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High Level Activation of Vitamin B1 Biosynthesis Genes in Haustoria of the Rust Fungus *Uromyces fabae*

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In the rust fungus *Uromyces fabae*, the transition from the early stages of host plant invasion toward parasitic growth is accompanied by the activation of many genes (*PIG*s = in planta induced genes). Two of them, *PIG1* (= *THI1*) and *PIG4* (= *THI2*), were found to be highly transcribed in haustoria, and are homologous to genes involved in thiamine (vitamin B1) biosynthesis in yeast. Their functional identity was confirmed by complementation of *Schizosaccharomyces pombe* thiamine auxotrophic *thi3* (*nmt1*) and *thi2* (*nmt2*) mutants, respectively. In contrast to thiamine biosynthesis genes of other fungi that are completely suppressed by thiamine, *THI1* and *THI2* expression was not affected by the addition of thiamine to rust hyphae grown either in vitro or in planta. Immunoblot analysis revealed decreasing amounts of THI1p in extracts from spores, germlings, and in vitro-grown infection structures with increasing time after inoculation. Immunofluorescence microscopy of rust-infected leaves detected high concentrations of THI1p in haustoria, and only low amounts in intercellular hyphae. In the sporulating mycelium, THI1p was found in the basal hyphae of the uredia, but not in the pedicels and only at very low levels in uredospores. These data indicate that the haustorium is an essential structure of the biotrophic rust mycelium not only for nutrient uptake but also for the biosynthesis of metabolites such as thiamine.

Additional keywords: plant-pathogenic, *Vicia faba*.

Thiamine diphosphate (vitamin B1) is a cofactor required for the activity of several enzymes of the central carbon metabolism, such as pyruvate dehydrogenase, pyruvate decarboxylase, α-ketoglutarate decarboxylase, and transketolase. In yeast cells, biosynthesis of thiamine occurs via two precursor molecules that are derivatives of thiazol and pyrimidine (Hohmann and Meacock 1998). These are phosphorylated and then condensed to form thiamine phosphate, which is subsequently converted to the enzyme cofactor thiamine diphosphate. Whereas the later steps of thiamine biosynthesis are well characterized, the origin of the precursors is not well established. In *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, several thiamine auxotrophic mutants have been isolated, and the corresponding thiamine biosynthesis genes have been characterized (Hohmann and Meacock 1998). In *S. cerevisiae*, biosynthesis and uptake of thiamine are strongly repressed by thiamine (Manetti et al. 1994). Repression occurs mainly at the transcriptional level and involves several regulatory genes (Hohmann and Meacock 1998).

Rust fungi are obligate plant parasites that depend on living host tissue for growth. Little is known about their nutritional demands during parasitic growth. Feeding experiments with radioactively labeled metabolites have shown that rusts take up sugars and amino acids from their host plants (Mendgen 1981). The establishment of axenic cultures from a few rust species such as *Puccinia graminis* f. sp. *tritici* and *Melampsora lini* have revealed their ability to grow on defined mineral media containing sugars and amino acids, supplemented either with low concentrations of yeast extract (Williams 1984) or peptone (Coffey and Shaw 1972), or with some vitamins (Fasters et al. 1993). Thus, the obligate parasitic lifestyle of rust fungi cannot be explained by strongly handicapped biosynthetic capabilities. Instead, it was proposed that development of rust fungi is tightly linked to essential signals provided by the host plant (Heath and Skalamera 1997).

To investigate the metabolism of rust fungi during biotrophic growth, a molecular genetic approach has been taken by our group. From a haustorium-specific cDNA library, isolated from the broad bean rust *Uromyces fabae*, 32 cDNAs were isolated that showed preferential expression during fungal growth in planta (Hahn and Mendgen 1997). Two of these cDNAs (corresponding to genes named *PIG1* (= *THI1*) and *PIG4* (= *THI2*)) are highly similar to fungal genes involved in thiamine biosynthesis (Hahn and Mendgen 1997). In this paper, we present a structural and functional analysis of these genes, including their regulation, and describe the expression of the *THI1*-encoded protein and its localization within the biotrophic mycelium.

RESULTS

Structural analysis of *THI1* and *THI2*.

The cDNA sequence of *THI1* (*PIG1*) has already been published (Hahn and Mendgen 1997). The sequence of *THI2* (*PIG4*) cDNA reveals an open reading frame of 1,014 bp, encoding a protein (THI2p) with a calculated molecular mass of 36.2 kDa. Similar to THI1p, THI2p shows unambiguous homology to fungal and plant proteins involved in thiamine biosynthesis (Fig. 1). For instance, THI2p is 63.0% identical to the *thi2* (*nmt2*)-encoded protein of *Schizosaccharomyces pombe*, and 49.5% identical to the *thi4*-encoded protein of *Arabidopsis*

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Nucleotide and/or amino acid sequence data are to be found at GenBank at accession numbers AJ250426 (*THI1*) and AJ250427 (*THI2*).

thaliana. Based on these homologies, THI1p is likely to be involved in the synthesis of the pyrimidine moiety, and THI2p in the synthesis of the thiazol moiety of thiamine (Fig. 2).

With *THI1* and *THI2* cDNAs as hybridization probes, the corresponding genomic clones were isolated from an EMBL3 bacteriophage library of *Uromyces fabae* total DNA. EMBL3 clones with inserts of about 17 kb (*THI1*) and 16 kb (*THI2*) were taken for sequencing, with subclones and polymerase chain reaction (PCR) fragments used as templates. From *THI1*, 5,180 bp were sequenced (accession no. AJ250426), covering 1,762 bp upstream of the transcriptional start site, 2,365 bp containing the transcribed region including introns, and 1,053 bp representing the 3′ region of the gene. From *THI2*, 4,833 bp were sequenced (accession no. AJ250427), covering 2,045 bp upstream of the transcriptional start site, 1,709 bp containing the transcribed region including introns, and 1,072 bp representing the 3′ region of the gene. *THI1* contains 9 introns ranging in size from 69 to 244 bp (average size 100 bp), and *THI2* contains 7 introns ranging in size from 73 to 102 bp (average size 85 bp). Transcriptional start points were mapped by primer extension 137 bp (*THI1*) and 96 bp (*THI2*) upstream of the presumptive start codons (Fig. 3). Searches for control elements revealed putative TATA boxes 35 bp (*THI1*) and 37 bp (*THI2*) upstream of the transcriptional start points, but no other apparent features or similarities in the promoter regions of *THI1* and *THI2*.

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U.fab MSPVATESMYKPTTINQTAHQQAMDPLKSKQQSNATVNKP 40
F.oxy .A.P.--AVSP.SRSAEL.TSTKL-.VM..NINTK..EEM 37
S.cer ..ATS.ATSTSASQLH-------LNSTPVTHCLSDI.K.E 33
S.pom .A.AT--AVVT.-------FKTDL-.V-E.TAH.TV.KSE 32
A.tha .AAI.STLSLSS.KPQRLFDSSFHGSAI.AAPISIGLKPR 40

U.fab A---FKPEPAVNLT-PIKFAPIKEHQVQRAMVRRYFQDME 76
F.oxy LGQW----------DDF.....R.S..S...T.....LD 67
S.cer --DWS----------DF.....R.ST.S...TS...K.LD 61
S.pom MGALS.AY.TYS.DESFS....R.ST.S...T....S.LD 72
A.tha SFSVRATTAGYD.-NAFT.D....SI.S.E.T...MT..I 79

U.fab ERAISDVIIVGAGSAGLSCAYALGTARPDLKITILESNVA 116
F.oxy NY.E..IV.I....C...A..I..KK.....A.I.AS.S 107
S.cer KF.V..........S...A..VIAKN.....VC.I..S.. 101
S.pom KY.E..IV.........TA..YI..R.....A.I.AS.. 112
A.tha TY.ET..VV.........A..EISKN-.NVQVA.I.QS.S 118

U.fab PGGGCWLGGQLMSAMVCRKPADEFLDQVGVPYEDEGNFVV 156
F.oxy ....A.....F...IM.....A..RE.........Y.. 147
S.cer ....S.....F....M....HL..QELEI.....DY.. 141
S.pom ....A.....F....V.....L..NEI.....DY.. 152
A.tha ....A.....F...IV....HL...EI..A.DEQDTY.. 158

U.fab VKHAALFTSTVLSKVLAMPNVKNFNATACEDLIIKPCPIN 196
F.oxy ..........IM....Q...I.L....CV....TR.S--E 185
S.cer .......I.........QL....L....CV....VTR.--TE 180
S.pom ..........MART..L....L....V....V.EG--K 190
A.tha ..........IM..L..R....L...V.A....V.G---- 194

U.fab PGVQRIAGCVTNWTLVSLNHDHQSCMDPSTITAP------ 230
F.oxy E.--.....V.........MH..D.....N..N.------- 218
S.cer K.EVTV..V.........TQA.GT.C....NV.ELAGYKNDG 220
S.pom D.K.....V.........GL.....N..N.H------- 224
A.tha ---N.VG.V....A..AQ..HT.....NVME.K------ 225

U.fab ----------LVCSFAGHDGPFGAFCVKRVASAGLSEGLGD 261
F.oxy ----------.II.TT.....M.....LV.MQRI.K..G 249
S.cer TRDLSQKHGVIL.TT.........A..IVDIDQNQK..G 260
S.pom ----------.V.AT.............L...Q.VSN.H. 255
A.tha ----------I.V.SC.....TG...LK.I.MIDHVPG 256

U.fab MRPLDMERAEDHIA--NKTREILPGLIVGGMELSEFDGSA 299
F.oxy ..G...NL...A.V--KG....V.............V..AN 287
S.cer .KG...NH..HDVVIHSGAYAGVDNMYFA...VA.L..LN 300
S.pom .....N....L.V--KG....VF..M.............AN 293
A.tha .KA...NT...A.V--RL...VV..M..T...VA.I..AP 294

U.fab RNGPTFGAMLLSGKRAAEVALQSLERVKIEEGEVVG---- 335
F.oxy .........A...LK...E..KIFDTR.KQND------- 319
S.cer .........A...VH...QI.KHFAA------------ 326
S.pom ----------------------------SV--------- 300
A.tha .........MI..QK.GQL..KA.GLPNAID.TL..NLSP 334

U.fab ---LA--------K. 339
F.oxy ---------------L 320
S.cer 326
S.pom ---------------V 301
A.tha ELV..AADSAETVDA 349
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Fig. 1. Alignment of the predicted *Uromyces fabae* THI2p amino acid sequence with protein sequences encoded by *Fusarium oxysporum THI4* (F.oxy, accession number P23618), *Saccharomyces cerevisiae THI4* (S.cer, P32318), *Schizosaccharomyces pombe THI2* (S.pom, P40998), and *Arabidopsis thaliana THI4* (A.tha, Q38814). Identical residues are indicated by dots and missing residues by dashes.

Functional complementation of *S. pombe* thiamine mutants.

To prove the identity of *THI1* and *THI2*, thiamine auxotrophic mutants of fission yeast were used for complementa-

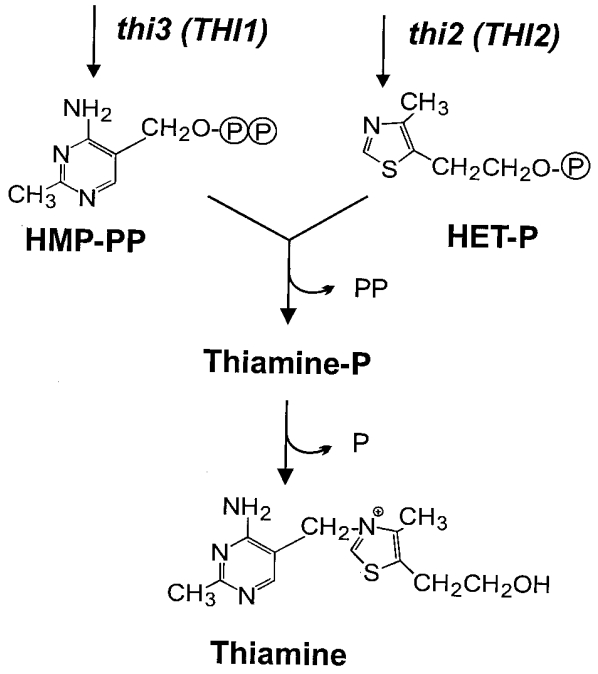

Fig. 2. Thiamine biosynthesis in fungi. The presumptive roles of the *Schizosaccharomyces pombe* genes *thi3* (*nmt1*) and *thi2* (*nmt2*), and of the corresponding *Uromyces fabae* genes *THI1* and *THI2* (in parentheses) are indicated. HMP-PP: 2-methyl-4-amino-5-hydroxymethylpyrimidine-pyrophosphate; HET-P: 4-methyl-5-(ß-hydroxyethyl)thiazol phosphate.

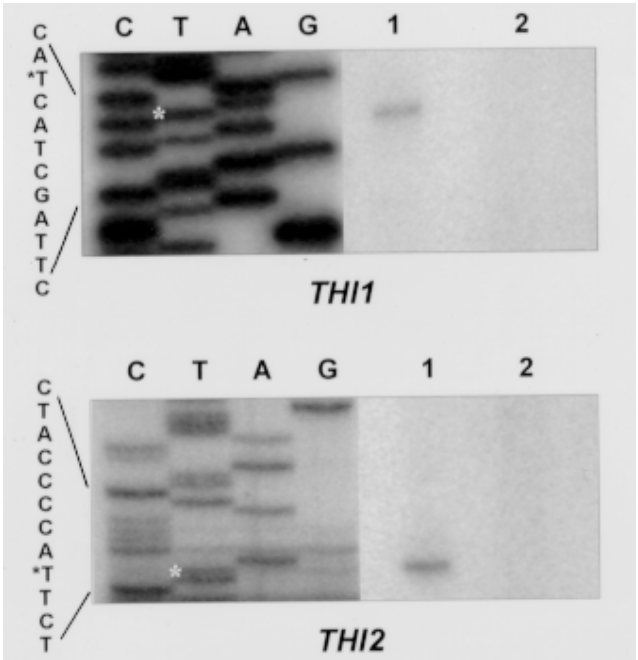

Fig. 3. Determination of the 5′ ends of *THI1* and *THI2* mRNAs by primer extension. In the neighboring sequence ladders, bases flanking the transcriptional start points (asterisks) are indicated. Lane 1: rust-infected leaves (6 days post inoculation); lane 2: noninfected leaves.

tion studies. cDNAs containing the complete coding regions of *THI1* and *THI2* were cloned into the *S. pombe* vectors pSAP-E and pSAP-N, in both expressing and nonexpressing orientations (see Materials and Methods). The recombinant plasmids, as well as the corresponding vectors, were transformed into *S. pombe* strains auxotrophic for leucine and vitamin B1: pSAP-N::*THI1* and pSAP-N::*THI1inv* into strain *thi3-1 leu1-32*, and pSAP-E::*THI2* and pSAP-E::*THI2inv* into strain *thi2-22 leu1-32*. Leucine prototrophic transformants were obtained and tested for their ability to grow on thiamine-free minimal medium. Only those transformants that carried the *THI1* (strain *thi3-1 leu1-32*) or *THI2* (strain *thi2-22 leu1-32*) cDNAs in the expressing orientation were able to grow in the absence of thiamine. In contrast, transformants carrying the vectors only or the cDNAs in inverse orientations did not grow unless thiamine was provided (Fig. 4). Thus, *THI1* and *THI2* were shown to complement their *S. pombe* homologs *thi3* and *thi2*, respectively.

Regulation of *THI1* and *THI2* expression.

The isolation of *THI1* and *THI2* cDNA clones was based on their phase-specific expression in haustoria. The pattern of *THI1* mRNA expression during rust development has been described (Hahn and Mendgen 1997). A very similar expression pattern was observed for *THI2:* No transcripts were detected in uredospores and in vitro-differentiated infection structures up to 18 h after germination. Low transcript levels were detected in 18-and 24-h-old in vitro infection structures, and very high mRNA levels in haustoria and rust-infected *Vicia faba* leaves (Fig. 5). Thus, *THI1* and *THI2* expression begins at a stage of rust development that normally occurs in planta, during formation of haustorial mother cells (Deising et al. 1991). While this initial induction is independent of signals from the plant, maximal expression occurs only in the biotrophic growth phase.

The following experiments were performed to test whether the expression of *THI1* and *THI2* can be suppressed by thiamine in a similar way as the homologous genes of other fungi, which are completely turned off at micromolar concentrations of vitamin B1 (Maundrell 1990; Cary and Bhatnagar 1995). Rust infection structures developed in vitro and in rust-infected broad bean leaves were treated with various concentrations of thiamine and the transcript levels of the *THI* genes were compared with the levels found in untreated controls (see Materials and Methods). In 24-h-old infection structures, which show easily detectable mRNA levels of *THI1* (Hahn and Mendgen 1997) and *THI2* (Fig. 5), treatment with 100 µM thiamine did not alter the transcript levels found with water-treated structures (Fig. 6A). Similarly, the high expression of the genes in rust-infected leaves remained unchanged after daily treatments of the leaves with thiamine concentrations up to 1 mM (Fig. 6B). These data demonstrate that, in *U. fabae*, expression of thiamine biosynthesis genes is unaffected by the presence of exogenous thiamine.

Immunological detection of THI1p.

The occurrence of the *THI1*-encoded protein in rust-infected leaves and in various stages of rust development was analyzed with polyclonal antibodies generated against histidine-tagged THI1p produced in *Escherichia coli* (see Materials and Methods). In a time course experiment, THI1p was not detected by Western blot (immunoblot) analysis in protein extracts from rust-infected leaves until 5 days after inoculation with *U. fabae* (Fig. 7A). THI1p was also detected in spores, and in progressively lower concentrations in germlings and infection

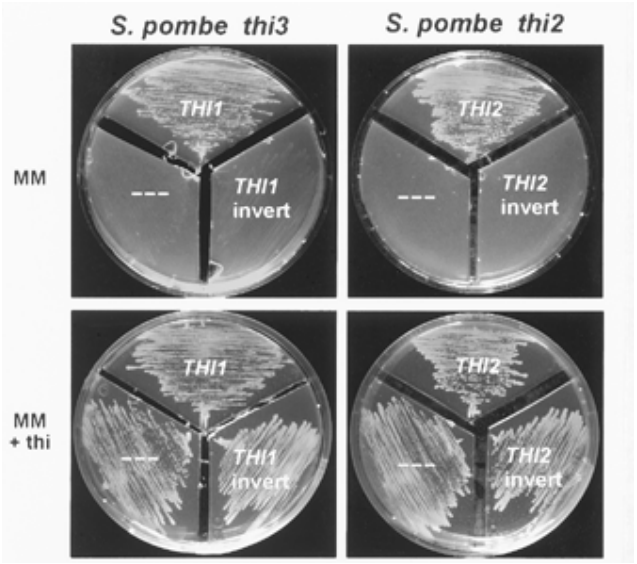

Fig. 4. Complementation of *Schizosaccaromyces pombe* thiamine auxotrophic mutants with plasmids carrying *THI1* or *THI2* cDNAs from *Uromyces fabae*. On each sector of the agar plates a strain carrying one of the following plasmids was streaked out: In the *S. pombe* strain *thi3-1 leu1-32* (left panels) pSAP-N (---), pSAP-N::*THI1* (*THI1*), and pSAP-N::*THI1inv* (*THI1* invert); in *S. pombe* strain *thi2-22 1eu1-32* (right panels) pSAP-E (---), pSAP-E::*THI2* (*THI2*), and pSAP-N::*THI2inv* (*THI2* invert). The plates contained minimal medium alone (MM) or supplemented with 50 nM thiamine (MM + thi).

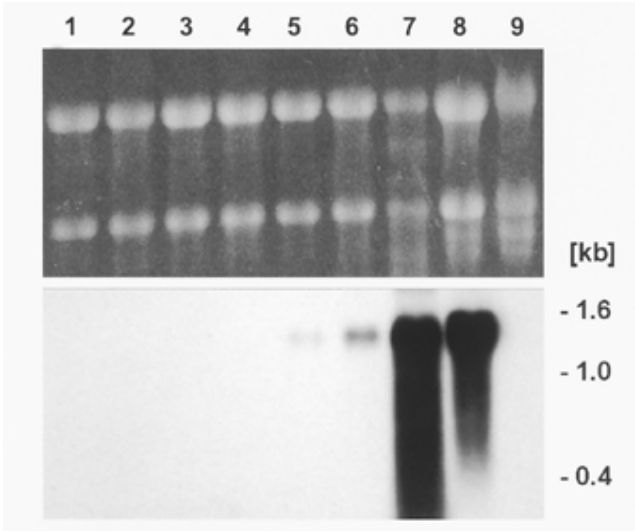

Fig. 5. *THI2* transcript levels during rust development. Top panel: ethidium bromide-stained agarose gel before transfer of the RNA. Bottom panel: autoradiograph with hybridization signals after chemiluminescent detection of digoxigenin-labeled *THI2*-cDNA probe. Lane 1: nongerminated uredospores; lane 2: 4 h germinated spores; lanes 3 to 6: in vitro-grown infection structures 6, 12, 18, and 24 h post inoculation (pi), respectively; lane 7: isolated haustoria; lane 8: rust-infected leaves (5 days pi); lane 9 : noninfected leaves. Five micrograms of total RNA was loaded per lane.

structures of increasing age (Fig. 7B). With a 25-fold lower protein concentration, THI1p was easily detected in haustoria (Fig. 7B, lane H). Immunofluorescence microscopy of rust-infected leaves also revealed very strong labeling of THI1p in haustoria and young haustorial mother cells, whereas intercellular hyphae were significantly weaker labeled (Fig. 8A, B). In leaves containing sporulating mycelium, THI1p was also found in the basal sporogenous hyphae of the uredia. In contrast, no fluorescence was detected in the adjacent pedicels and only barely detectable levels were found in the uredospores (Fig. 8C). Controls with pre-immune serum did not show any labeling of infected leaf tissue.

DISCUSSION

In rust fungi, the early stages of development, i.e., germination and infection structure formation up to the primary haustorial mother cell, can occur in the absence of the host plant and of any supplied nutrients, due to large nutrient reserves of the uredospores (Staples and Macko 1984; Deising et al. 1991). In contrast, growth of the biotrophic mycelium is dependent on metabolites provided by the host. The drastic changes in gene expression that are observed when *Uromyces fabae* enters the parasitic growth stage (Hahn and Mendgen 1997) probably reflect the molecular and cytological adaptations of the fungus to the biotrophic mode of growth. Among the in planta induced genes (*PIG*s), the thiamine biosynthesis genes *THI1* and *THI2* are the most highly expressed in haustoria, together representing approximately 5% of the haustorial mRNA (Hahn and Mendgen 1997). A similarly high level of expression of *S. pombe* genes homologous to *THI1* (*thi3* / *nmt1*) and *THI2* (*thi2* / *nmt2*) was observed when yeast cells were grown in the absence of thiamine (Maundrell 1990; Praekelt and Meacock 1992; Manetti et al. 1994). The most likely interpretation is that *U. fabae* performs thiamine biosynthesis very actively only during parasitic growth, presumably because this vitamin is growth limiting and not available from the host plant.

The expression patterns of *THI1* and *THI2* are very similar: No significant amounts of transcripts were detected until 18 h after germination. The mRNA levels increased in 18- and 24-h-old, in vitro-grown infection structures, and accumulated to very high concentrations in haustoria and rust-infected leaves. These data indicate that the activation of these genes is coupled to fungal development, but not directly induced by chemical host factors. In contrast to what occurs in several saprophytic fungi, thiamine biosynthesis in *U. fabae* is not suppressed by exogenously added thiamine. Our experiments could not distinguish whether this was due to the absence of a repressive effect or the inability of the rust hyphae to take up thiamine, possibly due to the lack of an appropriate transport system. In *S. cerevisiae*, thiamine uptake was shown to be mediated by a specific transporter belonging to the major facilitator superfamily (Enjo et al. 1997; Singleton 1997).

Sequence analysis of *THI1* and *THI2* revealed the presence of 9 and 7 introns, respectively. Based on the mRNA lengths of *THI1* (1,466 bp) and *THI2* (1,353 bp), this corresponds to an average exon length of 147 bp (*THI1*) and 169 bp (*THI2*). Because of their similar expression pattern and the involvement in the same biosynthesis pathway, it was expected that *THI1* and *THI2* share common regulatory elements. However, the sequences upstream of the transcriptional start sites of *THI1* and *THI2* did not reveal obvious promoter elements or any other similar sequences. To test whether these genes are subject to common regulation, DNA-protein binding studies need to be performed.

While no transcripts were observed in spores and early infection structures, *THI1*-encoded protein was detected in these samples by immunoblot analysis. Nevertheless, the decrease of THI1p during germination and infection structure formation indicates that the protein is subject to degradation during the

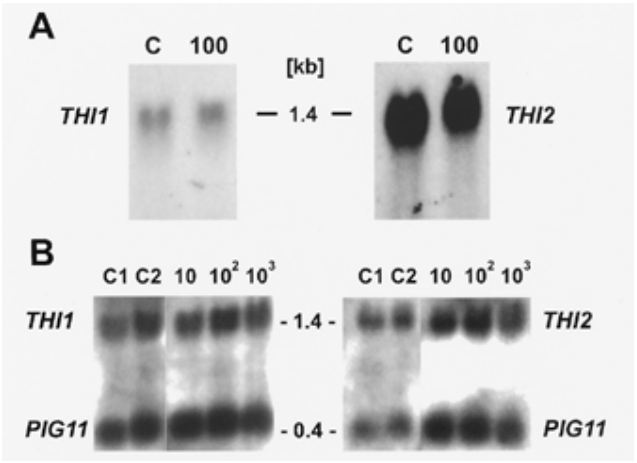

Fig. 6. *THI1* and *THI2* transcript levels after treatment with thiamine. **A,** Infection structures (24 h old) grown on polyethylene membranes. Distilled water (C) or 100 µM thiamine (100) was sprayed onto the spores before germination. Hybridization was performed with digoxigenin-labeled *THI1* (left) or *THI2* (right) cDNA probes. Ten micrograms of total fungal RNA was loaded per lane. **B,** Rust-infected leaves. Infiltration treatments were performed daily as described in Materials and Methods. To confirm similar loading and quality of RNA in all samples, *THI1* (left) and *THI2* (right) probes were mixed with a cDNA probe for *PIG11*, a gene encoding a putative metallothionein (Hahn and Mendgen 1997). *PIG11* was found to be a suitable marker for the amount of fungal mycelium in rust-infected leaves (J. Sohn and M. Hahn, *unpublished data*). C1: Noninfiltrated infected leaves. C2: Water-infiltrated infected leaves. 10, 10^2, 10^3: Infected leaves infiltrated with various concentrations (µM) of thiamine. Ten micrograms of total leaf RNA was loaded per lane.

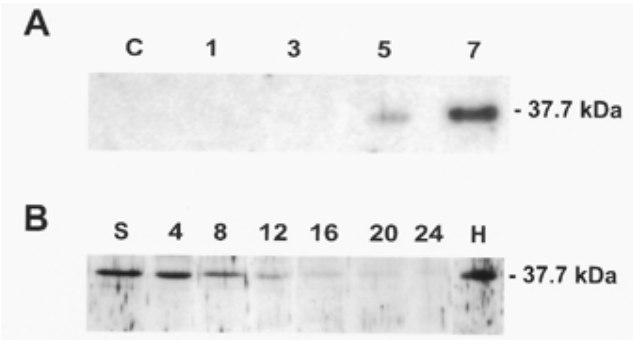

Fig. 7. Detection of THI1p by immunoblot analysis. **A,** Infected leaves. Numbers indicate days after inoculation with *Uromyces fabae*. C: Noninfected leaves. Twenty micrograms of total protein was loaded per lane. Antiserum dilution was 1:7.500. **B,** *U. fabae* at various stages of development. S: uredospores. Numbers indicate times after inoculation (4 to 24 h) of in vitro-grown infection structures. H: haustoria. Fifty micrograms of total protein was loaded on each lane, except for lane H (2 µg). Antiserum dilution was 1:1.000.

first hours of development. Apparently, the fungus concentrates its metabolic efforts during early development on the requirements of the invasion process, without investing in costly biosynthetic activities. In the biotrophic rust mycelium, THI1p levels are much higher, consistent with the very high *THI1* mRNA level found in planta. The particularly strong THI1p labeling in haustoria indicates that they are the major sites of thiamine biosynthesis. In contrast, intercellular hyphae showed only weak labeling, whereas an intermediate level of fluorescence was observed in the basal cell layer of uredia. The differences of labeling were partially due to vacuolization, which was pronounced in most hyphae but not in haustoria. Thus, THI1 shows a distinct pattern of cellular distribution.

There is evidence that plant-pathogenic fungi are under nutrient limitation during biotrophic growth within the plant, based on the low apoplastic metabolite concentrations (Lohaus et al. 1995) and on the observations that starvation-induced genes in *Cladosporium fulvum* and *Magnaporthe grisea* are also activated during growth in planta (Coleman et al. 1997; Talbot et al. 1997). Increased permeability of host membranes has been reported to be induced by powdery mildew infection (Aked and Hall 1993) but not by rust infection (Farrar and Lewis 1987). Low apoplastic concentrations could be one explanation for the evolution of haustoria if they form an interface at which increased metabolite efflux from the host cell occurs, due to an increase in cytoplasmic solute concentration or plasma membrane permeability (Patrick 1989). Evidence for the latter has been obtained by cytochemical studies that indicate that the host-derived extrahaustorial membrane might be depolarized due to the lack of plasma membrane ATPase activity (Spencer-Phillips and Gay 1981; Baka et al. 1995).

The observations of haustorium-specific expression of a putative amino acid transporter (Hahn et al. 1997) and a hexose transporter (R. Voegele, M. Hahn, and K. Mendgen, *unpublished data*) in *U. fabae* represent molecular evidence for a prominent role of rust haustoria in nutrient absorption. The data presented in this paper indicate that haustoria also perform biosyntheses of metabolites that are not available in sufficient amounts from the plant. Based on the very high and rather specific expression of *THI1* and *THI2* in haustoria, we hypothesize that they synthesize enough thiamine to supply it to other parts of the biotrophic mycelium. Such a privileged role of haustoria could explain the difficulties in establishing axenic cultures with rust fungi, which has only been achieved with a few species (Mendgen 1981; Fasters et al. 1993). Because such cultures never produce haustoria, they might be strongly restricted in both the uptake and the biosynthesis of

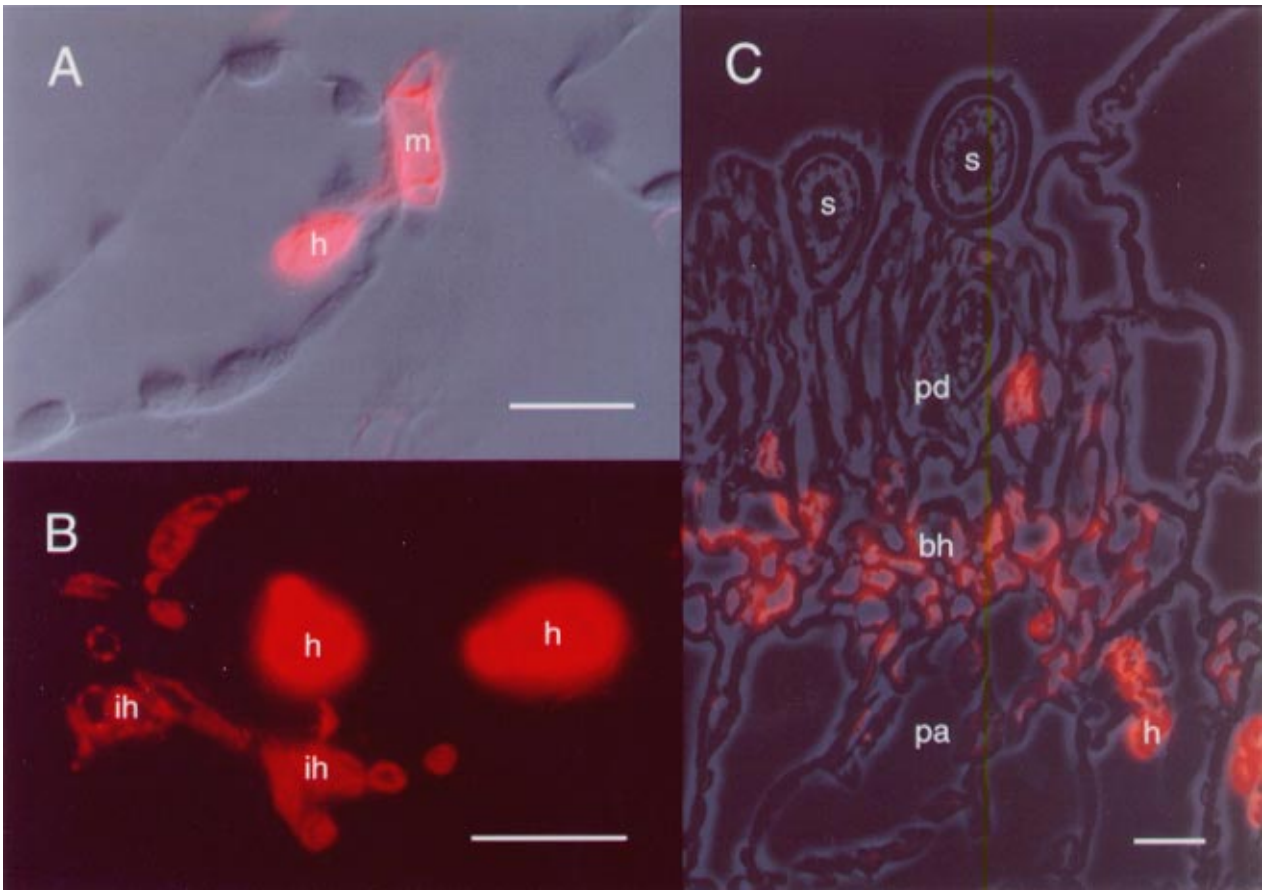

Fig. 8. Localization of THI1p in rust-infected leaves by immunofluorescence microscopy. **A,** Fluorescently labeled haustorial mother cell (m) and haustorium (h) in a mesophyll cell. Fluorescence and differential interference contrast superimposed. **B,** Overexposure of image reveals weakly labeled intercellular hyphae (ih) beside two strongly labeled haustoria (h). **C,** Sporulating lesion with palisade parenchyma cells (pa), basal hyphae (bh), pedicels (pd), and newly formed uredospores (s). Only basal hyphae and haustoria are labeled. Fluorescence and phase contrast superimposed. Antiserum dilution was 1:500. Scale bars: 10 µm.

growth-limiting metabolites. Thus, we speculate that the near-obligate nature of biotrophy in rust fungi could be due to the fact that differentiation of functional haustoria, which occurs only in living plant cells, is indispensable for effective growth.

MATERIALS AND METHODS

Cultivation and manipulation of plant and rust fungus.

Cultivation of broad bean plants (*Vicia faba* cv. con Amore) and inoculation with *Uromyces fabae* race I_2 uredospores were done as described (Hahn and Mendgen 1997). Germinated spores and in vitro-grown infection structures were obtained as described (Deising et al. 1991; Hahn and Mendgen 1997).

Thiamine treatments of rust infection structures and infected leaves were done as follows: Spores settled on scratched polyethylene membranes were sprayed with 100 µM thiamine/HCl or water, and incubated for 24 h at 20°C in the dark to allow infection structure formation. Broad beans leaves were spray inoculated with spores, and after 24 h, while being attached to the plant, infiltrated by pressing liquid with a blunt-ended syringe until they looked completely water soaked. The infiltration was repeated daily until 6 days post inoculation (dpi), and the leaves harvested 7 dpi. Infiltration was done with various concentrations of thiamine, or with water only. Another control plant remained untreated after inoculation.

Nucleic acid manipulations and plasmid constructions.

RNA isolations from rust-infected leaves, *U. fabae* infection structures, and isolated haustoria (Hahn and Mendgen 1992), and Northern (RNA) blot hybridization with digoxigenin-labeled DNA probes (Hahn and Mendgen 1997), were performed as described.

For complementation of fission yeast thiamine mutants, a 1,339-bp, near-full-length *THI2* cDNA was ligated as an *Eco*RI fragment into the *Schizosaccharomyces pombe* expression vector pSAP-E (Truernit et al. 1996), resulting in the *THI2*-expressing plasmid pSAP-E-*THI2*. In the plasmid pSAP-E-*THI2inv,* the fragment was inserted in the opposite orientation. For expression of the corresponding *THI1* constructs, the *Eco*RI cloning site of the vector pSAP-E was converted into a *Not*I site by *Eco*RI digestion followed by blunting and ligation to a *Not*I linker (Stratagene, La Jolla, CA), resulting in the new vector pSAP-N. Into this vector, a 1,412-bp, near-full-length *THI1* cDNA obtained from a haustorium-specific λgt10 cDNA library (Hahn and Mendgen 1997) was ligated as a *Not*I fragment in both orientations, resulting in plasmids pSAP-N-*THI1* (expressing) and pSAP-N-*THI1inv* (nonexpressing).

For overexpression of the *THI1* encoded protein in *E. coli*, another *THI1* clone of the haustorial cDNA library (cloned as a 1,426-bp *Sma*I fragment into plasmid pTZ19R) was amplified by PCR, with the sequence GAGATTCCTTCGTGC CATATGTCTACCGA as 5′ terminal primer, which contains an *Nde*I site (underlined) including the putative *THI1* start codon. The amplification product was digested with *Nde*I plus *Hin*dIII and the resulting 1,360-bp fragment ligated into *Nde*I plus *Hin*dIII-digested pET28a(+) (Novagen, Madison, WI). The resulting plasmid, pTHI1EXP1, expressed the complete THI1 protein, fused upstream to 20 vector-encoded amino acids including a 6xHis tag.

To produce templates for sequencing, the genomic clones λEMBL3-*THI1* and λEMBL3-*THI2* were digested with *Hin*dIII and *Bam*HI, respectively. A 4.0-kb *Hin*dIII fragment containing *THI1* and a 3.9-kb *Bam*HI fragment containing *THI2* were each ligated into pTZ19R (Amersham Pharmacia Biotech, Uppsala, Sweden). Sequencing was performed with fluorescently labeled ddNTP terminators and "walking" primers, with a 377 ABI sequencer (PE Applied Biosystems, Foster City, CA).

Determination of transcriptional start sites of *THI1* and *THI2* was done by primer extension analysis (Ausubel et al. 1995). Primer labeling was performed in 10 µl with 10 pmol of primer (*THI1*: TGAGAAGAACTGAAATTTTATCGGTA GACA; *THI2*: TGAGCAGTTTGATTGATAGTAGTAGGT TTG), 30 µCi adenosine 5′-[γ-^{32}P]triphosphate, T4 polynucleotide kinase buffer, and 15 U of T4 polynucleotide kinase (New England Biolabs, Beverly, CA), for 1 h at 37°C and stopped by 5 min of incubation at 65°C. The solution was precipitated three times with 200 µl of acetate buffer (0.5 M ammonium acetate, 10 mM EDTA) and 500 µl of ethanol and the resulting pellet dissolved in 50 µl of water. The primer extension reactions were carried out with 10 µg of poly(A)-RNA from rust infected leaves (6 dpi), and from noninfected control leaves. After annealing of the primer to the RNA in hybridization solution at 30°C overnight, the nucleic acids were ethanol precipitated, and the pellet dissolved in 20 µl of solution containing AMV-Buffer, 1 mM dNTPs, 24 U of RNasin ribonuclease inhibitor, and 4 U of AMV reverse transcriptase (Promega, Madison, WI). After incubation at 42°C for 1 h, the reaction was stopped by addition of 1 µl of 0.5 M EDTA and 1 µl of RNaseA (10 µg/µl) and incubation at 37°C for 30 min. After phenol extraction and ethanol precipitation, the DNA was dissolved in loading buffer and separated on a 6% polyacrylamide sequencing gel. For the sequencing reactions, the T7-Sequenase V.2.0 kit (Amersham) was used.

Fission yeast methods.

The *S. pombe* strains *thi2-22*(–), *thi3-1*(–) (Zurlinden and Schweingruber 1992), and *leu1-32*(+) were obtained from M. E. Schweingruber, Bern. All strains were cultivated at 30°C on minimal medium plates with the required supplements (Schweingruber and Edenharter 1990). With the protocol of Alfa et al. (1993), the *leu* mutant gene was crossed into the *thi* mutants, resulting in strains *thi2-22 leu1-32* and *thi3-1 leu1-32.* Plasmid transformation was done according to Okazaki et al. (1990). Transformants containing pSAP-E, pSAP-N, and their recombinant derivatives were selected on minimal medium agar plates without leucine, and subsequently tested for growth in the presence or absence of 50 nM thiamine.

Expression of His-tagged THI1p and antibody generation.

Overexpression of the recombinant, His-tagged THI1 protein encoded by plasmid pTHI1EXP1 (see above) was done with *E. coli* BL21(DE3) and induction with 0.5 mM isopropyl-β-D-thiogalactoside (Studier and Moffatt 1986). A one-step purification of THI1p was performed with immobilized metal ion affinity chromatography under denaturing conditions, according to the protocol of the manufacturer (Clontech, Palo Alto, CA). The protein was rebuffered in TBS (10 mM Tris/HCl, 150 mM NaCl, pH 7.4) and used for the immuniza-

tion of a rabbit. In immunoblots, the antiserum specifically recognized the THI1 protein whereas the preimmune serum did not.

Protein extraction and immunoblot analysis.

Spores were ground in a mortar with sea sand in 200 mM Tris/HCl, pH 7.4 at 4°C. Isolated haustoria were ground without sea sand in the same buffer. Rust infection structures grown on polyethylene membranes were sprayed with 1.5 ml of buffer (50 mM ammonium acetate, 1 mM PMSF [phenylmethylsulfonyl fluoride], 1 mM dithioerythreitol) and collected by scraping. The infection structures were homogenized on ice for 2 min with a polytron homogenizer (Ultraturrax T4; Janke & Kunkel, Staufen, Germany). Leaf tissue was ground in liquid nitrogen and dissolved in 10 mM Tris/HCl, pH 7.4. The resulting homogenates were centrifuged (16,000 × *g*, 5 min, 4°C). The clear supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970). Immunoblots were performed according to Towbin et al. (1979), with 15.6 mM Tris, 120 mM glycine, 20% (vol/vol) methanol, as electrode buffer. Proteins were electrotransferred to an Immobilon-P membrane (Millipore, Bedford, MA), probed with rabbit anti-THI1p serum, and visualized with a peroxidase-coupled goat anti-rabbit secondary antibody and ECL Western Blot Detection Reagent (Amersham).

Immunofluorescence microscopy.

Rust-infected leaf pieces (8 dpi) were vacuum infiltrated with 8% (vol/vol) methanol in water, high pressure frozen with the HPM 010 instrument (Balzers, Liechtenstein) and freeze substituted as described (Mendgen et al. 1991). Specimens were embedded in a resin consisting of 75% butyl methacrylate and 25% methyl methacrylate, 0.5% benzoin ethyl ether, and 10 mM dithiothreitol. The resin was infiltrated in mixtures with acetone (25, 50, 75, 100% resin) for 1 day each at 4°C. Polymerization took place at 4°C during 4 days under UV light. Sections were treated with acetone for 30 s, and three times for 15 min with blocking buffer (0.1% [wt/vol] bovine serum albumin in TBS [10 mM Tris/HCl, 150 mM NaCl, pH 7.4]) and incubated with anti-THI1p serum (1:500) in TBS for 2 h. To detect nonspecific binding, the pre-immune serum was used as control. After three washes for 15 min in TBS, sections were incubated with the secondary antibody (cyanin-3-conjugated goat-anti-rabbit; Rockland, Gilbertsville, PA), diluted 1:400 with TBS, for 1 h at 20°C. Samples were examined with a Zeiss Axioplan 2 microscope equipped for epifluorescence (filters BP 490, FT 510, LP565) with phase contrast, Nomarski differential interference contrast or fluorescence. Images were taken with Fujicolor 800 (Tokyo), digitized with an HP Photosmart Fotoscanner S20 (Hewlett-Packard, Palo Alto, CA) and superimposed with Photoshop software (Adobe Systems, Mountain View, CA).

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LITERATURE CITED

Alfa, C., Fantes, P., Hyams, J., McLeod, M., and Warbrick, E. 1993. Pages 577-580 in: Experiments with Fission Yeast. A Laboratory Course Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Aked, J., and Hall, J. L. 1993. Effect of powdery mildew infection on concentrations of apoplastic sugars in pea leaves. New Phytol. 123: 283-288.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. 1995. Current Protocols in Molecular Biology. John Wiley & Sons, New York.

Baka, Z. A., Larous, L., and Loesel, D. M. 1995. Distribution of ATPase activity at the host-pathogen interfaces of rust infections. Physiol. Mol. Plant Pathol. 47:67-82.

Cary, J. W., and Bhatnagar, D. 1995. Nucleotide sequence of a *Aspergillus parasiticus* gene strongly repressed by thiamine. Biochim. Biophys. Acta 1261:319-320.

Coffey, M. D., and Shaw, M. 1972. Nutritional studies with axenic cultures of the flax rust, *Melampsora lini*. Physiol. Plant Pathol. 2:37-46.

Coleman, M., Henricot, B., Arnau, J., and Oliver, R. P. 1997. Starvation-induced genes of the tomato pathogen *Cladosporium fulvum* are also induced during growth in planta. Mol. Plant-Microbe Interact. 10: 1106-1109.

Deising, H., Jungblut, P. R., and Mendgen, K. 1991. Differentiation-related proteins of the broad bean rust fungus *Uromyces viciae-fabae*, as revealed by high resolution two-dimensional polyacrylamide gel electrophoresis. Arch. Microbiol. 155:191-198.

Enjo, F., Nosaka, K., Ogata, M., Iwashima, A., and Nishimura, H. 1997. Isolation and characterization of a thiamin transport gene, THI10, from *Saccharomyces cerevisiae*. J. Biol. Chem. 272:19165-19170.

Farrar, J. F., and Lewis, D. H. 1987. Nutrient relations in biotrophic infections. Pages 92-132 in: Fungal Infection of Plants. G. F. Pegg and P. G. Ayres, eds. Cambridge University Press, Cambridge.

Fasters, M. K., Daniels, U., and Moerschbacher, B. M. 1993. A simple and reliable method for growing the wheat stem rust fungus, *Puccinia graminis* f. sp. *tritici*, in liquid culture. Physiol. Mol. Plant Pathol. 42: 259-265.

Hahn, M., and Mendgen, K. 1992. Isolation by ConA binding of haustoria from different rust fungi and comparison of their surface qualities. Protoplasma 170:95-103.

Hahn, M., and Mendgen, K. 1997. Characterization of in planta-induced rust genes isolated from a haustorium-specific cDNA library. Mol. Plant-Microbe Interact. 10:427-437.

Hahn, M., Neef, U., Struck, C., Göttfert, M., and Mendgen, K. 1997. A putative amino-acid transporter is specifically expressed in haustoria of the rust fungus *Uromyces fabae*. Mol. Plant-Microbe Interact. 10: 438-445.

Heath, M. C., and Skalamera, D. 1997. Cellular interactions between plants and biotrophic fungal parasites. Adv. Bot. Res. 24:195-225.

Hohmann, S., and Meacock, P. A. 1998. Thiamin metabolism and thiamin diphosphate-dependent enzymes in the yeast *Saccharomyces cerevisiae*: genetic regulation. Biochim. Biophys. Acta 1385:201-219.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lohaus, G., Winter, H., Riens, B., and Heldt, H. W. 1995. Further studies of the phloem loading process in leaves of barley and spinach. The comparison of metabolite concentrations in the apoplastic compartment with those in the cytosolic compartment and in the sieve tubes. Bot. Acta 108:270-275.

Manetti, A. G., Rosetto, M., and Maundrell, K. G. 1994. *nmt2* of fission yeast: A second thiamine-repressible gene co-ordinately regulated with *nmt1*. Yeast 10:1075-1082.

Maundrell, K. 1990. *nmt1* of fission yeast. J. Biol. Chem. 265:10857-10864.

Mendgen, K. 1981. Nutrient uptake in rust fungi. Phytopathology 71: 983-989.

Mendgen, K., Welter, K., Scheffold, F., and Knauf-Beiter, G. 1991. High pressure freezing of rust infected plant leaves. Pages 31-42 in: Electron Microscopy of Plant Pathogens. K. Mendgen and D. E. Lesemann, eds. Springer Verlag, Berlin.

Okazaki, K., Okazaki, N., Kume, K., Jinno, S., Tanaka, K., and Okayama, H. 1990. High-frequency transformation method and library transducing vectors for cloning mammalian cDNAs by trans-comple-

mentation of *Schizosaccharomyces pombe*. Nucleic Acids Res. 18: 6485-6489.
Patrick, J. W. 1989. Solute efflux from the host at plant-microorganism interfaces. Aust. J. Plant Physiol. 16:53-67.
Praekelt, U., and Meacock, P. 1992. *MOL1*, a *Saccharomyces cerevisiae* gene that is highly expressed in early stationary phase during growth on molasses. Yeast 8:699-710.
Schweingruber, M. E., and Edenharter, E. 1990. Thiamin regulates agglutination and zygote formation in *Schizosaccharomyces pombe*. Curr. Genet. 17:191-194.
Singleton, C. K. 1997. Identification and characterization of the thiamine transporter gene of *Saccharomyces cerevisiae*. Gene 199:111-121.
Spencer-Phillips, P. T. N., and Gay, J. L. 1981. Domains of ATPase in plasma membranes and transport through infected plant cells. New Phytol. 89:393-400.
Staples, R. C., and Macko, V. 1984. Germination of Urediospores and differentiation of infection structures. Pages 255-289 in: The Cereal Rusts, Volume I. W. R. Bushnell and A. P. Roelfs, eds. Academic Press, Orlando, FL.
Studier, F. W., and Moffatt, B. A. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. 189:113-130.
Talbot, N. J., McCafferty, H. R. K., Ma, M., Moore, K., and Hamer, J. E. 1997. Nitrogen starvation of the rice blast fungus *Magnaporthe grisea* may act as an environmental cue for disease symptom expression. Physiol. Mol. Plant Pathol. 50:179-195.
Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350-4354.
Truernit, E., Schmid, J., Epple, P., Illig, J., and Sauer, N. 1996. The sink-specific and stress-regulated Arabidopsis *STP4* gene: Enhanced expression of a gene encoding a monosaccharide transporter by wounding, elicitors and pathogen challenge. Plant Cell 8:2169-2182.
Williams, P. G. 1984. Obligate parasitism and axenic culture. Pages 399-430 in: The Cereal Rusts, Volume I. W. R. Bushnell and A. P. Roelfs, eds. Academic Press, Orlando, FL.
Zurlinden, A., and Schweingruber, M. E. 1992. Cloning and regulation of *Schizosaccharomyces pombe thi2*, a gene involved in thiamine biosynthesis. Gene 117:141-143.