 mg of RNA was obtained, with an estimated contamination by chloroplast RNA of 5.5%. Evaluation of the rRNA banding patterns after agarose electrophoresis revealed that only moderate degradation of the haustorial RNA had occurred (Fig. 1, top).

From haustorial RNA, poly A⁺ RNA was prepared and used for the construction of a cDNA library, with the bacteriophage vector λ gt10. A total of 3×10^7 recombinant clones was obtained, with an average insert size of about 1 kb.

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Nucleotide and/or amino acid sequence data are to be found at GenBank as the following accession numbers: U81789 (PIG1); U81790 (PIG8); U81791 (PIG11); U81793 (PIG16); U81792 (PIG28).

Differential hybridization of the cDNA library.

To search for cDNAs showing preferential expression in haustoria, a differential screening approach was followed. For quantitative evaluation of the hybridization results, individual cDNAs of the library were amplified directly by polymerase

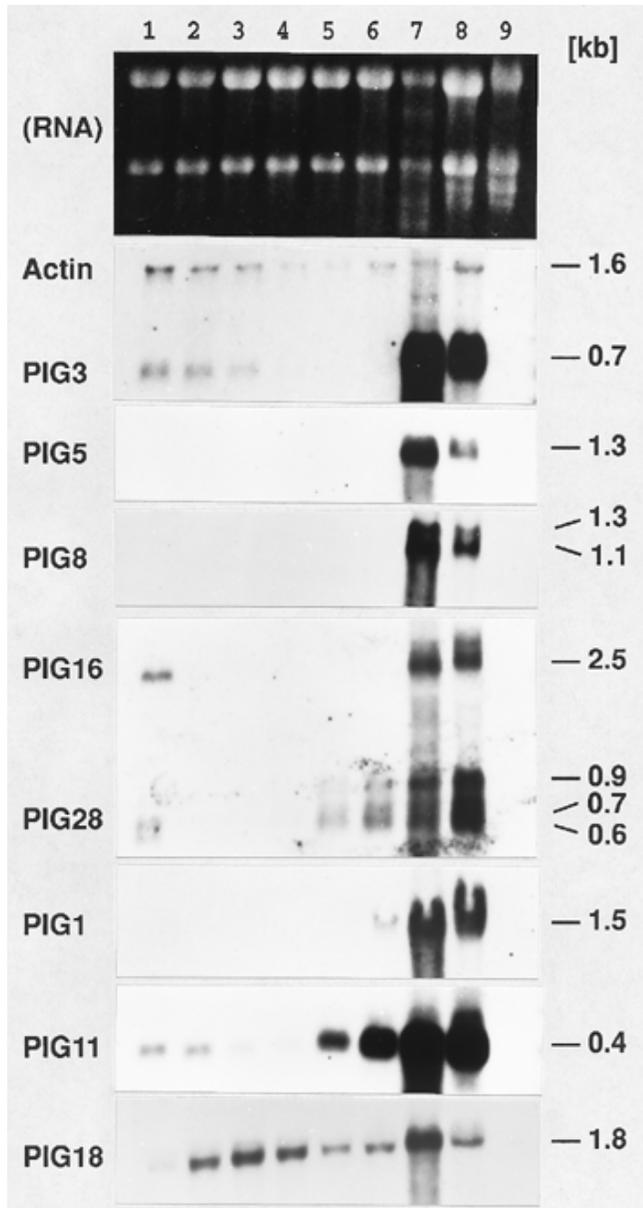


Fig. 1. Transcript levels of in planta-induced genes (*PIGs*) during rust development. Four micrograms (infected leaves: 8 μ g) of total RNA was loaded per lane. On the top, a representative agarose gel with ethidium bromide-stained samples demonstrates similar RNA amounts in all lanes; this was confirmed for each gel used for Northern (RNA) blotting. Two RNA filters were hybridized with two probes, with actin plus *PIG3*, and with *PIG16* plus *PIG28*, respectively. The remaining filters were hybridized with individual cDNA probes as indicated on the left side of the panels. Lane 1: nongerminated urediospores. Lane 2: 4 h germinated spores. Lanes 3 to 6: in vitro differentiated rust infection structures; lane 3: 6 h old; lane 4: 12 h old; lane 5: 18 h old; lane 6: 24 h old. Lane 7: isolated haustoria. Lane 8: rust-infected *Vicia faba* leaves (5 days after inoculation). Lane 9: *V. faba* leaves.

chain reaction (PCR) from 169 randomly selected plaques, with vector-specific primers flanking the *EcoRI* cloning site. Similar amounts of the PCR products were applied as dots to a nylon membrane. For successive rounds of hybridization, single-stranded cDNA probes derived from the following samples were used in the following order: (i) haustoria; (ii) 21-h-old urediospore-derived infection structures formed on thigmo-inductive polyethylene membranes (Deising et al. 1991); (iii) rust-infected broad bean leaves (5 days after inoculation); and (iv) urediospore germlings (Fig. 2A). A high proportion (32 out of 169, 19%; 168 clones shown) of the haustorial cDNA clones hybridized to the single-stranded cDNA probes from haustoria and infected leaves but did not hybridize, or hybridized only very weakly, to the probes representing in vitro growth stages of the rust (for instance E1, E2 and E3; Fig. 2). These differentially hybridizing cDNAs appeared to be specifically expressed during biotrophic growth. Therefore the corresponding genes were called *PIGs* (in planta-induced rust genes). In most cases, the *PIG* cDNAs showed stronger hybridization with the cDNA probe from haustoria than with the cDNA probe from rust-infected leaves, presumably due to the fact that the fungal mRNAs represented only a fraction of mRNA isolated from infected leaves. Seventeen (10%) of the 169 clones hybridized to the cDNA probe from noninfected leaves, and all of these clones hybridized with similar intensity to the rust cDNA probes (e.g., L5 and C9; data not shown). These data indicated that the vast majority of the clones of the "haustorial" cDNA library were of fungal origin.

Classification of *PIGs*.

A total of 145 independent *PIG* cDNA clones were purified, and their phase-specific expression was confirmed by repeating the differential hybridization in the same manner as shown in Figure 2. Cross-hybridization experiments of the filter-bound cDNAs were performed with individual *PIG* cDNA probes, followed by sequencing of cDNA clones from each hybridization group. It was found that the 145 cDNAs represent transcripts from 31 different genes or gene families (Table 1). Two clones were identified as false positives, containing cDNA sequences derived from chloroplast RNA (data not shown).

Based on their frequent occurrence among the collection of *PIG* cDNAs, and the strong hybridization signals they produced in the differential screening, it is concluded that expression of some of the *PIGs* is very high. This was confirmed by hybridizing individual *PIG* cDNAs with known numbers of clones from the non-amplified haustorial cDNA library. With a *PIG1* cDNA probe, 2.8% of the cDNA clones in the library hybridized, indicating that *PIG1* mRNA constitutes a similarly high proportion of haustorial transcripts. Other *PIGs* were detected with similar frequencies, indicating that they are also highly expressed in haustoria (Table 1).

PIGs are single or low copy number rust genes.

Genomic Southern hybridizations were performed with labeled cDNA probes obtained from 11 *PIGs*. In all cases, the probes hybridized only to fungal but not to plant DNA, except for *PIG1*, which showed a weakly cross-hybridizing band with DNA from *Vicia faba* (Fig. 3; data not shown). In the case of *PIG4*, only single hybridization bands were observed

in the lanes containing restriction-digested rust DNA, indicating the presence of a single copy gene. With *PIG11*, two hybridizing bands were observed, which are probably due to the presence of two genes. Probes representing other *PIGs* gave sometimes ambiguous results. For instance, with *PIG8* cDNA as a probe, single hybridization bands were observed in the lanes containing rust DNA digested with *Bam*HI, *Xba*I, and *Eco*RV. In contrast, *Eco*RI-digested DNA hybridized with two major (1.0 kb and 7 kb) and two minor (0.5 kb and 1.4 kb) bands, although no *Eco*RI site was present within the *PIG8* cDNA (Fig. 3). In summary, the results of the Southern hybridizations demonstrate that the *PIGs* are single or low copy number genes in *U. fabae*.

Different expression patterns of *PIGs*.

To study the expression of *PIGs* in more detail, Northern (RNA) hybridizations were performed with 13 of the *PIGs*. Similar loadings of the different RNA samples (except for RNA from infected leaves, which was loaded in twofold amount) were tested (i) by ethidium bromide staining, and (ii) by co-hybridizing with probes containing actin- or β -tubulin-cDNA from *U. fabae* (S. Wirsel, M. Wernitz, and M. Hahn, unpublished data). Whereas the probes for the cytoskeletal proteins showed a fairly similar (yet somewhat variable) degree of hybridization in all stages of rust development, by far the highest levels of *PIG* mRNAs were observed in haustoria

and/or rust-infected leaves (Fig. 1). Some of the *PIGs* (e.g., *PIG5* and *PIG8*) were expressed only in planta (i.e., in haustoria, lane 7, and in infected leaves, lane 8), whereas others also showed a lower, but significant, expression in spores (*PIG16*), germinated spores, or infection structures formed in vitro (*PIG1*, *PIG3*, *PIG11*, *PIG28*; lanes 1 to 6). The *PIG28* and *PIG1* mRNAs started to accumulate in infection structures after 12 and 18 h, respectively, reaching peak accumulation in haustoria and infected leaves. Remarkable differences were observed when transcript levels of haustoria were compared with those of infected leaves. Whereas *PIG5* showed a strong expression only in haustoria, the mRNA levels in infected leaves increased in the following order: *PIG16*, *PIG1*, and *PIG28*. Since rust mycelia within infected leaves consist of intercellular hyphae, haustorial mother cells, and haustoria, the expression of these *PIGs* appears to vary in different types of hyphae. *PIG18* mRNA was present in all stages of development, with highest levels in haustoria.

Sequence analysis of *PIG* cDNAs.

Sequencing of the cDNAs representing 31 *PIGs* was initially done from both ends, with PCR-amplified cDNAs as templates. To verify that no rearrangements of, or ligations between, different cDNAs during construction of the library had occurred, more than one cDNA clone, including the largest one, of each of these *PIGs* was sequenced. The sequences

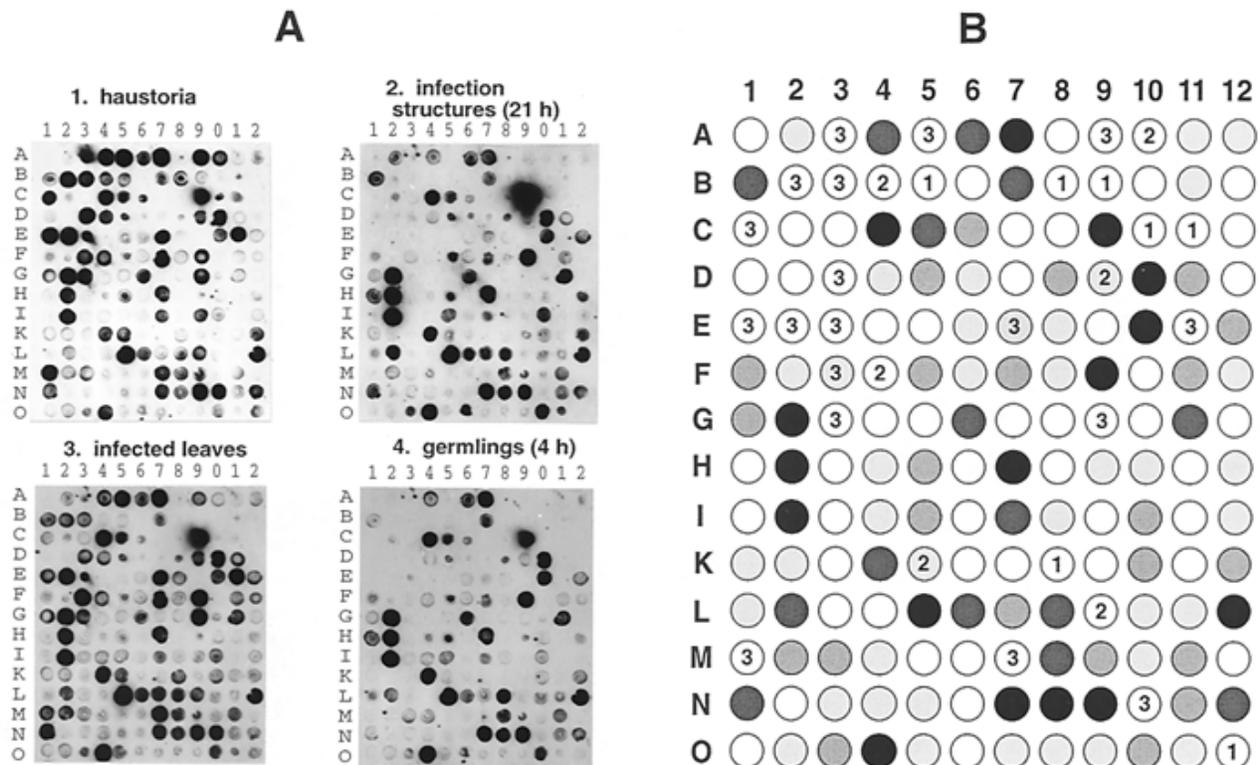


Fig. 2. A, Differential hybridization of a λ gt10 cDNA library from haustoria of *Uromyces fabae*. A nylon membrane containing randomly chosen cDNAs was successively hybridized with digoxigenin-labeled cDNA from 1: haustoria; 2: 21-h-old rust infection structures; 3: rust-infected *Vicia faba* leaves (5 days after inoculation); and 4: 4 h germinated rust spores. B, Schematic representation of the hybridization results shown in A. Clones hybridizing exclusively or preferentially with cDNA probes from haustoria and from infected leaves are indicated with numbers (1 to 3), according to the degree of hybridization. Clones hybridizing similarly against all rust cDNA probes are indicated by various degrees of shadowing, depending on the intensity of hybridization. Clones showing no significant hybridization to any of the probes are indicated as open circles.

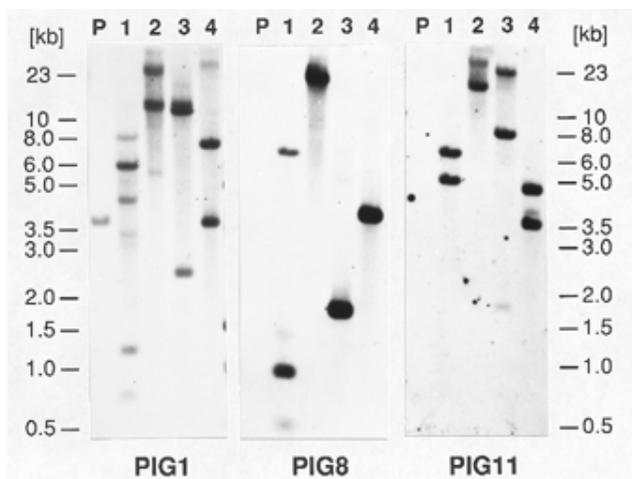


Fig. 3. Genomic Southern hybridizations with in planta-induced gene (*PIG*) cDNA probes. Five-microgram samples of restriction-digested total DNA were loaded per lane. The same 0.7% agarose gel was used for the membrane-bound DNA samples hybridized against the three probes. One of the filters was successively hybridized with *PIG8* and *PIG11* cDNA probes; some carryover of *PIG8* hybridization bands is visible in lanes 2 to 4 of the *PIG11* hybridization result. Lane P: *Vicia faba* DNA (*EcoRI*). Lanes 1 to 4: *U. fabae* DNA, digested with *EcoRI* (1), *BamHI* (2), *XbaI* (3), *EcoRV* (4).

obtained were compared with the sequence entries stored in public data bases. With BLAST or FASTA search programs, similarities with genes or proteins in the current data bases could be detected for 9 *PIGs*. In the following section, the analysis of cDNA sequences from *PIG1* (U81789), *PIG8* (U81790), *PIG11* (U81791), *PIG16* (U81793), and *PIG28* (U81792) is presented in detail. Data from other cDNAs encoding putative amino acid transporters (*PIG2*, *PIG27*), proteins involved in thiamine biosynthesis (*PIG4*), and chitinases (*PIG18*) will be published elsewhere.

Putative functions of *PIGs*.

A *PIG1* cDNA of 1,438 bp encodes an open reading frame of 341 amino acids corresponding to a protein with a molecular mass of 37,710 Da. The ATG in position 74 is likely to be the start codon as the alignment fits well with complete protein sequences from other fungi involved in vitamin B1 biosynthesis (not shown; Fig. 4). In *Schizosaccharomyces pombe*, the corresponding gene, *nmt1*, was shown to be required for the synthesis of the thiamine precursor pyrimidine (Maundrell 1990). The similarities (percent identities) between the *PIG1* protein and other fungal NMT1-like proteins range between 57.6% (to *S. pombe*) and 65.5% (to *Aspergillus parasiticus*).

A *PIG8* cDNA sequence of 1,063 bp was shown to contain an open reading frame of 256 amino acids corresponding to a protein with a molecular mass of 27,844 Da. The ATG (after

Table 1. Characterization of in planta-induced gene (*PIG*) cDNAs^a

Gene	Fraction of haustorium cDNA	Clones (no.)	cDNA size	mRNA size	Postulated function	Gene copies
<i>PIG1</i>	2.8%	26	1,437 bp	1.5 kb	Thiamine synthesis (<i>nmt1</i>)	1 to 2
<i>PIG2</i>	0.7%	3	1.6 kb	ND	Amino acid permease ^b	ND ^c
<i>PIG3</i>	1.3%	15	0.7 kb	0.7kb	???	2 to 4
<i>PIG4</i>	2.4%	20	1.3 kb	1.4 to 1.5 kb	Thiamine synthesis ^d	1
<i>PIG5</i>	2.0%	19	1,124 bp	1.3 to 1.4 kb	???	1 to 3
<i>PIG6</i>	1.0%	6	517 bp	0.6 kb	???	1 to 2
<i>PIG7</i>	0.7%	8	0.9 kb	0.95 kb	???	ND
<i>PIG8</i>	ND	3	1,044 bp	1.1/1.3 kb	Short chain dehydrogenase	1
<i>PIG9</i>	ND	2	1.55 kb	1.6 kb	???	1 to 2
<i>PIG10</i>	ND	2	1.4 kb	ND	???	2 to 3
<i>PIG11</i>	ND	12	365 bp	0.4 kb	Metallothionein	1 to 2
<i>PIG12</i>	ND	1	0.6 kb	ND	???	ND
<i>PIG13</i>	ND	4	0.25 kb	ND	???	ND
<i>PIG14</i>	ND	3	0.9 kb	ND	???	ND
<i>PIG15</i>	ND	3	1.1 kb	1.3 kb	???	ND
<i>PIG16</i>	ND	1	1.8 kb	2.5 kb	Cytochrome P-450	1 to 2
<i>PIG17</i>	ND	1	1.7 kb	ND	???	ND
<i>PIG18</i>	ND	1	0.7 kb	1.8 kb	Chitinase ^d	ND
<i>PIG19</i>	ND	1	1.2 kb	ND	???	ND
<i>PIG20</i>	ND	1	0.6 kb	ND	???	ND
<i>PIG21</i>	ND	1	1.0 kb	ND	???	ND
<i>PIG22</i>	ND	3	0.6 kb	ND	???	ND
<i>PIG23</i>	ND	1	0.95 kb	ND	???	ND
<i>PIG24</i>	ND	1	(2 cDNAs)	ND	ND	ND
<i>PIG25</i>	ND	1	0.9 kb	ND	???	ND
<i>PIG26</i>	ND	1	(2 cDNAs)	ND	ND	ND
<i>PIG27</i>	ND	1	1.0 kb	ND	Amino acid permease ^d	ND
<i>PIG28</i>	ND	1	575 bp	0.6 to 0.9 kb	Peptidyl-prolyl isomerase	1
<i>PIG29</i>	ND	1	(2 cDNAs)	ND	ND	ND
<i>PIG30</i>	ND	1	(2 cDNAs)	ND	ND	ND
<i>PIG31</i>	ND	1	(2 cDNAs)	ND	ND	ND

^a A total of 145 independent cDNA clones showing in planta-specific expression were classified into 31 groups (*PIGs*). If more than one cDNA clone was isolated representing the same *PIG*, the size of the largest cDNA is indicated. For clones containing two cDNA inserts, the insert representing the *PIG* cDNA remains to be determined. Further explanation is given in the text.

^b *PIG2* is described in Hahn et al. (1997).

^c Not determined.

^d Data not shown.

78 bp) is probably the start codon, as it is the first ATG in the cDNA, and it is preceded by three stop codons in the same reading frame (not shown). The PIG8 protein shows high sequence similarity to a family of enzymes called short-chain dehydrogenases (Fig. 5; Persson et al. 1991). For instance, 37.3% identity was found with D-arabitol dehydrogenase from *Candida tropicalis*. PIG8 mRNA was detected as a double band of 1.1 and 1.3 kb on a Northern blot, although PIG8 seems to exist as a single copy gene (Figs. 1 and 3).

A total of 13 cDNAs in the size range of 300 to 364 bp were found to belong to PIG11, corresponding well to the size of the PIG11 RNA of about 0.4 kb (Fig. 1). The first ATG following the assumed 5' end of the PIG11 cDNA marked the beginning of an open reading frame comprising 24 amino acids, six of them being cysteines, corresponding to a protein of 2,383 Da. The amino acid sequence (Fig. 6A) exhibits characteristic features of metallothioneins, such as the high proportion of cysteines and the lack of aromatic and hydrophobic amino acids (Hamer 1986). Alignment of the putative PIG11 protein with metallothioneins from other organisms is shown in Figure 6A.

A 1,849 bp cDNA representing PIG16 was found to contain an open reading frame, starting from one end of the cDNA, of

541 amino acids (data not shown). The approximate size of 2.5 kb of the PIG16 mRNA (Fig. 1) is considerably larger than the cDNA, indicating that the cDNA does not contain the complete coding region. Significant similarities of the deduced amino acid sequence were found to proteins that belong to the cytochrome P-450 monooxygenases (P-450s). For instance, 26.3% identity and 49.8% similarity were found after alignment of the PIG16 protein with pisatin demethylase, another fungal P-450 monooxygenase that detoxifies a phytoalexin plant defense compound. Part of this alignment is shown in Figure 6B.

The sequence of a 575-bp cDNA clone representing PIG28 revealed an open reading frame, starting with ATG, of 163 amino acids, corresponding to a protein of 17,994 Da. The amino acid sequence of the deduced protein showed high homology to peptidyl-prolyl *cis-trans* isomerases (Fig. 6C), enzymes that catalyze protein folding (Schmid 1993). Since the PIG28 protein apparently lacks an N-terminal targeting sequence, and as it has highest similarity to the cytoplasmic isoforms of yeast, *Arabidopsis*, and humans, it probably represents a cytoplasmic isoform. Similar to PIG8, the multiple transcripts of PIG28 appear to be encoded by a single copy gene (Figs. 1 and 3).

Ufab	1	MSTDKISVLLNWHATPYHLPIFVAQSKGFFAKEGIKVAILEPNDPSDVTTELIGSGKADLG	
Apar	1TF.T.....A.LYL.....Y.KE..L. .L.....I.....V.M.	
Spom	1	...N..TF.T..E.....L..TR.YYER...E.....TN.....A.....V.M.	
Scer	1TF....QP....I...L..T..Y.KEQ.LDM.....TN.....V.M.	
Ufab	61	CKAMIHTLAGKARGFPPIKISIGTLMDEPFTGVIYLESGITSDFRSLKGRIGYVGEFGKI	
Apar	61	F.....A...N..VT. .S.L.....V..KD....E.....K.....	
Spom	61	L.....A....Y.VT.F.S.LN.....L.T.K.N..-N..KDI.....	
Scer	61	L.....A.....VT.VAS.L.....L..K.....E..Q.....K.....	
Ufab	121	QIDELTKYYGMTSKDYTAVRCGMNVSKAIIIEGTIDAGIGLENIQQVELEEWCKANNRPAS	
Apar	121AD.....T...R.D.....V.M...A..LASQ...RD	
Spom	120	.L.D.CSKF.LSPS...I.....IAP...N.E..G...I.CM.....R..VSQG..R.	
Scer	121H...KPE.....A.Y...K.....I.CM.....YLAQOG....	
Ufab	181	DVKMLRIDELAEELGCCCFCSILYIANEDWLKDHDPKETAAFMRAVKAGADAMFADPRGSWA	
Apar	181	..QIV...Q.....DAF.AAN.EKVQK.....RAT.YVL.E.AAAFE	
Spom	180	..Q.....R..N.....T....HDEFIAK..DKIK..L..IHSATLD.LK..VQTYK	
Scer	181	.A.....K..C.....TV...C.DEF..KN.EKVRK.LK.I.KAT.YVL...VKA.K	
Ufab	241	EYCKVKPAMNTPLNRIMFDRSFNYMSQDLINVS RDWNKVTNYSKRLGIVPEDFVSNYTNE	
Apar	241	..VDM..I.G..V..KI.E...A.F.R..K.....A.....G.....LDA..QP....Q	
Spom	240	..IHF.RE.GSE.H.EQ.E.C.A.F.H.IS..P.....I.Q..EP.C..G	
Scer	241	..IDF..RL.ND.SYKQYQ.CYA.F.SS.Y..H...K...G.G...A.L.P.Y.....	
Ufab	301	FVQWEVAPEGGEAEGLAKQEQEMKTLQAHVAEHGGVLQAVTA	341
Apar	301	YLS.TLDAD--STDP.GD.KR.AE..KE..CE..FKRLQV.SSA	342
Spom	300	YLT..LD.D--.KDPMG..EAIAEI.DEIKQK...FSGNSLRVYEPANL	346
Scer	301	YLS.PEPE.--VSDP.EA.RL.AIH.EKCRQE.TFKRLALPA	340

Fig. 4. Alignment of the in planta-induced gene (PIG) PIG1-encoded protein from *Uromyces fabae* (Ufab) with NMT1-like proteins from *Aspergillus parasiticus* (Apar) (P42882), *Schizosaccharomyces pombe* (Spom) (P36597), and *Saccharomyces cerevisiae* (Scer) (P42883). Amino acids identical to those of the rust sequence are indicated by points. Positions where amino acids are missing are indicated by dashes.

DISCUSSION

Isolation of development-specific genes.

Screening of a haustorium-specific cDNA library by differential hybridization turned out to be a highly effective way for the isolation of a large number of developmentally regulated rust genes. Infection-related expression of genes or gene products has been described for various phytopathogenic fungi. Genes that are abundantly expressed in germinated spores have been isolated from the rust fungus *Puccinia graminis* (Liu et al. 1993), from the powdery mildew fungus *Erysiphe graminis* (Justesen et al. 1996), and from the downy mildew fungus *Bremia lactucae* (Judelson and Michelmore 1990). Several of these genes encode small proteins that seem to be secreted. In *Colletotrichum gloeosporioides* and *Uromyces appendiculatus*, genes or cDNAs induced during appressorium formation in vitro have been isolated (Xuei et al. 1992; Hwang et al. 1995). In *U. fabae*, synthesis and secretion of host cell-wall-degrading enzymes and chitin deacetylase were found to be dependent on infection structure development (Deising et al. 1995). From *Phytophthora infestans*, nine in planta-induced genes have been isolated. Two of them encoded ubiquitin and calmodulin, respectively (Pieterse et al. 1991, 1993). Two in planta-induced proteins that are secreted into the intercellular space of infected tomato leaves have been isolated from *Cladosporium fulvum* (Wubben et al. 1994). Also, expression of the race-specific elicitors AVR4 and AVR9 of *C. fulvum* was shown to be induced in the plant (De Wit et al. 1996).

An infection-specific gene encoding a hydrophobin was isolated from *Magnaporthe grisea*. Mutants with a disrupted

gene showed a strongly reduced ability to form appressoria and to cause disease (Talbot et al. 1993). Monoclonal antibodies against isolated haustorial complexes from pea powdery mildew have been used for the identification of a glycoprotein that is unique to the haustorial plasma membrane (Mackie et al. 1991). A similar approach with *Colletotrichum lindemuthianum* resulted in the identification of glycoproteins that are restricted to the plasma membranes of appressoria and to surfaces of fungal infection hyphae, respectively (Pain et al. 1994, 1995).

The ectomycorrhiza-forming basidiomycete *Pisolithus tinctorius* also shows an altered pattern of gene expression during development of the symbiotic interaction with the roots of a host plant. In particular, changes in the synthesis of major cell wall proteins including hydrophobins were observed (Tagu and Martin 1996).

From the examples cited above, a preliminary picture regarding the function of infection-related or in planta-induced fungal proteins emerges. Some appear to be directly involved in the interaction with the host, either by being secreted into the apoplastic space that separates both organisms or by forming an altered hyphal wall. Other proteins that are inserted into the fungal plasma membrane might be responsible for altered permeability or transport activities. The sequences of in planta-induced rust genes described in this paper indicate that they are involved in nutrient transport, carbohydrate metabolism, and other functions.

Based on the analysis of 169 randomly chosen cDNAs, 19% of the clones in the haustorial cDNA library were found to be differentially expressed in planta. This value, however, must be taken as a rough estimate, because of (i) the limited

PIG8	1	MTFTIDLKQDCIIVTGGNRGIGLAMSQACADAGA AVGIIY-----NS	
aradh	1	MDSSSYWSYDNIVPSFR.DGKLV.I...SG.LSAVV.R.LLAK..DIAL.DMNLERTQQA	
glcdh	1	MYK..EGKVVI..SST.L.KS.AIRF.TEK.K.VVN.RSKEDEA..	
hstdh	1	MNLVQ.KVT.I...T....F.AAKIFI.N..K.S.FGETQEEVDTA	
PIG8	43	AKDAEDRASEISK-KYG---VKCKAYQCDVGQ QHKVKEVFKKINEELGPVTG-LIANAGV	
aradh	61	.R.VLQWGE.QMKGKHESPIGQVS.WS.NI.DAEA.ELT..A...HH.K.ASV..NT..Y	
glcdh	48	VLEEIKKVGG-----EAI-.VKG..TVESD.INLVQSAIK.F.KLD-VM.N...L	
hstdh	47	LAQLKELYP-----EEVLGFAP.LTSRDA.MAAVGQVAQKY.RLD-VM.N...I	
PIG8	98	SVVKEALQYNKDDFNKIFDVNVFVFNCAQAMAQIWTDTGFGQSGSVVLISSMSSQICNRP	
aradh	121	AENFP.EE.PAKNAEN.MK..GL.S.YVS..F.RPLIQNNM-T..IIL.G...GT.V.D.	
glcdh	96	ENPVSSHEMSLS.W..VI.T.LT.A.LGSREAIKYFVENDI-K.T.INM..VHEK.PWPL	
hstdh	95	TSNNVFSRVSEEE.KH.M.I..T....G.WCAY.CMK.AK--K.VIINTA.VTGIFGSL	
PIG8	158	LTQCFYNSSKA AVSNLGKCLAEWA EKSI RVNMLSPGYVKTDQ TSHMDQKLRDFQAD---	
aradh	180	QP..M..M...G.IH.ARS..C...KYN....T....IL.PL.RNVISGHTEMKTEWES	
glcdh	155	FVH--.AA..GGMKLM TET..L.Y.P.G...NIG..AIN.PINAEKFAD-PEQR..VES	
hstdh	153	GVG--.PA...S.IG.THG.GR.IIR.N...VGVA..V.N..M-TNGNPP-EIM-EGYLK	
PIG8	215	GVPLKRFAEPEEMAGQAILLLSPKA-SYMTGGEYFVDGGNLVW	256
aradh	240	KI.M..M...K.FV.SILY.A.DS.S..T..HNLV...YEC.	282
glcdh	212	MI.MGYIG...I.AV.AW.A.SE--.V..ITL.A...MTQYPSFQAGRG	261
hstdh	208	AL.M..ML...I.NVYLE.A.DL-.GI.ATT--.SVDGAYRP	249

Fig. 5. Alignment of the in planta-induced gene (PIG) PIG8-encoded protein with short-chain dehydrogenases. aradh: *Candida tropicalis* arabinol dehydrogenase (JC4041). glcdh: *Bacillus megaterium* glucose 1-dehydrogenase DHG3 (P40288). hstdh: *Eubacterium* spp. 7- α -hydroxysteroid dehydrogenase (P19337). For explanations, see legend to Figure 4.

number of clones analyzed, and ii) the limits of sensitivity of a differential screening. Possibly, we have missed some of the weakly—yet differentially—expressed *PIG* cDNAs.

Up to now, 31 different cDNA clones were preliminarily characterized by cross hybridization and sequencing. We as-

sume that most of them represent *PIGs*, based on the following lines of evidence: (i) Northern hybridizations with 13 *PIGs* confirmed their phase-specific expression; (ii) the cDNAs representing 11 *PIGs* hybridized with rust genomic DNA, proving their fungal origin; and (iii) 15 *PIGs* were rep-

A

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1   aaaacattagccatcgcaatcaatcttccctcaatcctccaatttaaagcgtcttc
59  cccaaaa ATG AAC CCT TGC TCT TCA AAC TGC TCT TGC GGT GCT TCA
   1           Met Asn Pro Cys Ser Ser Asn Cys Ser Cys Gly Ala Ser
105 TGT ACC TGC AGC GGC TGC TCT TCT CAC AAG AAA tgaggcataaatcgg
   14          Cys Thr Cys Ser Gly Cys Ser Ser His Lys Lys
153 tgtcgggccctgataagcgagatgctggaccctttccatcatctctgagacaaaggaa
212 acaaatttcgtcatcagcggttcctgggacccgcaataagccgtatcctgctttctttt
271 atctacgatttccaattcctacatttggtgattagacgtcattgcatagcacttggcg
330 cgtaatccaacattcagttagagcttaaaaaaaaa

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C. gloeosporioides   MSGGCGASTGTCHCGKDCTCAGCPHK
                       | |||: : : | | | :|: | | |
N. crassa           MGDCGCSGASSCNCGSGCSCSNCGSK
                       : | | ||: | :| | | | |
U. fabae PIG11      MNPCSSNCSCGASCTCSGCSSHKK
                       : |||||: | :| | | |
T. aestivum        ...RANRRANCSCGAACNCASCGSTTA

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B

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PIG16:                380 YLNACLQETMRLQPPSPANLQRICPPGGAVICGRQIPEG 418
                       ||:| : | :|| | :| |: | || | | :|||
PIDEM:                364 YLQAVIKEALRLHPGVGTQLTRVVPKGLVIEGQFFPEG 402

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C

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Ufab 1 --MANCYFDVSSNGKPLGRIVFELYDDVPRRTTNNFRQLCLNPPGKG-----FKHSIFHRVIPDFMIQGGDFTNNGTGGESIYGKKF
Scer 1 ---SQV...EAD.Q.I.V.K.N.I.K.AE...A.TGEK.F-----YAG.P.....A.....G..
Atha 1 MAFPKV...MTID.Q.A...M...T.KT...AE...A.TGEK.V.GTGKPLH..G.K.....NF.C.....A.....S..
Hsap 1 -VNPTVF...IAVD.E...VS...FA.K..K.AE...A.STGEK.F-----Y.G.C...I..G..C.....RH.....E..

Ufab 86 EDENFQKKHVERGMLSMANAGPNTNGSQFFITVTKPWLDGKHVVFVKVIEGYDQVVKAMEKTGSQSGKTSVVKIEDCGTL- 163
Scer 85 P....K.H.DRP.L.....TVPC.....E.VD...-I..KV.SL..P..A.KARIVVAKS.E.- 161
Atha 91 .....ER..TGP.I.....A.....CTV..D.....Q.V..L.....I..V..S...PTKPVVVA...Q.S 172
Hsap 87 .....IL..TGP.I.....CTA..E.....K..MN-I.E...RF..RN....KKIT.A...Q.E 164

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Fig. 6. Sequences and alignments of proteins encoded by in planta-induced gene (*PIG*) *PIG11* (A), *PIG16* (B), and *PIG28* (C) cDNAs. **A**, Upper part shows the DNA and deduced amino acid sequence of *PIG11* cDNA. Lower part shows an alignment of the *PIG11*-encoded polypeptide and metallothioneins. *Colletotrichum gloeosporioides*: *cab3* gene product (S55029). *Neurospora crassa*: copper metallothionein (P02807). *Triticum aestivum*: zinc metallothionein, class II (P30570), partial sequence shown. **B**, Alignment of partial amino acid sequences from *PIG16* cDNA and from pisatin demethylase of *Nectria haematococca* (P38364). Amino acids are counted from the first translatable codon of the 1,849-bp *PIG16* cDNA and from the start codon of pisatin demethylase, respectively. **C**, Alignment of the *PIG28*-encoded protein with cytoplasmic peptidyl-prolyl *cis/trans* isomerases. Scer: *Saccharomyces cerevisiae* CYPH (P14832). Atha: *Arabidopsis thaliana* CYP1 (P34790). Hsap: *Homo sapiens* CYPH (P05092). For explanations, see legend to Figure 4.

resented by two or more independently isolated cDNA clones, which indicates that the screening has given reproducible results.

Genomic Southern hybridization with different *PIG* cDNAs revealed single or few copies of the corresponding genes in the *U. fabae* genome. A precise estimation of the copy number was not possible, because of the dikaryotic state of the *U. fabae* race I₂, and because of the possible presence of introns that might contain restriction sites for the enzymes used.

***PIG* expression patterns.**

Northern hybridization experiments revealed a variety of patterns of *PIG* mRNA accumulation (Fig. 1). Some of the *PIG*-encoded transcripts were detected only in haustoria and in rust-infected leaves (*PIG5*, *PIG8*). Others were also found to be present, at lower levels, in spores or hyphae formed in vitro. For *PIG1*, *PIG11*, and *PIG28*, a continuous increase in mRNA expression, starting in 12- or 18-h-old infection structures and culminating with peak levels in haustoria and infected leaves, was observed. Thus, the physical signal provided by the scratches in the polyethylene membranes (simulating the stomatal guard cell lip) is sufficient to trigger a cascade of morphogenetic and molecular events including those that naturally occur immediately before haustorium formation (Mendgen et al. 1996). A similar kind of regulation in this fungus has been observed for host cell-wall-degrading enzymes. Their synthesis and secretion were found to be dependent only on the degree of infection structure differentiation, except for polygalacturonate lyase, which in addition required the presence of its substrate (Deising et al. 1995). Within the plant, the expression of some genes is further increased, and new genes (e.g., *PIG5*, *PIG8*, *PIG16*) are induced. As in the case of infection structure formation, we assume that fungal morphogenesis in planta and activation of these *PIGs* are coordinately triggered by host signals.

The transcript levels of several *PIGs* also varied with respect to their relative distribution in haustoria and infected leaves. Since the biotrophic rust mycelium consists of intercellular hyphae (including haustorial mother cells) and haustoria, we interpret a high ratio of mRNA levels in haustoria versus infected leaves (e.g., *PIG5*) as a rather haustorium-specific expression.

Expression of some *PIGs* was also observed in non-germinated spores (e.g., *PIG3*, *PIG16*). An explanation for this could be that some of the highly expressed transcripts in the biotrophic mycelium remain in sporulating hyphae and spores. Detection of *PIG* mRNA in spores is dependent on the stability of these transcripts. After germination, the transcripts of *PIG16*, but not those of *PIG3* and *PIG11*, had disappeared, indicating differences in mRNA stability (and/or transcription).

Putative functions of *PIGs*.

Sequencing of the *PIG* cDNAs and data base searches for similar genes or proteins revealed relevant information for 9 *PIGs* so far (Table 1). Two of the most abundantly expressed genes, *PIG1* and *PIG4*, seem to be involved in thiamine (vitamin B1) biosynthesis (see below). *PIG2* and *PIG27* encode proteins similar to amino acid transporters, indicating that the capability for nutrient uptake increases when the rust fungus enters the biotrophic phase (Hahn et al. 1997; M.

Hahn, unpublished data). The role of *PIG18* (chitinase) in the biotrophic rust mycelium is unknown. *PIG11* (metallothionein), *PIG8* (short-chain dehydrogenase), *PIG16* (P-450), and *PIG28* (peptidyl-prolyl isomerase) are discussed below.

The majority of *PIGs*, however, did not reveal clear similarities to any of the sequences in the data base (M. Hahn, unpublished data). Although this is a preliminary conclusion until full-length cDNA sequences are available, it was somewhat unexpected, given the high mRNA levels of many *PIGs* in haustoria, and the fact that the complete genome sequence of another fungus, *Saccharomyces cerevisiae*, has recently been published (Dujon 1996). The lack of homology means that the translated regions of the *PIG* cDNAs do not contain "ancient conserved sequences" that are found in different phyla of eukaryotic and/or prokaryotic organisms (Green et al. 1993). Therefore, many of the *PIG*-encoded proteins either fulfill rather specialized functions, or they have not been strongly conserved during evolution.

PIG1 was found to be highly homologous to fungal genes involved in the biosynthesis of vitamin B1. In *S. pombe*, the corresponding gene, *nmt1* (or *thi3*), is required for synthesis of the thiamine precursor, pyrimidine (Schweingruber et al. 1991). In minimal medium, *nmt1* is one of the most abundantly expressed genes, but it is completely repressed by thiamine levels above 0.5 μ M (Maundrell 1990). Thiamine is an essential component of several enzymes involved in primary carbohydrate metabolism, such as pyruvate and α -ketoglutarate dehydrogenases, and transketolase. The in planta-induced expression of *PIG1* during parasitic growth may indicate that thiamine is not available from host cells. In addition, it gives evidence for normal biosynthetic capabilities of rust fungi. These data are in agreement with earlier work that demonstrated that *Puccinia graminis* f. sp. *tritici* can be grown in axenic cultures without any complex or unusual components (Williams 1984; Fasters et al. 1993).

PIG8 probably encodes a member of the short-chain dehydrogenases. Enzymes belonging to this group are characterized by sizes of about 250 amino acids; they perform NAD(P)-dependent redox reactions with a large variety of substrates, including pentitols, sorbitol, and glucose (Persson et al. 1991; Murray et al. 1995). The substrate of *PIG8* cannot be deduced from its sequence. Because of the presence of significant quantities of hexitols and pentitols in rust-infected leaves and in spores, *PIG8* might be involved in the metabolism of one of these sugar alcohols (Maclean 1982).

PIG11 seems to encode a metallothionein. Similar to the known or predicted metallothioneins from other fungi, the *PIG11*-encoded protein is much shorter than mammalian and plant metallothioneins; with 24 amino acids it would represent the smallest metallothionein described so far. Metallothioneins bind heavy metals such as copper, zinc, or cadmium. They have been shown to protect cells against toxic concentrations of heavy metals, and are active in the protection against free oxygen radicals (Hamer 1986; Tamai et al. 1993). In *C. gloeosporioides*, two genes encoding metallothionein-like proteins were found to be induced during appressorium formation (Hwang and Kolattukudy 1995).

PIG16 cDNA encodes a polypeptide similar to cytochrome P-450 monooxygenases (P-450s). P-450s catalyze a variety of oxygenation reactions on a large spectrum of substrates. While some of them are involved in the biosynthesis of sec-

ondary metabolites such as hormones and toxins, the majority of P-450s are involved in detoxification, often being referred to as drug-metabolizing P-450s (Gonzalez and Nebert 1990). An example of a fungal P-450 is pisatin demethylase from *Nectria haematococca*, which converts the pea phytoalexin, pisatin, into a less toxic product (VanEtten et al. 1995).

PIG28 cDNA encodes a protein strongly resembling peptidyl-prolyl *cis-trans* isomerases (PPI). In vitro, PPI catalyze the isomerization of proline-containing peptide bonds and are therefore believed to assist in the folding of proteins (Schönbrunner and Schmid 1992). In yeast, the expression of a cytoplasmic PPI was shown to be increased by heat shock (Sykes et al. 1993).

We have noticed that three of the *PIGs* presented in this work encode proteins that are potentially involved in the tolerance of various stress factors, namely heavy metal- or oxidative stress (*PIG11*), exposure to toxic plant metabolites (*PIG16*), and protein unfolding conditions (*PIG28*). Whether or not this means that the rust fungus experiences and responds to these kinds of stresses in its host plant remains to be studied.

MATERIALS AND METHODS

Plant and fungal materials.

Broad bean plants (*Vicia faba* cv. Con Amore) were cultivated in growth chambers (Deising et al. 1991). For infection, they were spray inoculated with urediospores of a compatible wild isolate (I_2) of *Uromyces fabae* (formerly referred to as *U. viciae-fabae*; Boerema and Verhoeven 1979). Spores were harvested and used either fresh or after storage at -70°C .

For isolation of rust haustoria, dense inoculation of leaves was performed with spores that had been washed for 5 min with distilled water containing 0.02% bovine serum albumin (1 liter per 1 g of spores). Washed urediospores were collected on a filter paper by vacuum filtration, gently resuspended with a glass homogenizer, and sprayed with a hand-operated glass atomizer onto both sides of the leaves. The plants were kept in a dark, moist chamber overnight and then cultivated as described (Deising et al. 1991).

Germinated spores were obtained by shaking washed spores submerged in washing solution (0.5 liter per 1 g of spores) for 3 to 4 h at 20°C . In vitro differentiated rust infection structures were obtained on scratched polyethylene membranes as described (Deising et al. 1991).

Isolation of haustoria.

Hauستoria of *U. fabae* were isolated from infected leaf tissue by ConA affinity chromatography (Hahn and Mendgen 1992), with minor modifications: For each preparation, 30 g of heavily rust-infected broad bean leaves (5 to 6 days after inoculation) was homogenized in 180 ml of homogenization buffer, using a Waring blender at maximum speed for 15 s. The homogenate was passed through a 20- μm nylon mesh and the filtrate centrifuged for 5 min at $5,000 \times g$. The pellet was resuspended (without a washing step) in 10 ml of suspension buffer. Two 1.7-ml aliquots of the suspension were loaded successively onto three columns (15 \times 2 cm), each filled with 5 ml of a Sepharose 6MB column (Pharmacia, Uppsala, Sweden) coupled to ConA. After the first aliquots had entered the columns, an incubation for 15 min was allowed before addi-

tion of the second aliquots and another incubation for 15 min. After extensive washing of the columns, the haustoria were released from the columns by agitation, using a wide-bore Pasteur pipette. The suspension was centrifuged for 1 min at $16,000 \times g$, and the pellet frozen in liquid nitrogen and stored at -70°C . From 30 g of infected leaves, a suspension containing an average of $7.8 \pm 2.2 \times 10^7$ haustoria and $1.6 \pm 0.5 \times 10^8$ chloroplasts was obtained (means of 19 experiments).

Isolation of RNA.

For RNA isolation, fungal spores, germinated spores, and leaves were frozen in liquid nitrogen and ground in a mortar before suspending them in denaturing solution (4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7.0; 0.5% [vol/vol] sodium laurylsarcosine; 0.1% [vol/vol] 2-mercaptoethanol). Rust infection structures generated in vitro were scraped from the polyethylene membranes at 4°C with a large spatula in the presence of denaturing solution and quickly transferred into a glass homogenizer.

The homogenates obtained were extracted 3 times with phenol/chloroform, and the nucleic acids precipitated with 2-propanol. The pellet was dissolved in a solution containing 10 mM Tris (pH 7.5), 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), extracted once with chloroform, and again precipitated by adjusting the solution to 2 M LiCl. After centrifugation, the RNA pellet was washed with 75% EtOH, dried, and dissolved in water. For storage, the RNA was kept as an ethanol precipitate at -20°C .

For the isolation of haustorial RNA, 2 to 3×10^8 haustoria were used, corresponding to three haustorial preparations. The frozen pellets were ground for 10 min in a small mortar filled with liquid nitrogen. The frozen powder was suspended in 1 ml of denaturing solution. The homogenate was vortexed for 1 min and centrifuged for 5 min at $16,000 \times g$. The supernatant was transferred into a new tube, and the pellet re-extracted with 200 μl of denaturing solution. The supernatants were combined and overlaid onto 3 ml of a cesium chloride solution (5.7 M CsCl; 10 mM EDTA, pH 7.5) in two 2.2-ml polyallomer ultracentrifugation tubes. The samples were centrifuged in a RP55S rotor for 24 h at 46,000 rpm, 20°C , in a RCM120 Micro-Ultracentrifuge (Sorvall, DuPont, Wilmington, DE). After centrifugation, the pellets were washed several times with 75% ethanol and air dried. The pellets were dissolved in 250 μl of buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 0.1% [wt/vol] SDS) and extracted once with chloroform/isoamylalcohol (24:1). The aqueous phase was precipitated with 1 volume of 2-propanol and 0.1 volume 2 M sodium acetate. The pellet obtained after centrifugation (15 min, $16,000 \times g$; 4°C) was washed with 75% ethanol, air dried, and dissolved in 200 μl of water.

Construction of a haustorial cDNA library.

From total haustorial RNA, poly A⁺ RNA was enriched by binding to Oligotex (dT) resin (Qiagen, Chatsworth, GA). Five micrograms of poly A⁺ RNA from isolated haustoria were used for the construction of a cDNA library, with the Time Saver cDNA synthesis kit from Pharmacia, with oligo d(T) as a primer for first-strand cDNA synthesis. The double-stranded cDNA was ligated to an *EcoRI* adapter carrying a *NotI* site, and then ligated into the *EcoRI*-digested, dephosphorylated bacteriophage vector $\lambda\text{gt}10$. In vitro packaging of

the DNA and transfection of *Escherichia coli* strain NM514 was performed according to standard protocols (Sambrook et al. 1989).

DNA manipulations.

For the isolation of total DNA from *U. fabae*, spores germinated in water were ground with liquid nitrogen in a mortar and the powder transferred into a buffer (5 ml per g of germinated spores) containing 10 mM Tris-HCl, pH 8.0, 100 mM EDTA, 50 µg of RNaseA per ml, 0.5% SDS, and gently shaken for 1 h at room temperature. Proteinase K was added to a final concentration of 100 µg/ml and incubation was continued at 50°C for 2 h. The homogenate was centrifuged (5 min, 3,000 × g), and the supernatant extracted once with phenol (pH 8.0) and twice with phenol/chloroform (1:1). The solution was adjusted to 0.3 M sodium acetate and 1 volume of isopropanol was added. The DNA precipitate was collected by centrifugation, redissolved in Tris-EDTA (TE) buffer and precipitated as before. The pellet was washed with 75% ethanol, air dried, and dissolved in TE buffer. For further purification, the solution containing the dissolved DNA was centrifuged for 20 min at 100,000 × g, and the supernatant recovered from the yellowish, gel-like pellet.

Inserts from the cDNA library were amplified with *Taq* DNA polymerase directly from plaques or liquid lysates, with λgt10-specific primers flanking the *Eco*RI cloning site. For dot blot hybridizations, the PCR products were denatured with 0.5 M NaOH for 5 min, and dotted as 1-µl aliquots onto a nylon membrane before hybridization.

Generation of DNA probes and filter hybridizations were performed with the digoxigenin (DIG) labeling and detection system, and the chemiluminescent substrate CSPD (Boehringer Mannheim, Germany). Labeled DNA probes were generated by PCR including DIG-dUTP into the dNTP mix, or by random-primed labeling, following the protocols of the supplier. DIG-labeled, first-strand cDNA hybridization probes were synthesized as follows: In a total reaction volume of 20 µl, 1 µg of poly A⁺ RNA was added to a mixture containing 0.2 mM each of dATP, dCTP, and dGTP; 0.125 mM TTP; 0.065 mM DIG-dUTP (alkali labile); 50 mM Tris-HCl (pH 8.3); 75 mM KCl; 3 mM MgCl₂; 10 mM dithiothreitol; 1.5 µg random hexamer oligonucleotides (dN₆; Pharmacia); 40 units RNase Block (Stratagene, La Jolla, CA); 50 units StrataScript Reverse Transcriptase (Stratagene). After incubation for 3 h at 37°C, 2 µl of 0.5 M EDTA was added and the mixture heated to 100°C for 5 min and quickly cooled on ice. RNaseA (Boehringer) was added to the sample at a concentration of 100 µg/ml and incubation continued overnight at 4°C.

For sequencing, PCR-amplified cDNAs were purified with a PCR purification kit (Qiagen). Sequencing reactions were performed with λgt10-specific or gene-specific primers and the ABI PRISM dye terminator sequencing kit (Perkin-Elmer, Foster City, CA). Gel electrophoresis and data collection were performed with an ABI 373 automatic sequencer. Sequence data were analyzed with the University of Wisconsin (Madison) Genetic Computer Group software. Homology searches in data bases were performed with FASTA (Pearson and Lipman 1989) and BLAST (Altschul et al. 1990) programs, supplied by the EMBL FASTA and the NCBI BLAST e-mail servers, respectively.

Filter hybridizations.

Filter hybridization experiments with membrane-bound DNA were performed according to standard protocols supplied by Boehringer Mannheim. For rehybridization, the probes were stripped from the nylon membranes by two 20-min incubations in 0.2 N NaOH, 0.1% SDS, at 42°C.

For Northern hybridizations, total RNA (in a loading buffer containing 25 µg/ml of ethidium bromide) was separated in 1.1 to 1.5% formaldehyde agarose gels, and transferred to nylon membranes (Boehringer) by downward alkaline transfer for 2 h (Chomczynski 1992). Hybridizations were performed as described (Engler-Blum et al. 1993), except that the concentration of SDS in the hybridization puffer was reduced to 17.5%.

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