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Cloning of β-tubulin and succinate dehydrogenase genes from *Uromyces fabae* and establishing selection conditions for their use in transformation

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Abstract

The obligate biotrophic nature of rust fungi calls for an *in planta* selection scheme as a means of developing a rust transformation technology. We show that the fungicides benomyl (used as its formulated product benlate) and carboxin suppress morphogenesis of the rust fungus *Uromyces fabae in vitro* and disease *in planta*, the latter without affecting the health of the host. The limits of their applicability were determined regarding concentration, method of application and optimal time intervals of treatment. Besides procedures for selection, a stable transformation system will also need to include genetic markers allowing to enrich for transformed cells within a large background of untransformed cells. Since the molecular targets of benlate and carboxin had been identified as β-tubulin and succinate dehydrogenase, respectively, the corresponding genes (*Uf-TBBI* and *Uf-SUCDHI*) were cloned and characterized. Molecular phylogenies demonstrate that both are typical homologs to those of other Basidiomycota. RT-PCR analysis confirmed that both genes are constitutively expressed in all developmental stages of the mitotic uredospore multiplication cycle. Since homologs of *Uf-TBB1* and *Uf-SUCDH1* have been successfully used as selection markers in other fungal systems, they provide valuable tools to develop additional corner stones of a stable transformation system for rust fungi.

Introduction

Rust fungi comprise more than 6000 species infecting a wide range of hosts including some important crop plants. Events leading to the establishment of disease have been particularly well documented by cytological methods (Hoch et al., 1987; Mendgen et al., 1996; Heath and Skalamera, 1997). However, the obligate biotrophic nature of these fungi has complicated the application of molecular genetics.

cDNA sequences from a library enriched for haustoria of *Uromyces fabae* have been determined (Hahn and Mendgen, 1997). The function of several genes was inferred from their sequences, thus permitting heterologous expression studies in yeast and *E. coli* to be devised. These confirmed their assumed roles as enzymes of the vitamin B1 biosynthetic pathway, a plasma membrane ATPase and a hexose transporter, respectively (Struck et al., 1998; Sohn et al., 2000; Voegele et al., 2001). On the other hand, BLAST searches revealed that many of the obtained ESTs did not find matches in contemporary databases, indicating the existence of genes in obligate biotrophic fungal plant pathogens that are missing in the currently sequenced model genomes. Their functional analyses and/or that of putative avirulence genes would tremendously

benefit from a transformation system for rust fungi since this would allow the application of gene inactivation and overexpression technologies in the homologous genetic background.

Transient rust transformation has previously been described by demonstrating the expression of the β-glucuronidase (GUS) reporter gene after particle bombardment of uredospores (Bhairi and Staples, 1992; Li et al., 1993; Schillberg et al., 2000). In these experiments, GUS expression was not observed beyond the stage of the substomatal vesicle. A more ambitious goal is the establishment of a stable transformation system leading to the permanent integration of a selection marker and finally to the production of recombinant fungal spores. Since rust fungi are obligate biotrophic such a selection has to proceed on the host. This can theoretically be achieved in two ways. First, one might use a dominant antibiotic affecting a broad range of organisms. Hygromycin B represents this type and the corresponding resistance gene originating from *E. coli* is a widely applied selection marker in fungal transformation systems (Fincham, 1989; Punt and Van den Hondel, 1992). Since this drug also affects plants (Waldron et al., 1985) it cannot be directly applied to rust transformation. An alternative approach, avoiding the efforts required to generate Hygromycin B resistant host lines would be to use antibiotics that specifically target fungi such as benomyl and carboxin. Fungal resistance to these drugs has been documented and it was found to be caused by mutations in genes encoding their molecular targets, i.e., β-tubulin (Jung et al., 1992; Reijo et al., 1994) and succinate dehydrogenase (Broomfield and Hargreaves, 1992; Skinner et al., 1998; Honda et al., 2000). Here, we investigate the suitability of these agents for selection in rust transformation and develop procedures for their application. We describe the cloning and characterization of the β-tubulin (*Uf-TBB1*) and the succinate dehydrogenase (*Uf-SUCDH1*) genes from *Uromyces fabae* as the base for selection schemes.

Materials and methods

Cultivation and manipulation of host plants and rust fungi

Procedures for cultivating *Vicia faba* cv. Con Amore and propagation of *Uromyces fabae* are outlined in (Deising et al., 1991). Large-scale *in vitro* differentiated rust infection structures for RNA preparations were produced in a custom-designed aluminium container. Liquid cultures for arresting uredospore differentiations in the germ tube stage were used for some control RNA preparations (Struck et al., 1996).

The effect of benomyl (used as the formulated product benlate) and carboxin on rust differentiation was studied in an *in vitro* system and *in planta*. The *in vitro* system used an 'inoculation tower' with an effective area of 16×16 cm to inoculate 18 mg of dry uredospores on four pieces of polyethylene sheets (PE) (1×4 cm) (Bender and Hobein GmbH, Ulm, Germany). After settling of spores (15 min) the PE pieces were laid on wetted filter paper in a glass petri dish. They were sprayed with aqueous suspensions containing benlate (Registered Trademark, Du Pont de Nemours SA, France) and carboxin (Riedel-de-Haen AG, Seelze, Germany); concentrations provided refer to the amount of active ingredient. The petri dishes were sealed and incubated in the dark at 24 °C for 22 h. Staining of fungal cells was performed with a solution containing 1 mg ml^{-1} Trypanblue, 0.3 g ml^{-1} Phenol, 30% glycerol and 30% lactic acid.

Treatment of rust-infected plants with benlate and carboxin was carried out in several ways. For all experiments, we used 2 ml of a suspension of uredospores that were washed twice for 30 min in 500 ml distilled water, harvested on filter paper and adjusted to a concentration of 1.25 mg ml^{-1}. This inoculum was evenly sprayed onto four to five 14 days old *Vicia faba* plants. Further incubation was as described (Deising et al., 1991). Antibiotics were either spray inoculated onto the leaves or systemically applied via the roots. In one series of spray inoculations, the fungicide was included with the spore suspension. In another, we sprayed the spores without the fungicide and then interrupted the incubation in a time dependent manner to additionally spray a defined volume of 2 ml of the respective drug onto the plants using the denoted concentrations. In systemic application experiments, the fungicide had been included in the watering scheme, each treatment applying a defined volume of 50 ml of the respective drug at the indicated concentrations. In one series, this was executed twice, i.e., 1 day before and about 4 h before the inoculation with the fungus, and then

ceased. In another, the application continued every other day until scoring of the symptoms. In a third, antibiotics were not applied before the inoculation with spores but rather started in a time dependent manner thereafter and then continued every other day until the end of the experiment.

Nucleic acid manipulations

Isolation of genomic DNA, RNA and DNA blot hybridizations with digoxigenin-labeled probes were done as outlined (Hahn and Mendgen, 1997). Lambda EMBL3 genomic and lambda GT10 cDNA libraries used to isolate *Uf-TBB1* and *Uf-SUCDH1* were described earlier (Hahn and Mendgen, 1997).

PCR reactions to initially retrieve *Uf-TBB1* gene fragments relied on degenerate primers that targeted conserved regions of fungal β-tubulin genes (Yarden and Katan, 1993). A lysate from a lambda GT10 cDNA library from 18 h old *in vitro* differentiated rust infection structures (Hahn and Mendgen, 1997) was used as template. The obtained PCR fragment was digoxigenin labeled and used as a probe to screen both the genomic and the cDNA phage libraries. Positive clones covering the whole coding region were identified in both cases. A 5 kb *Xba*I fragment from a recombinant EMBL3 clone was subcloned into the same site of pBluescript II KS (+) (Alting-Mees and Short, 1989). A 2.1 kb cDNA insert was recovered from a recombinant lambda GT10 clone by restriction with *Not*I and cloned into the respective site of the above plasmid.

Uf–SUCDH1 was identified as a partial clone (H130) in an EST sequencing project based on a haustorial lambda GT10 cDNA library (M. Hahn, unpublished). A digoxigenin labeled PCR fragment of this clone was used to screen both, genomic and the cDNA phage libraries. Positive clones covering the whole coding region were only obtained from the genomic library. A full length cDNA clone was obtained by nested PCR using uncloned cDNA prepared from 18 h infection structures as template. The second primer pair (SucDH1-5-*Sal*I: 5′ GCT CC**G TCG AC**C ATT CAA CAA CTA CCA TG 3′ and SucDH1-3-*Xho*I: 5′ GGT G**CT CGA G**GG TGT CTT CGC TTC ATT TC 3′) covered sequence start and stop sites (indicated by underlined letters) and also introduced unique restriction sites in the 5′- and 3′-untranslated region which are indicated in the sequence by bold letters. The PCR fragment was digested and ligated into vector pRV11a digested with the same enzymes yielding plasmid pRV11a::SucDH1. pRV11a is based on vector pET32a(+) (Novagene, Madison, WI), but has the short *Xba*I-*Xho*I fragment replaced with the short *Xba*I-*Xho*I fragment of vector pET28a(+) (Novagene, Madison, WI).

All clones were completely sequenced by primer walking, assembled, aligned, edited and deposited at the EMBL database. Accession numbers for *Uf-TBB1* and *Uf-SUCDH1* are AJ311552 and AJ575686, respectively. Procedures for RNA isolation and RT-PCR analysis were as described (Wirsel et al., 2001). Primer sequences for RT-PCR on *Uf-TBB1* were UfTBB1.RTF1 (5′ GCG ACC GAC GGA CAA TAC A 3′) and UfTBB1.RTR1 (5′ AAT TCC ATT TCG TCC ATT CCT TC 3′); those for *Uf-SUCDH1* were H130.F1 (5′ CCC TCA TAT GTA CAT AAT TAA GG 3′) and H130.R4 (5′ GAA GCG AGG TAA AAA GGA TG 3′), respectively.

Phylogenetic methods

Sequence alignments were created with the Clustal W algorithm within the program MegAlign of the DNAstar* V5.03 software package (DNAstar, Madison, WI). Alignments of β-tubulin amino acid sequences included all database accessions available for Basidiomycota, most of those for Chytridio- and Zygomycota, and a selection of those for Ascomycota. Many of the database entries originated from PCR approaches and the respective sequences were therefore not full length. For our analysis, we used 44 partial sequences including 375 characters originating from 34 species. In divisions where a subset of the available sequences had been used, preference was given to include those species where duplicated genes were described. Alignments of succinate dehydrogenase amino acid sequences used partial sequences from all seven fungal database accessions available and included 247 characters. Alignments were manually improved. Phylogenetic trees were constructed using the program ProtML of the MOLPHY package version 2.3 applying the ITT-F data frequencies and the local rearrangement option (Adachi and Hasegawa, 1996). Statistical support for internal nodes was evaluated by 1000

bootstrap replicates using the Neighbor Joining algorithm (Saitou and Nei, 1987) and the Gamma based distance estimates implemented in the program MEGA version 2 beta 3 (http://www.megasoftware.net/). The resulting trees were drawn in the program TreeView version 1.5.3 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) and transferred to Microsoft PowerPoint version 8.0 for further editing.

Results

Inhibition of Uromyces fabae *by benlate and carboxin*

Requirements for using benlate and carboxin as selective agents for generating stable transformed rust strains are the sensitivity of *U. fabae*, whereas the host plant should not be affected. As a first test, we analyzed the impact of these antibiotics on the differentiation of uredospore derived infection structures *in vitro* (Deising et al., 1991). In both cases, a dose-dependent effect on development was observed (Figure 1), i.e., the higher the dosage, the earlier the arrest of morphogenesis. If these drugs would have to be applied by spraying, rust morphogenesis would have to be blocked at stages differentiated on the surface of the host, i.e., before penetration had occurred (the substomatal vesicle is the first structure differentiated within the host). The minimum concentration necessary for preventing cell types that normally develop in the interior of the host was 10 μg ml^{-1} for carboxin, whereas for benlate it was 500 μg ml^{-1}. An earlier arrest at the germ tube stage could be achieved by carboxin concentrations of 250 μg ml^{-1}, whereas for benlate we needed to use concentrations above 500 μg ml^{-1}. Microscopy showed that the few spores still germinating at these concentrations produced short, deformed hyphae (data not shown).

Since future selection of transformants will have to rely on the host plant as the 'medium' for growth, we tested several methods for applying the fungicides to broad bean and studied the inhibition of rust development. Simultaneous spraying of benlate together with uredospores onto leaves at a concentration of 2.5 mg ml^{-1} prevented disease (Table 1). Systemical application exclusively before inoculation did not prevent disease even at the highest concentration tested (Table 1). By con-

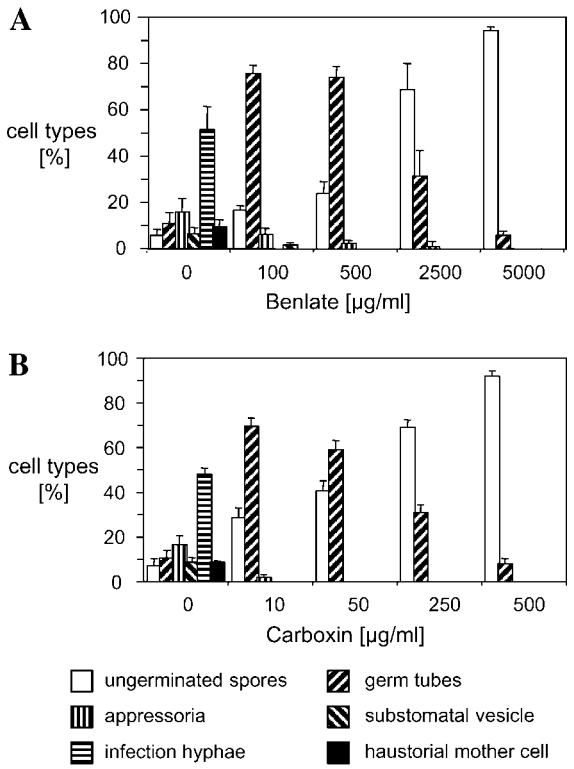

Figure 1. Inhibition of *Uromyces fabae* on polyethylene sheets by benlate (A) and carboxin (B). Uredospores were dry inoculated and then sprayed with the indicated concentrations of the chemicals. Incubation progressed for 22 h. Bars represent the percentages of *in vitro* differentiated cell types.

Table 1. Inhibition of *Uromyces fabae* development on *Vicia faba* by benlate

Benlate (μg ml^{-1})	Spray application together with inoculation[1]	Systemic application before inoculation[2]	Systemic application before & after inoculation[3]
0	+ + +	+ + +	+ + +
5	+ +	+ +	+ +
50	+ +	+ +	+ +
250	+	+ +	+ +
2500	–	+ +	–
5000	–	+ +	–
no spores, no benlate	–	–	–

+ + + wild type levels of uredosori, + + less than 50%, + less than 5% of wild type levels, respectively, – no uredosori.
[1] Benlate was mixed with the spore suspension then sprayed.
[2&3] Benlate was applied by watering the plants.
[2] Systemic application twice before inoculation.
[3] Systemic application twice before inoculation and thereafter every other day.

Table 2. Inhibition of *Uromyces fabae* development on *Vicia faba* by carboxin

Carboxin (μg ml^{-1})	Spray application together with inoculation[1]	Systemic application before & after inoculation[2]
0	+ + +	+ + +
2	+ +	+ + +
10	+	+ + +
50	–	+ + +
250	–	+ + +
no spores, no carboxin	–	–

+ + + wild type levels of uredosori, + + less than 50%, + less than 5% of wild type levels, respectively, – no uredosori.
[1] Carboxin was mixed with the spore suspension then sprayed.
[2] Carboxin was applied by watering the plants, systemic application twice before inoculation and thereafter every other day.

Table 3. Suppression of *U. fabae* development on *V. faba* after delayed fungicide application

Application of fungicides after inoculation [hpi][1]	Spray application of 2.5 mg ml^{-1} benlate[2]	Spray application of 0.25 mg ml^{-1} carboxin[2]	Systemic application of 2.5 mg ml^{-1} benlate[3]
0	–	–	–
3	–	–	(–)
6	(–)	(–)	+
9	+	+	+
12	+ +	+	+ +
16	+ +	+ +	n.d.
24	+ +	+ +	n.d.
48	+ + +	+ + +	n.d.
with spores, no drug	+ + +	+ + +	+ + +
no spores, no drug	–	–	–

+ + + wild type levels of uredosori; + + less than 50%; + less than 5% of wild type levels, respectively; (–) single uredosori, – no uredosori, n.d. not determined.
[1] Fungicides were applied after the indicated intervals with respect to inoculation.
[2] Application used a single spraying of the respective fungicide.
[3] Application started by watering the plants with the fungicides at the indicated time intervals and was repeated thereafter every other day.

trast, repeating benlate treatment every other day after inoculation proved to be effective at a concentration of 2.5 mg ml^{-1} (Table 1). Carboxin simultaneously sprayed with the inoculum prevented the emergence of rust uredosori at 50 μg ml^{-1} (Table 2). Attempts to use this drug by including it in the watering scheme remained unsuccessful at all concentrations tested, even when the treatment was repeated every other day throughout the experiment (Table 2). Control series where the fungicide was applied but the spores omitted did not indicate harmful effects of the fungicides to broad bean (data not shown). Additional experiments carried out with spray and systemical applications of the fungicides, the latter only for benlate, addressed the question for how long the treatment could be delayed without infection to occur. The results indicated that this delay should not last longer than about 6 h for spray inoculating the fungicide and only 3 h when applying benlate systemically (Table 3). In summary, these experiments demonstrated that carboxin is effective at relatively low concentrations but only when applied onto the plant surface whereas benlate would have to be used at considerably higher concentrations but could also be applied systemically.

Isolation and characterization of Uf-TBB1

A PCR fragment of β-tubulin cDNA was labeled with digoxigenin and used to screen lambda GT10 and EMBL3 phage libraries yielding four cDNA and two genomic clones, respectively. PCR fragments, obtained by using recombinant phage DNA as template and the same primers, were sequenced and showed only one type of sequence. BLASTX searches confirmed high similarity to fungal β-tubulins. The cDNA clone with the longest insert (2.1 kb) and one genomic clone (14 kb insert) were chosen for subcloning and establishing the gene sequence. From 5.0 kb of sequence determined from subcloned genomic DNA, 2.0 kb comprised 5′ and 0.3 kb 3′ non-coding sequences, respectively. Comparison of the cDNA sequence with that of the genomic clone revealed the presence of 9 introns ranging in size from 71 to 207 bp (average 95 bp). The translational start site was found by comparisons to β-tubulin sequences from other fungi and resided at position 1990 of the genomic *Uf-TBB1* sequence. The 5′ and 3′ untranslated regions present on the cDNA comprised 313 bp and 641 bp, respectively. BLASTP searches revealed closest similarities to β-tubulins from the Basidiomycota with best hits to

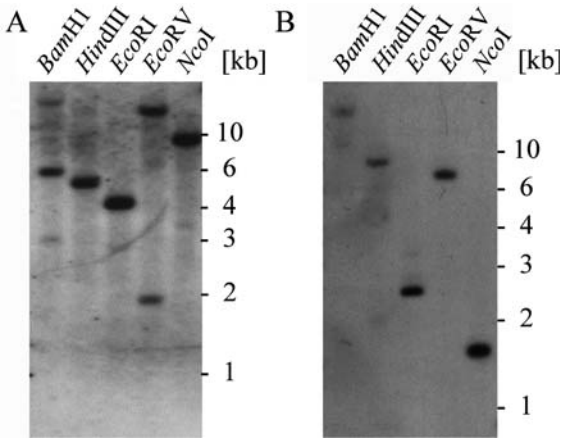

Figure 2. Uf-TBB1 (A) and *Uf-SUCDH1* (B) are single copy genes. Genomic DNA was prepared from germinated uredospores of *Uromyces fabae*, digested with the indicated enzymes and separated on a 0.8% agarose gel. Digoxigenin-labeled probes were amplified from genomic DNA (*Uf-SUCDH1*) and subcloned cDNA (*Uf-TBB1*), respectively. Probes covered the sequence from nucleotide 1229 to nucleotide 1636 for *Uf-SUCDH1* and from nucleotide 2772 to nucleotide 3353 for *Uf-TBB1*. Numbers on the right indicate the size in kb.

Melampsora lini, *Coprinus cinereus* (syn.: *Coprinopsis cinerea*) and *Pleurotus sajor-caju*, respectively. A Southern blot carrying genomic DNA isolated from uredospores and digested with several restriction enzymes was hybridized with the digoxigenin labeled *Uf-TBB1* cDNA. Results obtained indicated that there is no additional copy in the genome having high similarity to this gene (Figure 2A).

Isolation and characterization of Uf-SUCDH1

A PCR fragment of partial succinate dehydrogenase cDNA clone H130 was labeled with digoxigenin and used to screen lambda GT10 and EMBL3 phage libraries yielding one genomic clone. All positive cDNA clones identified were of the same size as H130 or smaller. The genomic clone was sequenced by primer walking using PCR fragments as templates, which were produced using gene specific and vector primers. A full length cDNA clone was obtained by nested PCR using uncloned cDNA prepared from 18 h infection structures as template and introducing unique restriction sites in the nested PCR step. Of the 3.2 kb of sequence determined from subcloned genomic DNA, 0.4 kb comprised 5′ and 1.6 kb 3′ non-coding sequences, respectively. Comparison of the cDNA sequence with that of the genomic clone revealed the presence of 5 introns ranging in size from 64 to 89 bp (average 78 bp). The translational start site was found by comparisons to succinate dehydrogenase sequences from other fungi and resided at position 380 of the genomic *Uf-SUCDH1* sequence. The size of the 5′ untranslated region could not be determined because of the PCR approach. The size of the 3′ untranslated regions present in the partial cDNA clones comprises 285 bp. BLASTP searches revealed closest similarities to succinate dehydrogenases from the Basidiomycota with best hits to *Ustilago maydis*, *Agaricus bisporus* and *Pleurotus sajor-caju*, respectively. A Southern blot carrying genomic DNA isolated from uredospores and digested with several restriction enzymes was hybridized with the digoxigenin labeled *Uf-SUCDH1* cDNA. Results obtained indicated that this gene is present as a single copy in the genome (Figure 2B).

Phylogenetic analysis of Uf-TBB1 *and* Uf-SUCDH1

In a minority of fungi two different β-tubulin genes have been discovered (May et al., 1987; Panaccione and Hanau, 1990; Gold et al., 1991; Goldman et al., 1993; Keeling et al., 2000). We established a molecular phylogeny with 44 β-tubulin amino acid sequences that represented all divisions of the Mycota in addition to that of *Uf-TBB1* (Figure 3A). In most fungi with duplicated genes one resided on a shorter branch that clustered within the 'expected' taxonomic context close to genes from related species that only have a single gene. In contrast, the additional gene often showed accelerated evolutionary rates as indicated by the longer branches, which is associated with positions in the phylogenetic tree that do not correspond with taxonomy. For one species (*Aspergillus*/*Emericella nidulans*) with duplicated tubulin genes a comprehensive functional analysis had been carried out (May et al., 1987; May and Morris, 1988; May, 1989). The gene (*benA*) residing on a short branch of the phylogeny in Figure 3A is needed during vegetative growth, mitosis and nuclear movement whereas the second gene (*tubC*) showing accelerated evolution appears to be only used during asexual sporulation but without being essential for this process (May et al., 1987; May and Morris, 1988; May, 1989). *Uf-TBB1* clustered

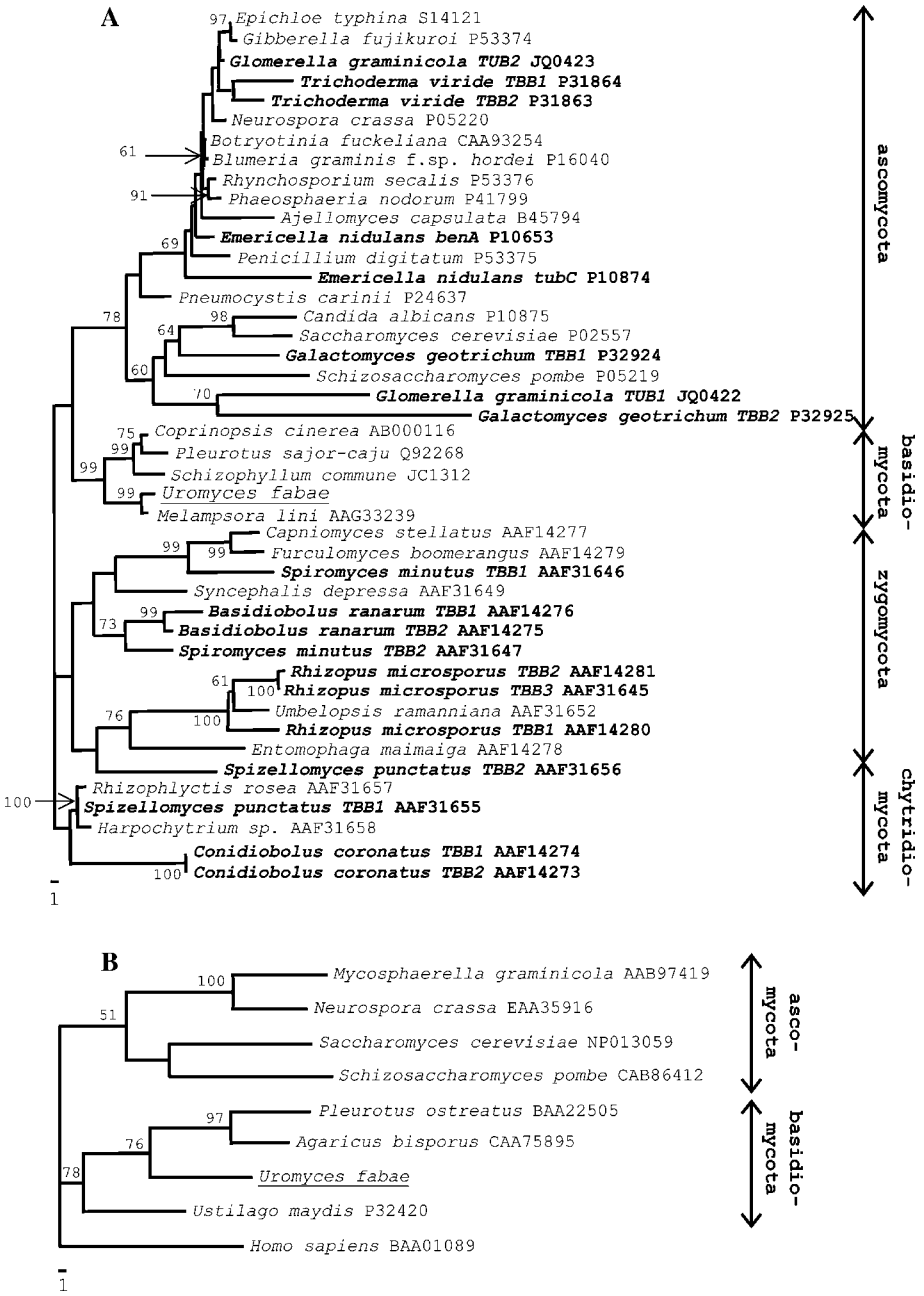

Figure 3. Phylogenetic analysis of Uf-TBB1p and Uf-SUCDH1p. (A) Phylogram displaying the relationships of β-tubulins from the four divisions of fungi. Proteins from organisms shown to have more than one β-tubulin gene are in bold type. (B) Phylogram displaying the relationships of the iron-sulfur protein subunit of succinate dehydrogenase from two divisions of fungi. Both trees were generated by ProtML and evaluated by 1000 bootstrap replicates with MEGA. Only bootstrap values above 50% are shown. Accessions numbers of reference sequences are provided behind their names. The names of organisms are used as annotated in the NCBI database and do not necessarily reflect current conventions.

with all the other basidiomycete sequences and resided on a short branch closely linked to an annotation for *Melampsora lini*, another member of the Urediniomycetes. The branch including these two was separated with high bootstrap support from the branch leading to the

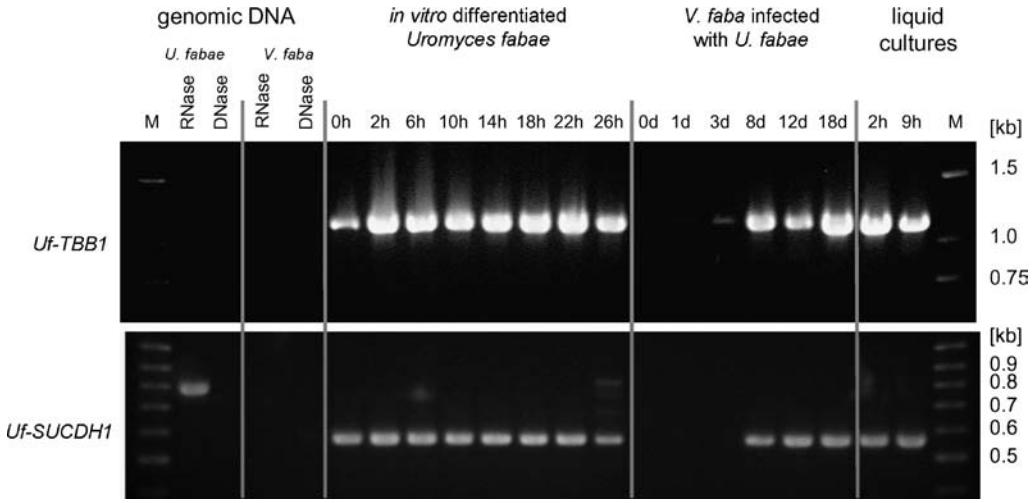

Figure 4. RT-PCR analysis for *Uf-TBB1* and *Uf-SUCDH1* transcripts *in vitro* and *in planta*. Reactions were fractionated on a 1% agarose gel. M: Molecular weight marker; genomic DNA: controls from *U. fabae* and *V. faba*, samples received treatment with RNase or DNase as indicated; *in vitro* differentiated *Uromyces fabae*: infection structures grown for the indicated period after inoculation (0h = ungerminated spores); *V. faba* infected with *U. fabae*: leaf samples taken on various days after infection (0d = non-infected leaves); liquid cultures: undifferentiated germ tubes 2 h, and 9 h old. All RNA samples received a DNase treatment to destroy any DNA contamination. For *Uf-TBB1*, primers spanned introns 5 and 8, respectively, thus preventing amplification of genomic DNA.

Hymenomycetes. The shortness and the pattern of the branches within this part of the tree indicated that *Uf-TBB1* represents a typical fungal β-tubulin and not a duplicated form that might had acquired specialized functions during evolution.

Succinate dehydrogenase (SDH) of eukaryotes is a membrane-associated complex in mitochondria of four distinct subunits. SDH takes part in the citric acid cycle where it oxidizes succinate to fumarate and in the respiratory electron transport chain where it transfers electrons to ubiquinone. *Uf-SUCDH1* is a homologue of yeast *SDH2* which encodes a catalytic iron-sulfur protein subunit. We have created a molecular phylogeny including the seven currently known fungal homologs of *SDH2* which all originate from the Ascomycota and Basidiomycota. The obtained tree reflected the major divisions within these taxa and *Uf-SUCDH1* clustered within the expected context (Fig. 3B). To our knowledge, currently available data indicate that fungi have just single copies of this gene.

Transcriptional regulation of Uf-TBB1 *and* Uf-SUCDH1

Selection markers used in transformation should be constitutively expressed so that their transcription is initiated as early as possible after the transformation event without the need for activation through endogenous or environmental signals. Therefore, we analyzed expression of these genes by RT-PCR on total RNA preparations by using a one-step protocol, where one of the primers used for the PCR step additionally primed the cDNA synthesis step. DNase pretreatment of the RNA prevented amplification from putative DNA contamination (Figure 4). *In planta*, *Uf-TBB1* transcripts were detectable from 3 dpi on, those of *Uf-SUCDH1* from 8 dpi on. However, it has to be emphasized, that at these early stages of infection RNA preparations mainly comprised RNA of plant origin. Therefore, we also monitored gene expression in an *in vitro* system simulating the very early phase of fungal development (0–26 h after inoculation) in the absence of the host. It utilized polyethylene sheets mimicking the plant surface and allowed differentiation from the uredospore to the haustorial mother cell stages (Deising et al., 1991; Wirsel et al., 2001). Here, *Uf-TBB1* and *Uf-SUCDH1* transcripts were discovered in ungerminated uredospores and at all developmental stages from 2 h through 26 h (Figure 4). As an additional control we included rust spores that germinated in water where development stopped at the germ tube stage. Again, we obtained clear bands,

confirming that *Uf-TBB1* and *Uf-SUCDH1* are constitutively expressed in all cell types and throughout the entire mitotic uredospore cycle.

Discussion

Technology for generating stable transformed rust fungi requires the development of several corner stones. Besides the transfection of vector molecules into rust cells which had been achieved earlier in transient assays (Bhairi and Staples, 1992; Li et al., 1993; Schillberg et al., 2000) one is faced with the challenge to find appropriate marker genes, to develop procedures allowing selection on the living host plant and to propagate transformants through the mitotic uredospore cycle. The work described here deals with two of these problems. First we assessed the range of applicability of two fungicides, benlate and carboxin, to select for putative transformants and second we cloned the genes encoding their respective molecular targets.

Both fungicides prevent the growth of *Uromyces fabae* on *Vicia fabae* without affecting the host, which is a prerequisite for their application as selecting agents in transformation schemes. Observation of *in vitro* differentiated rust infection structures revealed that development ceased earlier, with increasing doses of the drugs. For *in vitro* assays, concentrations of 10 μg ml^{-1} carboxin and 500 μg ml^{-1} benlate are effective to interrupt fungal development at the appressorial stage which is the last to appear on the surface of the host, whereas for both substances about five times higher concentrations are needed to completely prevent infection. Benomyl belongs to the class of benzimidazole fungicides. Upon dissolving in water it releases carbendazim (MBC) that binds to β-tubulin and thus prevents the assembly of microtubuli. Effective concentrations to inhibit growth of wild type isolates are 1–5 μg ml^{-1} of benomyl for *Blumeria graminis* assayed *in planta* (Chaure et al., 2000) and *Ustilago maydis* assayed *in vitro* (Gold et al., 1994) and 0.5 μg ml^{-1} MBC for *Botrytis cinerea* assayed *in vitro* (Yarden and Katan, 1993), given as examples for other plant pathogenic fungi. Thus it appears that *U. fabae* has a higher basal resistance towards this type of fungicide. In contrast, the concentration of carboxin needed to inhibit *U. fabae* was in the same range reported for other plant pathogens, e.g., 50 μg ml^{-1} for *Mycosphaerella graminicola* assayed *in vitro* (Skinner et al., 1998). Beyond the determination of the effective concentrations necessary to suppress growth of *U. fabae* we determined that the effective period for fungicide application is at most 6 h after inoculation, which roughly coincides with appressorium formation during rust development. This might be a critical detail in a transformation procedure, since transformed rust cells would have to phenotypically express resistance against the fungicide in order to survive the selection pressure thereafter.

The second advance towards a procedure for generating stable rust transformants is made by cloning and characterization of the genes encoding the molecular targets of benomyl and carboxin, i.e., β-tubulin and the iron-sulfur protein subunit of succinate dehydrogenase, respectively. Neither PCR experiments using degenerate primers, nor the hybridization of genomic DNA, nor an ongoing EST project (data not shown) indicated the presence of additional close homologs for both, *Uf-TBB1* and *Uf-SUCDH1*. In the case of *Uf-TBB1*, we cannot completely rule out the existence of a second gene given the precedence from a few other fungi. For example, it might be possible that a distantly related homolog could be expressed under very specific conditions, e.g., during the sexual cycle. This would not affect our claim that *Uf-TBB1* is the gene encoding the β-tubulin subunit, which is essential at least for the vegetative uredospore cycle. This claim is supported by the molecular phylogeny and by the RT-experiments. The same is true for *Uf-SUCDH1* which also resided at its expected taxonomical position in a molecular phylogeny and which was also found to be constitutively expressed. The latter will facilitate the development of transformation vectors where their native promoters will drive the expression of the respective selection markers. Constitutive expression of the marker would allow for an early establishment of resistance which might be critical as discussed above.

Previous studies have identified the β-tubulin subunit of microtubules as the binding site for benomyl. Extensive research has revealed that single amino acid substitutions at several conserved positions bestow benomyl resistant phenotypes in many fungal species (Jung et al., 1992; Yarden and Katan, 1993; Reijo et al., 1994). Alignments including the *Uf-TBB1* sequence

established during this study show that amino acids at and surrounding these positions are well conserved when compared with other fungal β-tubulins where the corresponding mutations had been shown to lead to benomyl resistance (data not shown).

In the case of carboxin, resistance results from mutations within the iron-sulfur-protein subunit of succinate dehydrogenase. In all cases where it had been determined, a single amino acid substitution within the third Cys-rich cluster that takes part in binding of iron-sulfur centers, was found responsible (Broomfield and Hargreaves, 1992; Skinner et al., 1998; Honda et al., 2000). The alignment used to generate Figure 3B shows that all sequences together with *Uf-SUCDH1* and even the outgroup *Homo sapiens* exhibit perfect conservation of a stretch of eight amino acids around this residue (data not shown). We therefore argue that the *in vitro* generation of the corresponding mutations in *Uf-TBB1* and *Uf-SUCDH1* will produce alleles conferring resistance to select for rust transformants on benlate or carboxin treated host plants. The next challenge is to combine technology developed here with that of introducing DNA to generate transformed cells that are viable enough to establish disease upon selection in the host, which should lead then to the production of recombinant spores.

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