

ORIGINAL ARTICLE

Evolution of host resistance in a toxin-producing bacterial–fungal alliance

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The rice seedling blight fungus *Rhizopus microsporus* harbors endosymbiotic *Burkholderia* sp. for the production of the virulence factor, the antimetabolic agent rhizoxin. Since the toxin highly efficiently blocks mitosis in most eukaryotes, it remained elusive how self-resistance emerged in the fungal host. In this study, rhizoxin sensitivity was systematically correlated with the nature of β -tubulin sequences in the kingdom Fungi. A total of 49 new β -tubulin sequences were generated for representative species of Ascomycota, Basidiomycota and Zygomycota. Rhizoxin sensitivity assays revealed two further amino acids at position 100 (Ser-100 and Ala-100), in addition to the known Ile-100 and Val-100, which convey rhizoxin resistance. All sensitive strains feature Asn-100. This hot spot was verified by modeling studies, which support the finding that rhizoxin preferentially interacts with the tubulin molecule in a cavity near position 100. Ancestral character state reconstructions conducted in a Bayesian framework suggest that rhizoxin sensitivity represents the ancestral character state in fungi, and that evolution of rhizoxin resistance took place in the ancestor of extant resistant Zygomycota. These findings support a model according to which endosymbiosis became possible through a parasitism–mutualism shift in insensitive fungi.

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Introduction

One of the key questions regarding symbiotic life forms is how stable associations have developed and persisted during evolution. From a very general point of view symbioses result from a series of subsequent stages, such as contact of the organisms, internalization and initiation of nutrient transfer (Dale and Moran, 2006). In many cases, mutualistic symbioses are considered to evolve from parasitic relationships (Moran and Baumann, 2000; Moran, 2006). However, a particular complex situation emerges when a pathogenic alliance against a third party is formed. If one of the partners produces a toxin or virulence factor, it is a prerequisite that not only the producing organism, but also the symbiotic partner is resistant or insensitive to the chemicals released. Thus, another important issue is raised:

did self-resistance of the partners develop prior to endosymbiosis?

We recently discovered a persistent phytopathogenic alliance of the bacterium *Burkholderia* spp. and the fungus *Rhizopus microsporus* (Partida-Martinez and Hertweck, 2005; Partida-Martinez *et al.*, 2007a). It has been known for decades that the fungus employs a highly potent antimetabolic agent, the polyketide macrolide rhizoxin (1, Figure 1), to attack rice plants and cause rice seedling blight (Sato *et al.*, 1983; Iwasaki *et al.*, 1984). However, in the course of our biosynthetic studies it became apparent that this virulence factor is in fact not produced by the fungus, but by bacteria that live within the fungal cytosol (Partida-Martinez and Hertweck, 2005). By curing the fungus from endosymbiotic bacteria and re-infection with the symbionts rhizoxin biosynthesis was clearly correlated to endofungal *Burkholderia* sp. (Partida-Martinez *et al.*, 2007c). The ultimate proof was provided by the cultivation of the endofungal bacteria in the absence of the fungal host and by the analysis and characterization of a complex of highly active rhizoxin derivatives (Scherlach *et al.*, 2006) and identification of the biosynthesis gene cluster (Partida-Martinez and Hertweck, 2007).

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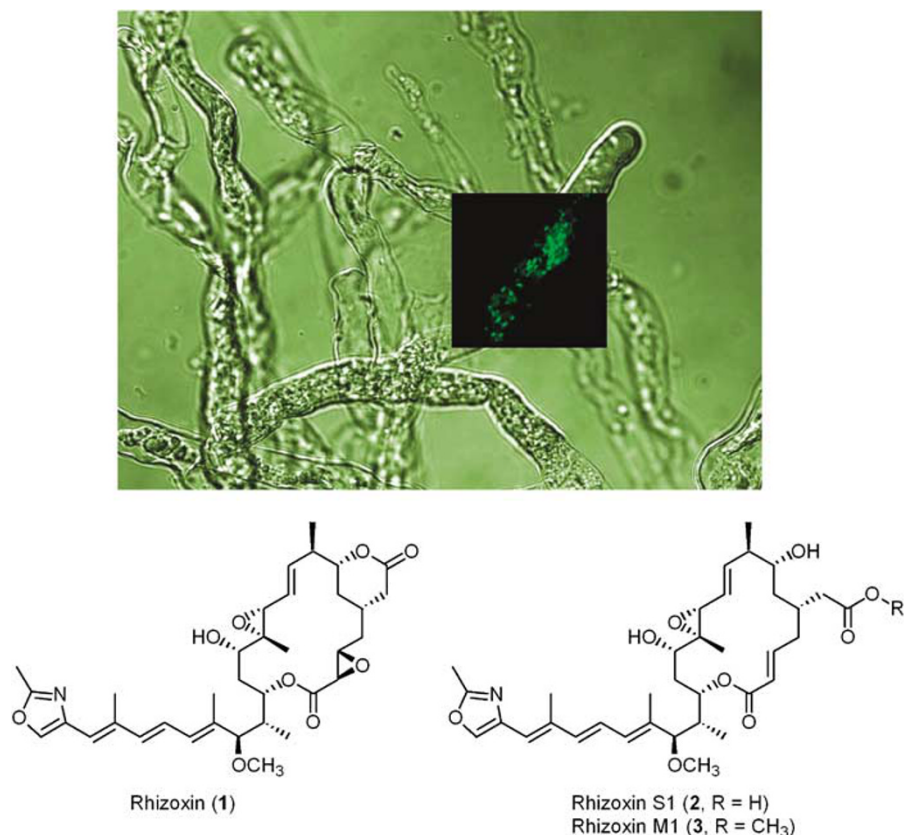


Figure 1 Confocal laser scanning (CLS) micrograph of mycelium of *Rhizopus microsporus* and SYTO9-labeled green fluorescent endobacteria (inset). Structures of the antimitotic rhizoxin (1), the causative agent of rice seedling blight and derivatives (2, 3).

The toxin exhibits its potent antimitotic activity against most eukaryotes including vertebrates, vascular plants and fungi by binding to β -tubulin (Tsuruo *et al.*, 1986; Takahashi *et al.*, 1987; Sullivan *et al.*, 1990). Some groups of fungi, however, appear to be rhizoxin resistant. These include the baker's yeast *Saccharomyces cerevisiae* (Iwasaki *et al.*, 1984) and the fission yeast *Schizosaccharomyces pombe* (Takahashi *et al.*, 1987). Furthermore, obviously *R. microsporus* needs to be insensitive toward the compounds produced by its endosymbionts (Partida-Martinez and Hertweck, 2005).

The amino-acid sequences of β -tubulin surrounding the 100th amino-acid residue from the *N* terminus are highly conserved, typically showing asparagine (Asn) at position 100 in a limited number of rhizoxin-sensitive organisms. Experimental evidence involving site-directed mutagenesis altering Asn-100 to Ile-100 confirmed that this residue confers resistance to rhizoxin-sensitive fungi, for example, *Aspergillus nidulans* (Takahashi *et al.*, 1989). Conversely, altering Ile-100 or Val-100 to Asn-100 in the naturally resistant *S. pombe* and *S. cerevisiae* confers rhizoxin sensitivity to these organisms (Takahashi *et al.*, 1990). The character state at residue 100 in β -tubulin genes thus appears to relate to rhizoxin resistance or sensitivity in the Ascomycota. Gaining deeper insight into this sequence–function relationship would provide an

ideal opportunity to trace the evolution of rhizoxin resistance across a phylogeny of β -tubulin genes. Reconstruction of ancestral sequences at selected nodes in the phylogeny allows direct inference of the character states rhizoxin resistance or sensitivity in the ancestral genes.

The current study focuses on rhizoxin sensitivity in multiple fungal species from different phyla, especially the Zygomycota, to corroborate the predictive potential of β -tubulin residue 100. Furthermore, we explored patterns of β -tubulin evolution across fungi and assessed the phylogenetic origins of rhizoxin resistance in the kingdom Fungi. These findings have significant implications for the evolution of the rare *Rhizopus*–*Burkholderia* symbiosis.

Materials and methods

Fungal strains, media and growth conditions

We determined 49 new β -tubulin sequences of 36 fungal strains. All strains are deposited and maintained in the Fungal Reference Centre Jena (FSU, Jena, Germany) or at the Hans Knöll Institute (HKI, Jena, Germany) and are available upon request. With the exception of chytridiomycetes, all fungi were cultivated on MEX medium containing 30 g l⁻¹ malt extract (Serva, Heidelberg, Germany). Chytrids were grown on ARCH medium as previously reported

(Einax and Voigt, 2003). For solidification, the media were supplemented with 20 g l⁻¹ agar (Roth, Karlsruhe, Germany). Petri dishes were incubated at 25 °C until sporulation or development of vegetative thalli for about 3–6 days. The fungal biomass served as inoculum for the mycelial growth for DNA extraction procedures. Liquid cultures with malt extract broth were inoculated in 500 ml round flasks and incubated for 2–6 days at 25 °C under constant shaking (120 r.p.m.).

Purification of genomic DNA and PCR amplification

Genomic DNA was purified and amplified according to Einax and Voigt (2003) using primer pair F-tub1 (5'-CARGCYGGTCARTGYGGTAACCA-3') and F-tub4r (5'-GCCTCAGTRAAYTCCATYTCRTCCAT-3'). PCR products were electrophoretically separated on 1.2% agarose gels (SeaKem LE; BMA, Rockland, ME, USA) and visualized on a TL-312A transilluminator (Spectrolin) after staining in 0.5 mg ml⁻¹ ethidium bromide.

Cloning and sequencing of PCR products

Amplified PCR products were purified by adsorption of DNA to glass particles (GeneClean II, BIO 101, Vista, CA, USA) based on an established procedure (Vogelstein and Gillespie, 1979). The purified PCR products were ligated into the pCR4-TOPO vector (Invitrogen, Karlsruhe, Germany) and, after heat shock, cloned in chemically competent Top10 one shot cells of *Escherichia coli* (Invitrogen). Plasmids were purified after a modified protocol of (Birnboim and Doly, 1979) using additional purification steps (additional removal of proteins with potassium acetate to a final concentration of 1.0 M and chloroform extraction in a second step). Cycle sequencing with the universal primers T3 (5'-ATTAACCCTCACTAAAGGGA-3') and T7 (5'-TA ATACGACTCACTATAGGG-3') with the BigDye fluorescent-labeled terminator dye deoxy protocol applying AmpliTaq polymerase (PE Applied Biosystems, Warrington, Cheshire, UK) was conducted in accordance with Einax and Voigt (2003). Each sequencing reaction mixture was precipitated with isopropanol and resuspended in formamide (20 µl) before running on an ABI Prism 310 (PE Applied Biosystems, Foster City, CA, USA) automated sequencer. Sequence fragments were assembled with TSE, a DOS text software program (SemWare; Marietta, GA). Tubulin sequences were submitted to GenBank at <http://ncbi.nlm.nih.gov> under accession numbers AY944765–767, 769, 772–773, 776–777, 783–784, 786–793, 795, 797, 800–803, 805–811, 813–814, 816–819, 824, 827, 829, 836–837, 852–856, 863).

Phylogenetic analysis and ancestral state reconstruction

We aligned β-tubulin sequences from representatives of all phyla in the kingdom fungi (Ascomycota,

Basidiomycota, Chytridiomycota, Glomeromycota, Zygomycota) using CLUSTAL W (Thompson *et al.*, 1994). As basal lineages, we included sequences of four members of Choanoflagellida and Stramenopila using *Phytophthora palmivora* as outgroup. For information on taxonomic placement of taxa see Table 1. Positions showing single amino-acid insertions were excluded from the alignment (final alignment, 1161 bp). Bayesian phylogenetic analysis was carried out using MRBAYES 3.1.1 (Huelsenbeck and Ronquist, 2001). The program was set to produce five Mio generations using the GTR + I + G model of nucleotide evolution, as estimated using MODELTEST 3.06 (Posada and Crandall, 1998), sample every 100th tree, run eight parallel chains and discard 5000 trees as burn-in. First, second and third codon positions were treated as separate partitions and estimated independently. Two analyses were run simultaneously. Ancestral nucleotide sequences were inferred for seven selected nodes in a Bayesian framework. Constraints were set to keep all of the descendants of a node together in a clade. The ancestral nucleotide sequence for these clades was inferred in separate analyses using the 'report ancstates' option in MRBAYES 3.1.1. For these analyses, we used the same settings as above, with the exception of producing one Mio generations, sampling every 1000th tree, running four chains and discarding 50 initial trees. The probabilities for each codon position at amino-acid residue 100 are averages of 1900 sampled generations (Table 2).

Rhizoxin sensitivity assay

To test whether the selected fungal strains were Rhizoxin sensitive or resistant, the rhizoxin complex from cultured endosymbiont *Burkholderia* sp. B4 was prepared as reported (Partida-Martinez and Hertweck, 2005; Partida-Martinez *et al.*, 2007b). The extract was dissolved in methanol to give a concentration of 2.5 mg l⁻¹ of rhizoxins. For every test, a small mycelial pellet was collected from a potato-dextrose agar petri dish and dissolved in 0.5–1 ml sterile saline solution (0.85% NaCl) supplemented with 0.3% Tween20 (Roth). Malt extract peptone agar was melted and left to cool to about 48 °C and then mixed with the diluted mycelium. The mixture was then plated in a 14-cm diameter petri dish. After drying, two 10-mm holes were cut out. Rhizoxin (50 µl) solution raw extract or methanol (used as negative control) was then applied. The petri dishes were incubated at 30 °C or room temperature for a couple of days until fungal growth could be observed. The degree of inhibition of hyphal growth around the toxin source was monitored.

Ligand docking into rice tubulin

Rice tubulin sequences (*Oryza sativa* subsp. japonica, P28752|TBA1_ORYSJ and Q43594|TBB1_ORYSJ)

Table 1 Materials used in the current study

<i>Species</i>	<i>Classification</i>	<i>β-tubulin accession nos.</i>	<i>Strain nos.</i>	<i>Rhizoxin test</i>	<i>Amino acid at residue 100</i>
<i>Aspergillus fumigatus</i>	Ascomycota, Pezizomycotina	AY048754		NT	N
<i>Aspergillus nidulans</i>	Ascomycota, Pezizomycotina	XM_653694, XM_659350		Sensitive (Takahashi <i>et al.</i> , 1989)	N
<i>Botryotinia fuckeliana</i>	Ascomycota, Pezizomycotina	U27198		NT	N
<i>Cochliobolus heterostrophus</i>	Ascomycota, Pezizomycotina	AY749036		NT	N
<i>Neurospora crassa</i>	Ascomycota, Pezizomycotina	AABX01000718		NT	N
<i>Ashbya gossypii</i>	Ascomycota, Saccharomycotina	AY944852	FSU 2677	Resistant	V
<i>Candida albicans</i>	Ascomycota, Saccharomycotina	M19398	HKI 2500	Resistant	V
<i>Kluyveromyces lactis</i>	Ascomycota, Saccharomycotina	CR382124		NT	V
<i>Saccharomyces cerevisiae</i>	Ascomycota, Saccharomycotina	NP_116616		Resistant (Takahashi <i>et al.</i> , 1990)	V
<i>Yarrowia lipolytica</i>	Ascomycota, Saccharomycotina	AY944854	FSU 2683	Sensitive	N
<i>Protomyces inouyei</i>	Ascomycota, Taphrinomycotina	AF170967		NT	N
<i>Schizosaccharomyces pombe</i>	Ascomycota, Taphrinomycotina	AL031743	FSU 2492	Resistant	I
<i>Taphrina deformans</i>	Ascomycota, Taphrinomycotina	AY944853	FSU 2554	Sensitive	N
<i>Coprinus cinereus</i>	Basidiomycota	AB000116		NT	N
<i>Cryptococcus neoformans</i>	Basidiomycota	XM_568244		NT	N
<i>Hericium erinaceus</i>	Basidiomycota	AY944855	FSU 2660	NT	N
<i>Piriformospora indica</i>	Basidiomycota	AY944856	FSU 2647	Sensitive	N
<i>Trametes versicolor</i>	Basidiomycota	AY131274	FSU 2571	Sensitive	N
<i>Ustilago maydis</i>	Basidiomycota	XM_756882		NT	N
<i>Acaulospora laevis</i>	Glomeromycota	AJ717327, AF158390		NT	N
<i>Glomus intraradices</i>	Glomeromycota	AY326320, AY326321		NT	N
<i>Glomus mosseae</i>	Glomeromycota	AF158387, AF159109		NT	N
<i>Glomus proliferum</i>	Glomeromycota	AJ717322, AJ717321		NT	N
<i>Chytridium confervae</i>	Chytridiomycota	AY131272		NT	N
<i>Nowakowskiella elegans</i>	Chytridiomycota	AY138800, AY138799		NT	N
<i>Powellomyces variabilis</i>	Chytridiomycota	AY138796, AY138797		NT	N
<i>Rhizophlyctis rosea</i>	Chytridiomycota	AF162078		NT	N
<i>Spizellomyces punctatus</i>	Chytridiomycota	AF162076, AF162077		NT	N
<i>Basidiobolus ranarum</i>	Zygomycota, Entomophthorales	AF162060, AF162059	FSU 770	Sensitive	N
<i>Conidiobolus coronatus</i>	Zygomycota, Entomophthorales	AF162058		NT	N
<i>Smittium commune</i>	Zygomycota, Harpellales	AY944827	FSU 2649	Sensitive	N
<i>Smittium culisetae</i>	Zygomycota, Harpellales	AF162069		NT	N
<i>Smittium simuli</i>	Zygomycota, Harpellales	AY944829	FSU 2652	NT	N
<i>Dissophora decumbens</i>	Zygomycota, Mortierellales	AY944863 , AY944836	FSU 801	Sensitive	N
<i>Lobosporangium transversale</i>	Zygomycota, Mortierellales	AY944837	FSU 806	Resistant	S
<i>Absidia corymbifera</i>	Zygomycota, Mucorales	AY944772 , AY944773	FSU 787	Resistant	S
<i>Absidia glauca</i>	Zygomycota, Mucorales	AY944776	FSU 659	Resistant	A

Table 1 Continued

<i>Species</i>	<i>Classification</i>	<i>β-tubulin accession nos.</i>	<i>Strain nos.</i>	<i>Rhizoxin test</i>	<i>Amino acid at residue 100</i>
<i>Actinomucor elegans</i>	Zygomycota, Mucorales	AY944783 , AY944784	FSU 276	NT	S
<i>Blakeslea trispora</i>	Zygomycota, Mucorales	AY944767 , AY937397	FSU 391	NT	S
<i>Chaetocladium brefeldii</i>	Zygomycota, Mucorales	AY944765 , AY944766	FSU 284	NT	S
<i>Chlamydoabsidia padenii</i>	Zygomycota, Mucorales	AY944785	FSU 791	Resistant	A
<i>Ellisomyces anomalus</i>	Zygomycota, Mucorales	AY944813 , AY944814 , AY944816	FSU 2525	NT	S
<i>Fennellomyces linderi</i>	Zygomycota, Mucorales	AY944817 , AY944818	FSU 2527	NT	S
<i>Gongronella butleri</i>	Zygomycota, Mucorales	AY944786 , AY944787	FSU 266	Resistant	A,S
<i>Halteromyces radiatus</i>	Zygomycota, Mucorales	AY944788	FSU 299	NT	A
<i>Mucor hiemalis f. hiemalis</i>	Zygomycota, Mucorales	AY944789 , AY937401	FSU 697	NT	S
<i>Mucor mucedo</i>	Zygomycota, Mucorales	AY944791	FSU 621	NT	S
<i>Mucor racemosus</i>	Zygomycota, Mucorales	AY944790	FSU 624	NT	S
<i>Mycotypha africana</i>	Zygomycota, Mucorales	AY944805	FSU 296	NT	S
<i>Parasitella parasitica</i>	Zygomycota, Mucorales	AY944792 , AY944793	FSU 327	NT	S
<i>Phycomyces blakesleanus</i>	Zygomycota, Mucorales	AY944795	FSU 674	NT	S
<i>Pilaira anomala</i>	Zygomycota, Mucorales	AY944806	FSU 268	NT	S
<i>Poitrasia circinans</i>	Zygomycota, Mucorales	AY944769 , AY937399	FSU 889	NT	S
<i>Radiomyces spectabilis</i>	Zygomycota, Mucorales	AY944809 , AY944808	FSU 839	NT	S
<i>Rhizopus microsporus</i> var. <i>oligosporus</i>	Zygomycota, Mucorales	AF162064, AF162065, AF162066		NT	S
<i>Rhizopus microsporus</i> var. <i>microsporus</i>	Zygomycota, Mucorales	new: FSU 5255	FSU 5255	Resistant	S
<i>Rhizopus oryzae</i>	Zygomycota, Mucorales	AY944797	FSU 757	NT	S
<i>Spinellus fusiger</i>	Zygomycota, Mucorales	AY944800 , AY944801	FSU 857	NT	S
<i>Syncephalastrum racemosum</i>	Zygomycota, Mucorales	AY944811 , AY944810	FSU 290	NT	S
<i>Syzygites megalocarpus</i>	Zygomycota, Mucorales	AY944802 , AY944803	FSU 728	NT	S
<i>Thamnostylum piriforme</i>	Zygomycota, Mucorales	AY944819	FSU 278	NT	S
<i>Umbelopsis isabellina</i>	Zygomycota, Mucorales	AY944824	FSU 265	Resistant	A
<i>Umbelopsis ramanniana</i>	Zygomycota, Mucorales	AF162073		NT	S
<i>Utharomyces epallocaulus</i>	Zygomycota, Mucorales	AY944807	FSU 854	NT	S
<i>Phytophthora palmivora</i>	Stramenopiles	AY729822		NT	N
<i>Thalassiosira pseudonana</i>	Stramenopiles	AAFD01001166		NT	N
<i>Thalassiosira weissflogii</i>	Stramenopiles	AF374483		NT	N
<i>Monosiga brevicollis</i>	Choanoflagellida	AY026071		NT	N

Abbreviations: A, alanine; I, isoleucine; N, asparagine; NT, not tested; S, serine; V, valine.

All strains used for β-tubulin sequencing or rhizoxin sensitivity tests are deposited in the Fungal Reference Centre Jena (FSU, Jena, Germany). GenBank accession numbers of new sequences are indicated in bold print. Results of rhizoxin sensitivity tests for 17 species are given. The last column shows the amino-acid state of residue 100 in β-tubulin.

Table 2 Ancestral character state reconstructions of β -tubulin amino-acid residue 100 for seven selected clades in Figure 1 inferred in a Bayesian framework

Clade	codon position 1	Codon position 2	codon position 3	Ancestral amino acid
1	T (0.99)	C (1.00)	T (0.95)	S (Ser)
2	A (0.99)	A (1.00)	T (0.80)	N (Asn)
3	A (1.00)	A (1.00)	C (0.52), T (0.48)	N (Asn)
4	G (0.98)	T (1.00)	G (0.46), T (0.45)	V (Val)
5	A (1.00)	A (0.99)	C (0.97)	N (Asn)
6	A (1.00)	A (0.97)	C (0.96)	N (Asn)
7	A (1.00)	A (1.00)	C (1.00)	N (Asn)

Probabilities for ancestral nucleotide states at each codon position are given in parentheses.

were sent to the CPHmodels 2.0 server hosted at the Technical University of Denmark DTU. The crystal structure of 1IA0 was found to be the most suitable template with 83.3% (tubulin A) and 86.7% (tubulin B) sequence identity. Ligands were drawn in CS ChemDraw Ultra 6.0 and imported to CS Chem3D Pro 5.0. After minimizing the energy, the resulting structure was saved as a SYBIL2 file. This ligand file was preprocessed with corina (Molecular Networks GmbH, Erlangen, Germany) to obtain a mol2-file suitable for usage in flexX (BioSolveIT GmbH, St Augustin, Germany). Both, the modeled tubulin B of rice sequence and the ligand were imported into FlexX 3.0 pre. The possible receptor site was defined as 6.5 Å around residue Asn-100 of tubulin B. After docking, the best hit was chosen for visualization in PyMOL (DeLano Scientific, Palo Alto, CA, USA).

Results and discussion

Rhizoxin resistance in fungi is predictable

We first aimed at a systematic correlation of rhizoxin sensitivity with the nature of β -tubulin sequences. Thus, we retrieved sequences from databases and generated new ones from members of our strain collection to cover a broad range of fungal specimens. In total, we generated 49 new β -tubulin sequences for 36 species of Ascomycota, Basidiomycota and Zygomycota. Each fungal species contained 1–3 paralogs of the β -tubulin gene family. Selected fungal strains were then tested for rhizoxin sensitivity or resistance. A 2.5 mg l⁻¹ solution of the rhizoxin complex from endosymbiont *Burkholderia* sp. B4 was applied to a representative number of species from all major phyla in the kingdom fungi using a standardized agar diffusion assay. Rhizoxin sensitivity tests (Figure 2) and alignment of the β -tubulin sequences (Table 1) revealed that rhizoxin sensitivity in all cases correlates with the character state Asn-100 in the conserved AGN-x-WA motif of β -tubulin. Yeasts of the subphylum Saccharomycotina display Val-100 and are rhizoxin resistant, while yeasts of the subphylum Taphrinomycotina

exhibit Ile-100 or Asn-100 and are either resistant or sensitive accordingly. Members of the zygomycete order Mucorales, including the *R. microsporus* group, are rhizoxin resistant and their β -tubulin paralogs display Ser-100 or Ala-100. Other groups in the phylum Zygomycota, such as Entomophthorales and Harpellales show Asn-100 and are rhizoxin sensitive. It is apparent that even unrelated groups of organisms such as Viridiplantae (for example, *Oryza*) and Metazoa (for example, animals including humans) that feature Asn-100 are rhizoxin sensitive and thus the same applies to Basidiomycota, Chytridiomycota and Glomeromycota. From these results, we conclude that rhizoxin resistance is predictable according to the character state of β -tubulin residue 100: organisms displaying Asn-100 are rhizoxin sensitive, while fungi showing Ala-100, Ile-100, Ser-100 or Val-100 are rhizoxin resistant.

Mapping β -tubulin residue 100 critical for rhizoxin docking

The results from bioassays and sequence comparisons clearly established residue 100 as a hot spot for resistance or sensitivity. Yet, the rationale for this observation is lacking because the molecular basis of the antimetabolic action of rhizoxin and its binding to β -tubulin binding is not well understood. To rationalize the experimental finding, we mapped the critical amino-acid site in the α - and β -tubulin heterodimer and modeled the interaction of the rhizoxin molecule with the β -tubulin subunit. The structure of rice tubulin was modeled using the amino-acid sequences (*O. sativa* subsp. japonica, P28752 and Q43594) and crystal structure 1IA0 as a template (83.3% (tubulin A) and 86.7% (tubulin B) sequence identities). Notably, the model position 100 is situated at the interface of the β - and α -tubulin subunits (Figure 3). Consequently, ligands binding to this moiety would significantly alter the interaction between both proteins. Related eukaryotic tubulin sequences can be superimposed onto this model. To demonstrate a potential interaction between the most potent antimetabolic rhizoxin derivatives in the complex (2, 3, Figure 1), the ligand docking to the modeled β -tubulin was emulated by FlexX 3.0 pre. Figure 3 shows a potential interaction between a receptor site defined as 6.5 Å around residue Asn-100. Although extensive X-ray and NMR studies will be required to scrutinize the exact chemical binding of rhizoxin to β -tubulin, our modeling studies already provide strong evidence that ligand binding to Asn-100 and its vicinity would have an impact on the formation of the tubulin heterodimer and thus affect mitosis.

Phylogenetic origins of rhizoxin resistance in the kingdom fungi

Next, we aimed at revealing how rhizoxin resistance emerged in the kingdom Fungi. Therefore, we

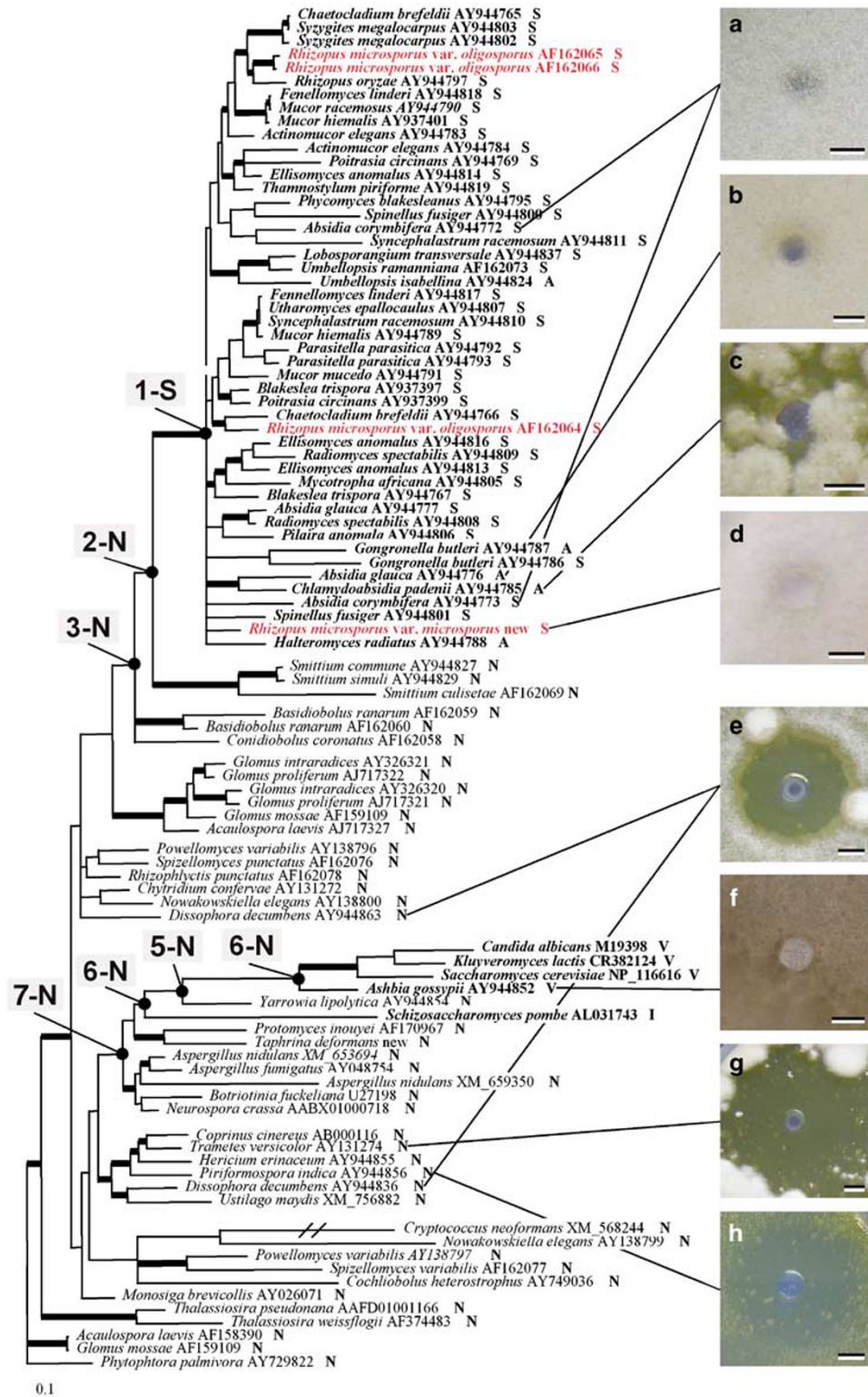


Figure 2 Evolution of β -tubulin paralogs in the kingdom Fungi. This is a 50% majority rule consensus tree from 90 000 trees sampled in a Bayesian Markov chain Monte Carlo (MCMC) analysis. Branches supported by posterior probabilities > 94 are indicated in bold. Amino-acid states at position 100 are mapped on the tree (A, alanine; I, Isoleucine; N, asparagine, S, serine, V, valine). Ancestral character states of amino-acid residue 100 were reconstructed in a Bayesian framework, and are indicated for nodes 1–7. Taxa predicted to be resistant against rhizoxin are typed in bold. Photographs on the right show selected species subjected to a rhizoxin sensitivity test: hyphal growth is inhibited around the toxin source in fungi sensitive to rhizoxin (e, g and h); growth is not affected in resistant species (a–d and f) (scale bar = 5 mm). Fungal species associated with rhizoxin producing bacterial endosymbionts are indicated in red.

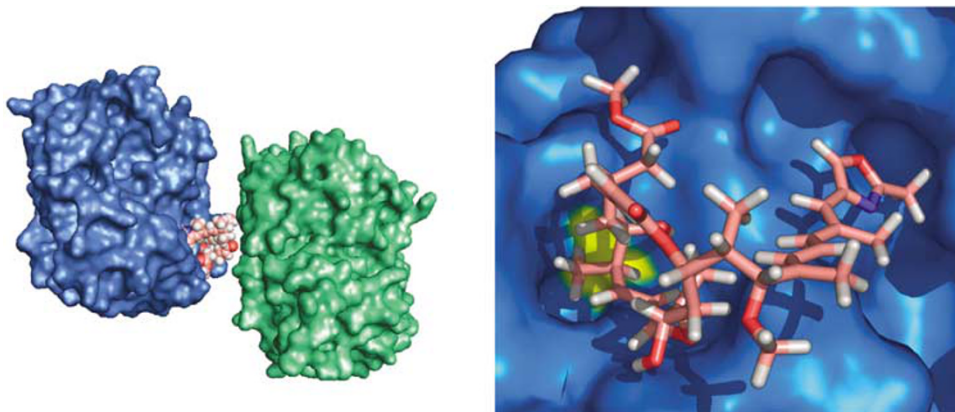


Figure 3 Modeled rhizoxin binding to β -tubulin at the interface of the α - and β -heterodimer. Position Asn-100 is marked in yellow.

aligned β -tubulin sequences from representatives of all major phyla and conducted a Bayesian phylogenetic analysis. Additionally, we inferred ancestral nucleotide sequences for seven selected nodes in a Bayesian framework. Phylogenetic analyses show that β -tubulin paralogs from fungi predicted to be rhizoxin resistant can be found in two independent clades (Figure 2). Yeasts of the subphyla Saccharomycotina (*Ashbya*, *Candida*, *Kluyveromyces*, *Saccharomyces*, *Yarrowia*) and Taphrinomycotina (*Protomyces*, *Schizosaccharomyces*, *Taphrina*) form an unsupported monophyletic group and contain some taxa, which are rhizoxin resistant (Val-100 or Ile-100). Selected Zygomycota—belonging mainly to the order Mucorales—form a highly supported group (clade 1), containing only resistant species (Ser-100 or Ala-100), including both symbiotic and asymbiotic strains of the *R. microsporus* group. This suggests that there are at least two phylogenetic origins of rhizoxin resistance in fungi, one within the Ascomycota and one within the Zygomycota. To assess whether rhizoxin sensitivity or resistance is the evolutionary original condition in fungi, we calculated the probabilities of ancestral character states (of β -tubulin residue 100) at nodes basal to the groups, which contain resistant taxa (Figure 2, clades 1–7). We used the Bayesian method of ancestral state reconstruction, which reports the probability of the character state at each individual residue in an ancient protein sequence (Ronquist and Huelsenbeck, 2003). It takes into account phylogenetic uncertainty as well as mapping uncertainty (Ronquist, 2004), and has been shown to outperform parsimony and maximum likelihood reconstructions of ancestral characters in a study using simulated sequences (Hall, 2006). Our analyses showed with statistical significance that Asn-100, that is, rhizoxin sensitivity, is the most probable character state in β -tubulin sequences ancestral to clades containing resistant taxa (Figure 2, Table 2). Within the Zygomycota, there is a single evolutionary origin of rhizoxin resistance achieved by a mutation from Asn-100 to Ala-100 or Ser-100 (clade 1), while within the Ascomycota

there are probably two origins of resistance, one attained by a mutation from Asn-100 to Ile-100 (clade 6), and a second achieved by a mutation from Asn-100 to Val-100 (clade 4). Rhizoxin sensitivity thus appears to be the ancestral character state in fungi. This is supported by the fact that basal groups within the Fungi, such as Chytridiomycota (James *et al.*, 2006), and organisms suggested to be fungal ancestors, such as Stramenopiles (Steenkamp *et al.*, 2006) are also rhizoxin sensitive.

Evolution of rhizoxin resistance and endosymbiosis in Fungi

In the *Rhizopus*–*Burkholderia* association, rhizoxin resistance of the fungal partner is a precondition for the maintenance of a successful symbiosis. For the evolution of this system, several different scenarios are conceivable: (1) rhizoxin produced by bacteria exerted a selection pressure on fungi, or the endosymbiosis event caused rhizoxin resistance of the fungus; (2) rhizoxin resistance evolved first and enabled endosymbiosis. In the first scenario, bacteria and fungi presumably shared the same habitat, until bacteria gained the ability to produce the antimetabolic agent rhizoxin, kill the fungi and use them as source of food. Under the influence of the toxin, some fungi developed resistance and eventually harbored the bacteria persistently. Thus, the fungi were able to use rhizoxin as a biological weapon without synthesizing it themselves. In the alternative scenario, certain groups of fungi developed rhizoxin resistance independently. Some species then took up rhizoxin producing bacteria to gain the ecological advantage of becoming plant pathogens.

Results of the present phylogenetic analyses of β -tubulin genes suggest that the latter scenario is more likely. We showed that a large number of Zygomycota, mainly belonging to the order Mucorales, is rhizoxin resistant (Figure 2). Within this group, however, the *R. microsporus* group appears to be the only taxon hosting endosymbiotic bacteria. To validate this, we screened (meta)genomic total

DNA samples of over 300 zygomycete strains other than *R. microsporus* for the presence of bacterial endosymbionts. The PCR-based assay revealed no detectable traces of prokaryotic DNA. Thus, the occurrence of bacterial endosymbionts appears to be an exception rather than an universal rule within the Zygomycota.

Our findings imply that evolution of rhizoxin resistance took place in the ancestor of extant resistant Zygomycota, and as a consequence endosymbiosis became possible. Furthermore, phylogenetic relationships of β -tubulin sequences of resistant Zygomycota are unresolved, that is, *R. microsporus* sequences are not basal to other sequences in this group, indicating it was not the symbiosis event, which triggered rhizoxin resistance in Fungi. This hypothesis is supported by the fact that certain yeast species, which are apparently endosymbiont free, have also developed rhizoxin resistance. Since the biological reason for these organisms to evolve rhizoxin resistance is not obvious, we assume that the character states at β -tubulin residue 100, and other as yet undefined factors, possibly confer evolutionary advantages other than rhizoxin resistance.

In conclusion, we have systematically correlated rhizoxin sensitivity with the nature of β -tubulin sequences in the kingdom Fungi. For this purpose, we generated 49 new β -tubulin sequences from representative species of Ascomycota, Basidiomycota and Zygomycota, and performed rhizoxin sensitivity assays. In addition to the known sites Asn-100 (rhizoxin sensitive), Ile-100 and Val-100 (rhizoxin resistant), Ser-100 and Ala-100 were identified as two new amino acids at residue 100 in rhizoxin-resistant fungi. This hot spot was verified by modeling studies, which support the finding that rhizoxin preferentially interacts with the tubulin molecule in a cavity near position 100. Bayesian reconstructions of ancestral sequences indicate that rhizoxin sensitivity represents the ancestral character state in Fungi. Obviously evolution of rhizoxin resistance took place in the ancestor of extant resistant Zygomycota. However, PCR screening of total DNA samples of over 300 zygomycete strains other than *R. microsporus* indicated that bacterial endosymbionts are scarce and were only found within a few strains of *R. microsporus*. Our findings support a model according to which endosymbiosis became possible through a parasitism–mutualism shift in insensitive fungi. Eventually, fungi and bacteria formed an alliance against rhizoxin-sensitive organisms such as rice seedlings for nutrient acquisition. Studies of the molecular and microbial interactions in this intriguing tripartite system are ongoing in our laboratories.

Note added in proof

The term Zygomycota in this article refers to the traditional classification used until the final review

of the manuscript. For a more recent classification, see Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson OE *et al.* (2007). A higher-level phylogenetic classification of the Fungi. *Mycol Res* **111**: 509–547.

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Conflict of interest

The authors declare no conflict of interest.

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