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ORIGINAL PAPER

Modulation of ethanol stress tolerance by aldehyde dehydrogenase in the mycorrhizal fungus *Tricholoma vaccinum*

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Abstract We report the first mycorrhizal fungal aldehyde dehydrogenase gene, *ald1*, which was isolated from the basidiomycete *Tricholoma vaccinum*. The gene, encoding a protein Ald1 of 502 amino acids, is up-regulated in ectomycorrhiza. Phylogenetic analyses using 53 specific fungal aldehyde dehydrogenases from all major phyla in the kingdom of fungi including Ald1 and two partial sequences of *T. vaccinum* were performed to get an insight in the evolution of the aldehyde dehydrogenase family. By using competitive and real-time RT-PCR, *ald1* is up-regulated in response to alcohol and aldehyde-related stress. Furthermore, heterologous expression of *ald1* in *Escherichia coli* and subsequent in vitro enzyme activity assay demonstrated the oxidation of propionaldehyde and butyraldehyde with different kinetics using either NAD^+ or $NADP^+$ as cofactors. In addition, overexpression of *ald1* in *T. vaccinum* after *Agrobacterium tumefaciens*-mediated transformation increased ethanol stress tolerance. These results demonstrate the ability of Ald1 to circumvent ethanol stress, a critical function in mycorrhizal habitats.

Keywords *Tricholoma vaccinum* · Aldehyde dehydrogenase · Alcohol and aldehyde stress tolerance · *Agrobacterium tumefaciens*-mediated transformation · Basidiomycetes

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Introduction

The mushroom forming basidiomycete fungus *Tricholoma vaccinum* forms ectomycorrhizal interactions with the roots of its natural host spruce (Singer 1986). In axenic co-cultures, *Tricholoma* needs 3 to 5 months for mycorrhiza formation (Scheidegger and Brunner 1993; Krause and Kothe 2006). The slow growth is in accordance with the description of *Tricholoma* ecology as late stage organisms, which is well in accordance with the 60 European *Tricholoma* species showing host specificity. Differential gene expression of hydrophobin *hyd1* has been linked to this trait for *Tricholoma terreum* comparing compatible and low compatibility host interactions (Mankel et al. 2002). This success linking molecular biology to ecological features of ectomycorrhizal fungi prompted us to investigate specifically expressed genes in another species, *T. vaccinum*, in order to gain insight in the specific interactions during ectomycorrhizal symbiosis.

Over the past 15 years, our understanding of ectomycorrhiza has been increased by studies on differential gene expression (Tagu et al. 1993; Tagu and Martin 1995; Duplessis et al. 2005), carbohydrate metabolism (Nehls et al. 2010), and nitrogen transport (Müller et al. 2007). Recent demonstrations showed that some of the ectomycorrhizal fungi can be transformed using *Agrobacterium tumefaciens*-mediated transformation (Hanif et al. 2002; Pardo et al. 2002; Kemppainen and Pardo 2010). This technique presents an opportunity to unravel the possible biological function of aldehyde dehydrogenase identified as one of the genes differentially expressed during ectomycorrhizal interaction between *T. vaccinum* and spruce (*Picea abies*; Krause and Kothe 2006).

Aldehyde dehydrogenases (ALDHs) belong to a superfamily of enzymes that have divergent roles in metabolism, primarily, but not limited to, metabolic roles resulting from NAD(P)-catalyzed oxidation of different aldehydes to their

corresponding carboxylic acids. Of the 14 different ALDH families, for instance glyceraldehyde-3-phosphate dehydrogenase, aromatic metabolizing ALDH and fungal ALDH family members differ in their sequence similarities, structures, substrate specificities, and coenzyme used (Perozich et al. 1999). Members of the fungal ALDH family are described as variable in substrate specificity, although this family is not well investigated so far.

ALDHs are commonly regarded as detoxification enzymes (Jakoby and Ziegler 1990) acting along with superoxide dismutases and thioredoxins (Morel et al. 2008). However, alternative functions show ALDHs to be involved in abiotic stress tolerance, male sterility, embryo development, seed viability, and maturation in plants (Kotchoni et al. 2010). To our knowledge, no report on expression changes during mycorrhiza formation is available.

Several roles for ALDHs are potentially possible in mycorrhization. ALDH is involved in alcohol metabolism with a position central to detoxification of aldehydes and alcohols, both of which are abundant in nature, especially when an organism is exposed to stress. Specifically, acetaldehyde is known to be produced as a result of ethanol accumulation in roots, typical for trees with reduced defense, diseases, or anoxia, depending on the species, developmental stage, and environment (Kelsey and Joseph 1998; Kreuzwieser et al. 2004, 2009). Deployment of ALDH-aided aldehyde and alcohol detoxification could therefore be a prerequisite for the plants' survival under these conditions. This is, probably, one of the underlying mechanisms for flood tolerance, as evidenced by up-regulation of rice ALDH under submerged conditions (Nakazono et al. 2000).

Interestingly, it has been shown that mycorrhiza increases flood tolerance of plants, mainly through suppression of toxic products of anaerobic respiration including ethanol (Osundina 1998; Rutto et al. 2002). Although these observations were made on endomycorrhiza using *Glomus clarum* and *Gigaspora margarita*, the same role in ectomycorrhiza is suggested, considering many other shared physiological roles between these mycorrhizal types. Mycorrhiza-enhanced flood tolerance of plants by suppression of ethanol accumulation thus awaits analysis of ethanol stress tolerance related to ALDH expression levels in both fungus and host tree. Since a fungal ALDH gene, *ald1*, was differentially expressed in *T. vaccinum*–spruce ectomycorrhiza, we tested the hypothesis that this gene is involved in mycobiont–plant association by mediating alcohol- and aldehyde-mediated stress. Here, we present the cloning and functional characterization of the novel ectomycorrhiza specifically expressed gene. We link *ald1* to the ability of *T. vaccinum* to circumvent ethanol stress. Since the sequences of the basidiomycete fungal ALDHs were not represented in the last detailed phylogenetic analyses (Perozich et al. 1999; Tanaka and Tanaka 2008), we re-investigated fungal ALDH phylogeny to include all major phyla within the kingdom of fungi. Compared to most of the families in the ALDH superfamily, the fungal ALDH family has been poorly investigated, with only three well studied ALDHs. Feeding experiments using mutants of Iad1 of *Ustilago maydis* (Reineke et al. 2008) and *ALD2/ALD3* of *Saccharomyces cerevisiae* (Rao et al. 2010) showed the involvement of these enzymes in the indole-3-acetic acid biosynthesis from a tryptophan precursor. The regulation of *aldA* in the ascomycete *Aspergillus nidulans* and its function within the ethanol utilization pathway were investigated by Flipphi et al. (2001). They showed that the pathway-specific transcription activator AlcR is essential for the ethanol-induced expression of the two involved genes, the alcohol dehydrogenase *alcA* and aldehyde dehydrogenase *aldA*, whereas the CreA repressor in the presence of glucose directly represses the *alcR* and *alcA* genes. Alcohol dehydrogenase AlcA oxidizes ethanol into acetaldehyde converted by aldehyde dehydrogenase AldA into acetate being further metabolized into acetyl-CoA by acetyl-CoA synthetase (Flipphi et al. 2003).

Materials and methods

Strains and culture conditions

The *Escherichia coli* strain DH5α (Gibco Life Technologies, Karlsruhe, Germany) was routinely used as a host for cloning purposes while strains ArcticExpress (Stratagene, Waldbronn, Germany) and BL21-AI (Invitrogen, Karlsruhe, Germany) were used for heterologous expression of *ald1*. *E. coli* cultures were grown overnight at 37°C shaking in LB medium (Sambrook et al. 1989). The cells that were used for heterologous gene expression studies were grown at 18°C to facilitate induction of gene expression.

A. tumefaciens strain AGL-1, kindly provided by M. Raudaskoski (University of Turku, Finland), was used for *A. tumefaciens*-mediated fungal transformation. It was grown at 28°C shaking in LB medium for 24 h while plate cultures were incubated for about 2 days.

T. vaccinum GK6514, a wild-type strain isolated from Norway spruce, was kindly provided by G. Kost (University of Marburg, Germany), and the derived *ald1*-overexpressing strains (Tvaldh1-eGFP^{-}1 to Tvaldh1-eGFP^{-}3) and *ald1*-overexpressing strains fused with *egfp* (Tvaldh1-eGFP^{+}1 to Tvaldh1-eGFP^{+}3) were grown on Modified Melin Nokrans (MMN) b medium adapted for mycorrhizal fungi (Kottke et al. 1987). In cultures used for real-time RT-PCR, malt extract content of MMNb medium was increased from 5 to 20 mg/l.

In vitro *T. vaccinum*-spruce ectomycorrhiza synthesis was performed to check the ability of mycorrhization of the transformed *T. vaccinum* strains. Surface-sterilized seeds of spruce (*P. abies*) from "Thüringer Forstamt Schmalkalden" (Germany) were germinated under sterile conditions on germination

medium (Chilvers et al. 1986). Three- to 4-week-old seedlings were transferred into petri dishes (diameter of 12 cm) where the root and the inoculated fungus were placed between two cellophane membranes on MMNa medium without glucose and malt extract (Kottke et al. 1987). The cultivation occurred under a day/night regime of 23/17°C with 80% humidity and 12 h of light. The development of mycorrhiza was checked visually at regular intervals.

Nucleic acid manipulation

Mycelium from 3-week-old liquid cultures of *T. vaccinum* was ground with mortar and pestle in liquid nitrogen to isolate genomic DNA (Krause and Kothe 2006) while total RNA was extracted using "RNeasy plant mini kit" (Qiagen, Hilden, Germany) for real-time RT-PCR or according to Sambrook et al. (1989) for competitive RT-PCR. Primers used in this study are listed in Supplemental Table S1. Standard protocols for cloning and transformations in *E. coli*, most of which are according to Sambrook et al. (1989), were used. For sequencing purposes, plasmid DNA was isolated using "PeqGOLD plasmid miniprep kit I" supplied by PeQlab (Erlangen, Germany) while alkaline lysis (Sambrook et al. 1989) was used for routine plasmid preparation. DNA fragments were excised and purified from agarose gels using JetsorbGel Extraction Kit (Genomed, Bad Oeynhausen, Germany) and "Gel extraction kit QIAquick PCR purification kit" (Qiagen, Hilden, Germany). Routine PCR reactions were performed with Taq DNA polymerase (New England BioLabs, Schwalbach, Germany) while a proofreading polymerase Pfu-x (Jena Bioscience, Jena, Germany) was used for high fidelity DNA amplification tasks. DNA modifications and restrictions were done using enzymes and restriction endonucleases supplied by New England BioLabs (Schwalbach, Germany). DNA was sequenced at GATC Biotech (Konstanz, Germany) and Jenagen (Jena, Germany).

To investigate *T. vaccinum* fungal ALDH, we isolated the full-length gene, *ald1*, using different PCR techniques. Two fragments of 1,600 and 800 bp were amplified with primers rf13a and rf-a3. Using an arbitrary degenerated primer AD3 and gene-specific primers T1 and T2, designed in the *ald1* partial sequence, a 307-bp fragment was amplified at the 5′ end of the gene by Thermal Asymmetric Interlaced (TAIL)-PCR (Liu et al. 1995, 1997). Subsequent 5′ TAIL-PCR with gene-specific primers T1b, T2b, and T3b and an arbitrary degenerated primer AD1 yielded an 847-bp new fragment, which included the promoter region. The 3′ sequence was isolated by using gene-specific primers T13b, T23b, and T33b in combination with an arbitrary degenerated primer AD6, which yielded a 487-bp fragment, including the putative translation stop codon (TAA). A special PCR, the "Poly (A) region PCR", was used to isolate the characteristic 3′ flanking sequences with a gene-specific, Poly1, and a modified oligo (dT18) dT1 primer pair. Localization and the apparent molecular mass of the Ald1 translation product were predicted using WoLF PSORT (http://wolfpsort.org) and TagIdent tools (http://www.expasy.org), respectively.

By using genomic DNA with the primer pair rf13a and rf13b, a fragment of another *T. vaccinum* ALDH sequence, *ald2*, was amplified. The third ALDH sequence, *ald3*, was amplified from mRNA using 3′ RACE PCR with gene-specific primer rf-a3 in combination with 3′Race1n, 3′Race2, and 3′Race3n. Southern blot analyses were performed using the protocol "DIG system for membrane hybridization" (Roche Diagnostics, Mannheim, Germany). The sequences were deposited at GenBank (http://www.ncbi.nlm.nih.gov/) with the accession numbers HM363121 for *ald1*, HM363122 for *ald2*, and HM363123 for *ald3*.

Reconstructing fungal ALDH phylogeny

Protein Id and GenBank accession numbers for ALDH amino acid sequences used in the phylogenetic analysis can be found at http://genome.jgi-psf.org (*Phanerochaete chrysosporium*, *Sporobolomyces roseus*, *Schizophyllum commune*, *Phycomyces blakesleeanus*, and *Batrachochytrium dendrobatidis*), http://blast.jcvi.org (*Cryptococcus neoformans*), and http://www.broadinstitute.org (*Puccinia graminis*) and http://www.ncbi.nlm.nih.gov. The sequences were aligned with MAFFT v6 (Katoh and Toh 2008), under assumption of a BLOSUM80 amino acid substitution matrix using the E-INS-i option, which assumes multiple conserved domains and long gaps. The alignment was then checked and manually edited. Phylogenetic reconstruction was performed under Bayesian inference using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). Two runs, each with 2,000,000 generations in four chains, were performed, sampling every 100 generations, with a burn-in of 25%. Results were evaluated with TRACER v1.4 (Rambout and Drummond 2007); all analyses had log likelihood ESS values above 100. Maximum likelihood using TREEFINDER version of October, 2008 (Jobb 2008) was used for validation of MrBayes tree, with 500 replicates of LR-ELW branch support. Both unpartitioned data and a dataset partitioned into conserved motif regions and unconserved regions were analyzed. In fact, more validation of the results was done by performing additional analyses using maximum likelihood with RAxML (Stamatakis 2006) and with maximum parsimony using PAUP (Swofford 2002) in a heuristic search with "tree bisection and reconnection" branch swapping. Phylogenetic trees were evaluated using FigTree v. 1.2.3 (Rambout 2009).

Recombinant protein expression

In order to express *ald1* as a His_6-tagged protein, its cDNA was cloned in pET101/D-TOPO (V5, 6 × His) vector (Invitrogen,

Groningen, The Netherlands), under the control of T7 promoter, after which the transforming plasmid was introduced into *E. coli* strains ArcticExpress and/or BL21-AI by electroporation. *ald1* expression was induced with 0.9 mM IPTG. Cells were collected by centrifugation and resuspended in 0.1 M sodium pyrophosphate solution. The cells were then sonicated at 3 cycles for 3 min on ice and centrifuged to collect crude proteins. For protein analysis, proteins were separated on SDS gel and stained with Coomassie Brilliant Blue (Sigma Aldrich, Steinheim, Germany). Identity of Ald1 was confirmed by a western blot analysis targeting Ald1-His_6 fusion protein. Western blotting was carried out on total soluble crude proteins separated on an SDS gel, where immunodetection of Ald1 was performed using anti-His antibodies (Miltenyi Biotech GmbH, Germany).

Enzyme activity analysis

Ald1 activity was carried out according to Guru and Taranath-Shetty (1990) and Isobe et al. (2007) with modifications. A typical enzyme reaction mixture consisted of 100 μl crude protein extracts, 10 mM aldehyde substrates, 0.1 M sodium pyrophosphate buffer, pH 7.4, and 0.5 mM cofactor β-NAD^+ or β-$NADP^+$ in a final volume of 1 ml. The reactions, which were carried out at 30°C, except acetaldehyde reactions carried out at 25°C because of its high volatility, were started by the addition of aldehydes, which was done after about 30 s of pre-reaction. To eliminate non-enzymatic reaction of NAD^+ or $NADP^+$ with aldehydes, which contributes to increase in absorbance at 340 nm (Gurr et al. 1987), enzyme activity was measured against a substrate blank (cofactor + aldehyde), as opposed to enzyme blanks (cofactor + enzyme) used in many studies.

Competitive and real-time RT-PCR

For analysis of *ald1* expression induction, *T. vaccinum* mycelium was grown in liquid MMNb medium supplemented with various concentrations of alcohols and aldehydes. After total RNA extraction from lyophilized mycelium, cDNA was synthesized either by using the Oligotex mRNA Batch Protocol of Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany), SUPERSCRIPT™ II RNase H Reverse Transcriptase (Invitrogen, Karlsruhe, Germany), or "iScriptTM cDNA Synthesis Kit" supplied by Bio-Rad Laboratories (Hercules, CA, USA).

For competitive RT-PCR, primers rf13a and rf13b were used to simultaneously amplify an 826-bp cDNA fragment and a 1,297-bp competitor fragment, resulting from cloned genomic DNA with seven introns. The signal intensity of the resulting PCR products was monitored on agarose gels to estimate the relative amount of transcript. Each treatment had two biological replicates. PCR was carried out on four replicates. Real-time RT-PCR was performed by using a kit supplied by Fermentas (St. Leon-Rot, Germany), which uses SYBR Green as a DNA intercalating dye. Transcription was monitored, in real time, by amplification of a 132-bp *ald1* cDNA fragment using aldRTPCRF and aldRTPCRR primers, which were designed to span an intron. The *ald1* mRNA accumulation was quantified in absolute terms (Bustin 2000) by comparing transcript levels to a standard curve generated using serial dilutions of plasmid DNA of an *ald1* cDNA cloned in pDrive (Qiagen, Hilden, Germany).

Construction of *ald1*-overexpressing *T. vaccinum* transformants

Two plasmids carrying *ald1*-overexpressing cassettes, one with *gpd* promoter and 35S terminator, designated pBGaldh1, and the other with the same features but with an additional *egfp* fusion, designated pBGaldh1SeGFP, were constructed (Fig. 1). Both plasmids consist of a pCAMBIA 1300 backbone (CAMBIA, Australia) containing the kanamycin and hygromycin resistance genes. The expression from the native promoter was expected to be under control of environmental factors which might not be useful for the investigation of overexpression. Therefore, the constitutive strong glyceraldehyde-3-phosphate dehydrogenase promoter (P*gpd*) from *Agaricus bisporus* was amplified using gpdF and gpdR primers on pBGgHg plasmid DNA (Chen et al. 2000) as a template. *ald1* genomic DNA was amplified with ORFF and ORFR primers. A fusion PCR was carried out using both fragments as the new template, with the primers gpdF forward and ORFR reverse, which resulted in a fragment of about 2.8 kb corresponding to P*gpd* promoter fused to *ald1*. An approximately 1.1-kb *egfp*-cauliflower mosaic virus terminator (35S) fragment was amplified from pBGgHg plasmid with EGFPFII and EGFPRII primers. A 35S fragment was generated by a PCR using 35SF forward and EGFPRII reverse primers on pBGgHg plasmid.

Different restriction and ligation steps using intermediate plasmid were performed to construct the plasmids pBGaldh1 and pBGaldh1SeGFP. In vector pNEB193 (New England Biolabs, Schwalbach, Germany), the *Sac*I–*Asc*I fragment was replaced by the P*gpd-ald1* PCR product via *Sac*I–*Asc*I ligation followed by restriction with *Asc*I and *Pme*I to insert the 35S terminator PCR fragment. The resulting P*gpd-ald1*-35S construct was ligated instead of the excised P*gpd-egfp*-35S fragment in plasmid pBGgHg using *Sac*I and *Pme*I restriction enzymes to obtain plasmid pBGaldh1 with genotype Kan^R, Hyg^R, pBGgHg::*ald1*, P*gpd* from *A. bisporus* and 35S terminator from CaMV. To obtain plasmid pBGaldh1-SeGFP, in vector pNEB193 with the replaced *Pgpd-ald1* PCR product, the excised *Asc*I and *Pme*I fragment was replaced by the *egfp*-35S PCR fragment. The resulting P*gpd-ald1-egfp*-35S was excised by *Sac*I and *Pme*I and used to replace the

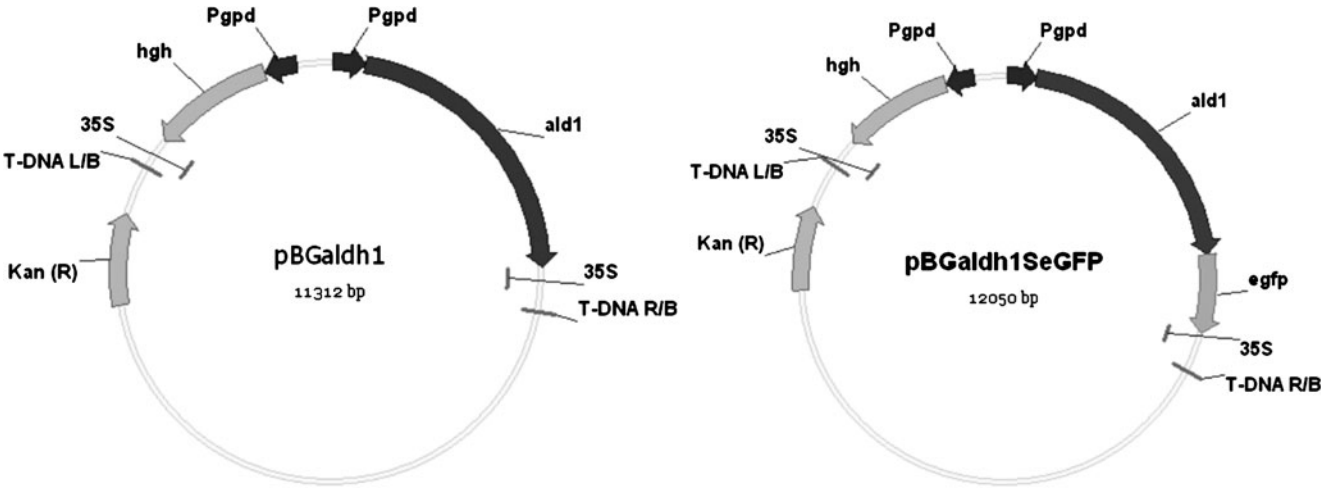

Fig. 1 Construction of plasmids pBGaldh1 and pBGaldh1SeGFP for overexpression of *ald1*

entire P*gpd-egfp*-35S fragment in plasmid pBGgHg to obtain plasmid pBGaldh1SeGFP with genotype Kan^R, Hyg^R, pBGgHg::*ald1-egfp* fusion, P*gpd* from *A. bisporus* and 35S terminator from CaMV. Cloned constructs were verified by PCR, restriction analysis, and sequencing.

A. tumefaciens-mediated transformation was used to deliver *ald1*-overexpressing constructs to *T. vaccinum* genome using an optimized protocol based on Hanif et al. (2002) and Pardo et al. (2002). Briefly, mycelia of *T. vaccinum* were grown on MMNb plates to a diameter of approximately 1 cm. *A. tumefaciens* pre-culture was prepared by growing the bacteria in minimal medium with kanamycin for 2 days at 28°C, harvested by centrifugation, and resuspended in induction medium containing 200 μM acetosyringone. After 6 h of induction, the cell density was adjusted to an optical density at 600 nm of 0.1. *T. vaccinum* was then co-cultivated with the pre-culture for 2 days. After co-cultivation, the mycelia were transferred to selection plates containing 25 μg/ml hygromycin B and 200 μg/ml cefotaxime. After the mycelia had started to grow, they were transferred to MMNb plates containing 25 μg/ml hygromycin B for the second round of antibiotic selection. The same conditions were used for propagation of the mycelia.

Results

The gene *ald1* possesses signature sequence characteristic of stress-induced genes

Sequence analysis of *ald1*, based on fragments obtained by different PCR approaches, revealed an open reading frame of 2,448 bp. This was confirmed by comparison to cDNA amplified using primers aldh1-F1 and aldh1-R1. The sequence is interrupted by 16 introns of 48–76 bp with the typical conserved 5′ GT and 3′ AG residues (Gurr et al. 1987; Supplemental Fig. S1) resulting in a 502 amino acid translation product, Ald1, predicted to be localized in the cytosol. Promoter analysis revealed the presence of seven stress response elements (STREs; Moskvina et al. 1999). Transcriptional regulatory sequences, including four TATA boxes (−118, −128, −175, −189), two CAAT boxes (−326, −333), and a GC box (−515), were identified in the promoter region. The 3′ flanking sequence analysis showed that the region immediately following the stop codon is AT-rich (80% for the first 10 bp), which is the preferred condition for transcription termination by mRNAs with TAA stop codons (Cavener and Ray 1991). Also, a putative polyadenylation signal AATTAT (consensus AATAAA) and a polyadenyl tail were identified (Fig. S1).

Ald1 is closely related to other fungal ALDHs

Ald1 possesses all 10 conserved ALDH motifs (Perozich et al. 1999; Table 1, Fig. S2). Additional conserved amino acid residues were observed; most of them had earlier been reported for this enzyme superfamily (Hempel et al. 1993; Perozich et al. 1999). One putative signature sequence, GxTxxG, proposed for the NAD-binding site of ALDHs (Liu et al 1997), and two sequences, for the general glycine motif GxGxxxG for NAD(P) binding (Cobessi et al. 2000; Jung and Lee 2006), were identified (see Table 1), suggesting the possibility of both NAD^+ and $NADP^+$. The comparison of Ald1 with other fungal ALDHs shows that it clusters with other fungal ALDHs with up to 74% amino acid identity, the closest ALDH being from *Laccaria bicolor* (Xp_001889968), which is also an agaricoid ectomycorrhiza fungus (Fig. S2). Interestingly, a significant amino acid identity of 58% was observed with the heterobasidiomycete *U. maydis* indole-3-acetaldehyde dehydrogenase Iad1 (Basse et al. 1996). Ald1 shows a relatively high similarity of the first 16 amino acids with most other fungal ALDHs (Figs. S2 and S3), supporting

Table 1 Sequence information of Ald1

Motif definition[a]	Motif[b]
ALDH 1	^{168}P-W-**N**-F-P^{172}
ALDH 2	^{183}A-L-A-T-G-N-T-I-V-L-K-P-S-E^{196}
ALDH 3	^{213}G-I-P-P-G-V-V-N-I-I^{222}
ALDH 4	^{242}V-A-F-T-G-S-T-V-I-G^{251}
ALDH 5	^{268}L-E-L-**G**-G-K-S-P-T-I-I-F-D-D-A-D^{283}
ALDH 6	^{296}Y-F-N-M-**G**-Q-V-**C**^{303}
ALDH 7	^{381}G-Y-F-I-Q-P-T-I-F^{389}
ALDH 8	^{401}E-**E**-I-**F**-G-P-V^{407}
ALDH 9	^{424}N-N-T-T-Y-G-L-A-C-S-V-F-S-Q-N^{438}
ALDH 10	^{467}P-F-G-G-Y-K-Q-S-G-I-G-R^{478}
NAD-binding domain	^{246}GxTxxG^{251}
NADP-binding domain	^{224}GxGxxxG^{230}
	^{475}GxGxxxG^{481}

[a] ALDH motifs after Perozich et al. (1999), NAD-binding domain after Liu et al (1997), and NADP-binding domains after Cobessi et al. (2000) and Jung and Lee (2006)

[b] Positions in amino acid sequence of Adl1

the proposed transcription/translation start. Intron position analysis confirmed that 10 of the 16 *ald1* intron positions are identical with a *L. bicolor* ALDH and 12 with a *Coprinopsis cinerea* ALDH (Fig. S2).

Duplication events characterize fungal ALDH evolution

ALDHs of fungi belong to different families of ALDH superfamily like γ-glutamyl semialdehyde dehydrogenase family, glyceraldehyde-3-phosphate dehydrogenase family, class 3 ALDH family, and fungal ALDH family. In contrast to other families, all members of the "fungal ALDH family" are exclusively of fungal origin while other families are mixed with respect to clades represented. A survey of fungal genomes for ALDH orthologs using the 10 most conserved sequence motifs in ALDHs (Perozich et al. 1999) could show that only a small part of the ALDH sequences clusters in the specific family of fungal ALDHs (Table S2). We carefully examined the available genomes from selected basidiomycete, ascomycete, zygomycete, and chytridiomycete lineages for a possible ALDH duplication. All fungi, with the exception of the chytridiomycete *B. dendrobatidis*, show evidence for duplications, which is more pronounced in higher fungi. Southern blot analyses resulted in more than one hybridization signal per lane of restricted genomic DNA of *T. vaccinum* (data not shown), suggesting the existence of additional ALDH genes. This could be verified by PCR amplification of two additional partial sequences, *ald2* (1,289 bp encoding 271 amino acids, Fig. S4) and *ald3* (721 bp encoding 191 amino acids, Fig. S4). The original *ald1* has a nucleotide sequence similarity of 88% and 79% with *ald2* and *ald3*, respectively.

The widely observed duplication of fungal ALDHs prompted us to investigate the evolutionary origin of this enzyme family. Fungal ALDH phylogeny was reconstructed with a total of 53 fungal ALDHs from all major phyla within the kingdom of fungi and four outgroup ALDHs (Fig. S3). The resulting phylogram (Fig. 2), which was confirmed by three independent phylogenetic reconstruction methods, shows a well supported clustering of fungal ALDHs in two groups each of basidiomycota and ascomycota, with a chytridiomycota ALDH being basal to all other fungal ALDHs. The three ALDH sequences from a zygomycete, *P. blakesleeanus*, also clustered in two different clades. The phylogram indicates that fungal ALDH duplication events probably happened two times during the course of evolution. The first major duplication event happened after the split of Chytridiomycetes, since only one ALDH sequence was found in the genome of *B. dendrobatidis*. This resulted in two major fungal lineage groups: subfamily 1 (zygomycote 1/ascomycote 1/basidiomycote 1) and subfamily 2 (zygomycote 2/ascomycote 2/basidiomycote 2). The second major duplication event happened within the basidiomycote 1 group, primarily in the Agaricomycetes clade, resulting in representative paralogs in the two new clades. Interestingly, Ald1 clustered together with the other two partial ALDH sequences of *T. vaccinum*, Ald2 and Ald3. The three ALDHs from *T. vaccinum* form a common cluster with other Agaricomycetes, with a distinct, fully supported branch, typical for a duplication after the separation of this phylogenetic clade. No transposable elements were detectable up- or downstream of 10 randomized fungal ALDH genes, which would be in accordance with duplication with subsequent differential functionalization.

No correlation between the phylogenetic clustering of fungal ALDHs and their cytosolic or mitochondrial localization was found. Apart from *S. cerevisiae* (EDN63047), *Pichia angusta* (AAA83769) and *P. graminis* (PGTG_05992), all other fungal ALDHs were predicted to be cytosolic. The putative signature sequences for the NAD-binding site, GxTxxG, and the two NAD(P)-binding sites, GxGxxxG identified in Ald1 of *T. vaccinum*, are generally found in the subfamily of fungal ALDHs (see Table 2), with the exception of the lack of the NAD-binding motif in *S. roseus* (24603), in *P. chrysosporium* (138693), and Ald2 of *T. vaccinum.* The first NAD(P)-binding motif is not visible in the clade of filamentous basidiomycete 2.

The 10 characteristic sequence motifs conserved in fungal ALDHs (Table 2) show slight differences between the two identified subfamilies 1 and 2, visible for motif ALDH 1 with ^{240}PWN**F**P^{244} and ^{240}PWN**Y**P^{244}. Further analysis revealed that five of the protein sequences used, *C. cinerea* (EAU84708, EAU92000), *A. alternata* (P42041), *P. graminis* (PGTG_15016), and *U. maydis* (EAK_83677), did lack at least one of the 10 characteristic motifs.

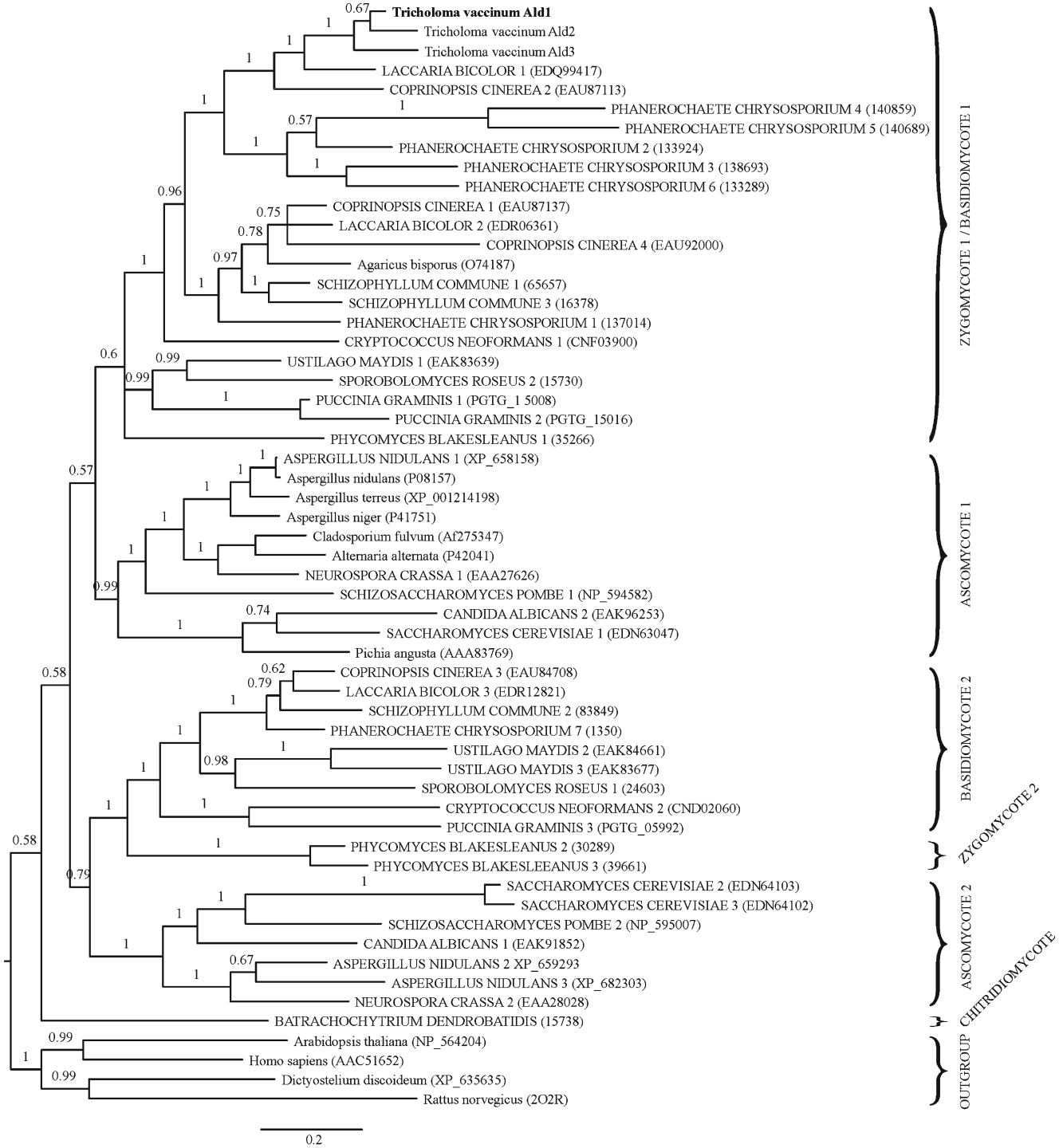

Fig. 2 A semistrict consensus phylogram of fungal ALDHs, created with MrBayes 3.1.2. Bayesian posterior probability values are shown above corresponding branches. Branch lengths are proportional to evolutionary distances. Mammalian, plant, and mycetozoa ALDHs were included as an outgroup. The first single digit number immediately behind a binomial represents the ALDH sequence of a given species, in a descending order of similarity to Ald1. After the binomials, GenBank accession numbers (*in brackets*) are given. *Binomials in capitals* indicate that this fungal ALDH originates from fungal genome sequences and was picked up together with all identified fungal ALDHs from the available genome and included in the phylogram

Alcohols and aldehydes induce ald1 expression

Studies from other *ald* genes, mainly, but not restricted to, higher eukaryotic origin, show up-regulation following exposure to alcohol, especially ethanol, suggesting their involvement in alcohol detoxification. We chose to investigate the effect of different alcohols and aldehydes on expression of *ald1* by using competitive and real-time RT-PCR.

Table 2 Consensus motifs in fungal aldehyde dehydrogenase family and subfamilies 1 and 2

Motif definition[a]	Consensus motif[b]
ALDH 1	
Fungal family	^{240}P-W-**N**-F-P^{244}
Subfamily 1	^{240}P-W-**N**-F-P^{244}
Subfamily 2	^{240}P-W-**N**-Y-P^{244}
ALDH 2	
Fungal family	^{263}A-L-A-x-G-N-T-V-V-L-K-P-A-E^{276}
Subfamily 1	^{263}A-[Lvi]-A-[Ta]-G-N-[Tca]-[Vi]-[Vi]-[Lmvi]-K-[Pt]-[SA]-E^{276}
Subfamily 2	^{263}A-L-A-[Ac]-G-[Nc]-[Tc]-[IV]-[Vi]-[MLif]-K-[Pt]-[AS]-E^{276}
ALDH 3	
Fungal family	^{293}G-F-P-P-G-V-V-N-x-V^{300}
Subfamily 1	^{293}G-[Fil]-P-[Pak]-G-[Va]-[Vifl]-N-[VI]-[IVl]^{300}
Subfamily 2	^{293}G-[Fy]-P-[pak]-G-V-[Vifl]-N-[ivt]-[Vi]^{300}
ALDH 4	
Fungal family	^{322}V-A-F-T-G-S-T-x-V-G^{331}
Subfamily 1	^{322}[Vil]-[As]-F-T-G-S-T-[LAiv]-[Vt]-G^{331}
Subfamily 2	^{322}[ivm]-[As]-F-T-G-S-T-[Tvak]-[Tv]-G^{331}
ALDH 5	
Fungal family	^{348}L-E-L-**G**-G-K-S-P-N-I-V-F-x-D-A-D^{363}
Subfamily 1	^{348}L-E-L-**G**-G-K-S-[Pa]-[Ntv]-[Iv]-[VI]-F-[Dna]-D-A-D^{363}
Subfamily 2	^{348}L-E-[Lct]-**G**-G-K-S-[Pa]-[lqnhva]-[ILv]-[Vi]-[Fc]-[Edp]-[Ds]-A-D^{363}
ALDH 6	
Fungal family	^{376}F-x-N-x-**G**-Q-x-**C**^{383}
Subfamily 1	^{376}[Fy]-[Fwy]-[Nh]-[Hsma]-**G**-Q-[cvamt]-**C**^{383}
Subfamily 2	^{376}[fmcl]-[fysne]-N-[qstm]-**G**-Q-[dis]-**C**^{383}
ALDH 7	
Fungal family	^{471}G-Y-F-I-x-P-T-I-F^{480}
Subfamily 1	^{471}G-[Yf]-F-[Iv]-[QEk]-P-T-[Ivl]-F^{480}
Subfamily 2	^{471}G-[Yf]-[Fy]-[Iv]-[edqap]-P-T-[Iv]-F^{480}
ALDH 8	
Fungal family	^{491}E-**E**-I-**F**-G-P-V^{497}
Subfamily 1	^{491}[Ed]-**E**-I-**F**-G-P-V^{497}
Subfamily 2	^{491}[Ed]-**E**-[Iv]-**F**-G-P-V^{497}
ALDH 9	
Fungal family	^{520}N-D-T-x-Y-G-L-A-A-A-V-F-T-x-D^{534}
Subfamily 1	^{520}N-[Dns]-[Ts]-[vtdsn]-Y-G-L-[As]-[Asc]-[Agns]-[Vi]-[FHy]-[TS]-[QKretn]-[NDs]^{534}
Subfamily 2	^{520}N-[Dn]-[Ts]-[tendc]-Y-G-L-[AG]-[Ags]-[AG]-[Vl]-[Fhy]-[Ts]-[Kern]-[Dn]^{534}
ALDH 10	
Fungal family	^{563}P-F-G-G-x-K-Q-S-G-I-G-R^{574}
Subfamily 1	^{563}P-F-G-G-[Yfv]-K-[Qe]-S-G-[Ilfm]-G-[Rk]^{574}
Subfamily 2	^{563}P-F-G-G-[yvkfm]-K-[Qm]-S-G-[Ifw]-G-[Rk]^{574}
NAD binding	^{326}GxTxxG^{331}
NADP binding	^{304}GxGxxxG^{310}
	^{571}GxGxxxG^{577}

[a]ALDH motifs after Perozich et al. (1999), NAD-binding domain after Liu et al. (1997), and NADP-binding domains after Cobessi et al. (2000) and Jung and Lee (2006)

[b]Positions in alignment Fig. S3; motifs are ordered in their occurrence: dominant in capitals, with lower frequency but two in minimum with small letters

Using competitive RT-PCR, we could show that spiking of *T. vaccinum* cultures with various concentrations of different alcohols and aldehydes significantly increased *ald1* transcript levels (Table 3). Interestingly, ethanol (0.01%) and leaf oil from Norway spruce (0.005%), the compatible host of *T. vaccinum*, induced *ald1* expression 50-fold. The addition of the ALDH substrates isobutanol, glutardialdehyde, pine, and spruce leaf oil into the medium resulted in a growth inhibition of the mycelium, which was even stronger for dodecyl alcohol, salicyl aldehyde, benzaldehyde, and cinnamic aldehyde

Table 3 Substrates that induce *ald1* expression, as quantified by competitive RT-PCR

Modification of MMNb medium	Transcript accumulation (pg) using 1 ng cDNA
Control (MMNb)	1
MMNb − glucose	3–5
MMNb − glucose + ethanol (1%)	<1
MMNb + ethanol (1%)	10
MMNb + ethanol (0.01%)	50
MMNb + butanol (0.1%)	25
MMNb + isobutanol (0.01%)	5
MMNb + glycerol (0.1%)	10
MMNb + formaldehyde (0.1%)	7
MMNb + glutardialdehyde (0.01%)	20
MMNb + pine leaf oil (0.01%)	10
MMNb + spruce leaf oil (0.005%)	50

(all 0.005%), where mRNA isolation repeatedly failed (data not shown).

The analyses were validated using real-time RT-PCR to determine the expression profile of *ald1*, as influenced by ethanol and a few selected aldehydes. By monitoring the amplification of a carefully selected 132-bp *ald1* cDNA sequence, we could confirm that ethanol and some aldehydes induced *ald1* expression; 0.01% ethanol and 0.1 mM indole-3-acetaldehyde increased *ald1* transcript levels by about fourfold and threefold, respectively (Fig. 3), while benzaldehyde

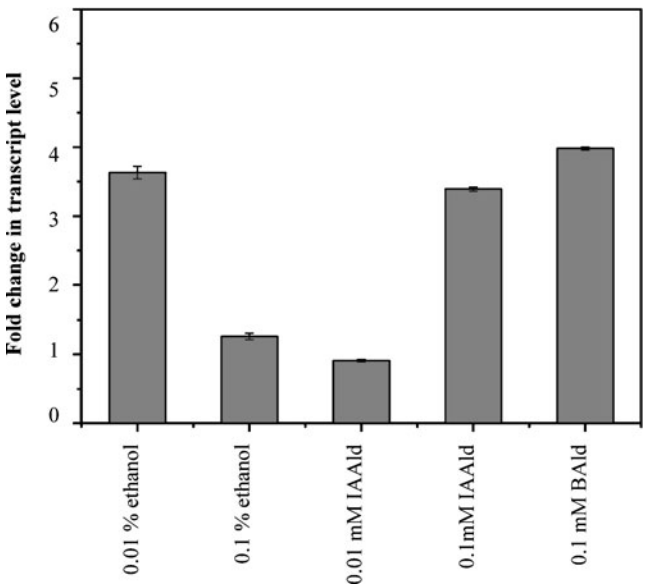

Fig. 3 Induction of *ald1* expression by ethanol and aldehyde stress as assayed by real-time RT-PCR. RNA was extracted from fungal mycelium of cultures incubated with 0.01% and 0.1% ethanol, 0.01 and 0.1 mM indole-3-acetaldehyde (*IAAld*), and 0.1 mM benzaldehyde (*BAld*). Each treatment had two biological replicates. PCR was carried out on three cDNA replicates in three reactions per cDNA replicate. *Bars* denote standard error

(0.1 mM), which was not tested with competitive RT-PCR, also induced *ald1* expression by about fourfold. A concentration of 0.01 mM indole-3-acetaldehyde was probably too low to increase the *ald1* mRNA amount. On the other hand, ethanol treatments showed a concentration-dependent *ald1* transcriptional inhibition, with 0.01% ethanol showing approximately fourfold transcript accumulation, while 0.1% ethanol failed to significantly induce *ald1* expression. However, the levels of induction were much lower, compared to the levels reported using competitive RT-PCR, where lower malt extract content was used for cultivation. The results, nonetheless, indicate that low, most likely physiologically relevant ethanol concentrations are able to strongly induce *ald1* expression.

Ald1 activity is aldehyde-specific, with a dual cofactor binding possibility

In order to verify the function of the cloned *ald1* cDNA and to investigate Ald1 activity in vitro, we heterologously expressed *ald1* in *E. coli*. We targeted Ald1-His_6 fusion protein to determine protein size on western blots using anti-His antibody. Ald1 has an apparent molecular mass of approximately 53 kDa, which corresponds to the predicted size of 53.5 kDa.

Using in vitro enzyme activity assays, we tested the ability of Ald1 to oxidize a wide range of aldehydes, including aliphatic and aromatic aldehydes. Ald1 oxidized short chain aliphatic aldehydes, notably propionaldehyde and, to some extent, butyraldehyde (Table 4). Although there was no enzyme activity detected with the tested aromatic aldehydes, indole-3-acetaldehyde seems to be a substrate for Ald1, at least to some extent, since the reaction showed consistently higher absorbance than the control, albeit loss of activity with time occurred (data not shown). The extremely volatile nature of acetaldehyde made it impossible to conclude the enzyme activity assay for the aldehyde.

Since both NAD and NAD(P)-binding sites were identified in the Ald1 amino acid sequence, the heterologously expressed enzyme was tested for both cofactors. By using propionaldehyde as a substrate, the enzyme activity with β-$NADP^+$ was approximately 13-fold higher than with β-NAD^+, suggesting that Ald1 prefers β-$NADP^+$ as a cofactor. However, butyraldehyde was only oxidized using β-NAD^+. These results suggest a possibility of Ald1 to utilize both cofactors, albeit with different affinities, depending on different substrates. As expected, Ald1 exhibited no enzyme activity on ethanol.

Transformation of *T. vaccinum* with *ald1* overexpression cassettes

We first developed an efficient *A. tumefaciens*-mediated transformation system for *T. vaccinum*. The wild-type strain, grown

Table 4 Enzyme activity, substrate, and cofactor specificity of Ald1

Substrate	Relative activity (fold change in relative absorbance)	
	β-NADP$^+$	β-NAD$^+$
Propionaldehyde	13.1	1.1
Butyraldehyde	0.0	2.7
Acetaldehyde	n.d.[a]	n.d.[a]
Benzaldehyde	0.0	0.0
Indole-3-acetaldehyde	n.d.[b]	n.d.[b]
Ethanol	0.0	0.0

n.d. not detected

[a] Enzyme activity, using acetaldehyde as a substrate, was not conclusive due to its high volatility

[b] Absorbance higher than in controls, but declined over time

on MMNb plates, was inoculated with *A. tumefaciens* AGL-1 containing the plasmid pBGgHg (Chen et al. 2000). After a 2-day co-cultivation, mycelia were transferred to selection plates containing hygromycin B. Transformed fungal colonies were detected after approximately 2 weeks of growth under selective conditions. A transformation rate of about 30% was observed. The transformation did not impair growth or morphology of the fungus. Moreover, the transformants maintained their ability to form ectomycorrhiza (data not shown).

Genomic DNA of putative transformants was isolated and tested with PCR using primers Hph-F and Hph-R spanning the hygromycin resistance gene (*hph*) and with primers Egfp-1 and Egfp-2 spanning the *egfp* gene. Both genes were integrated in the genome of all the transformants while no PCR signal was detected in the wild type (data not shown). The integration pattern, tested with Southern hybridization using the PCR amplification product of *hph* as a probe, indicated 20% to 50% of multicopy integrations in independent transformation assays (Fig. S5). To test the mitotic stability of the integrated genes, the mycelia of the transformants were grown on hygromycin selective plates following two growth cycles under non-selective conditions. The transformants were still able to grow, indicating that the *hph* gene was stably integrated. Using both PCR and Southern hybridization, we could show that the *egfp* gene was still present in the genome of 7 out of 10 transformants after a period of 5 years.

The developed transformation system was then used to deliver plasmids containing *ald1* overexpression cassettes, with (pBGaldh1SeGFP) or without (pBGaldh1) *egfp* fusion, to *T. vaccinum* genome. Transformants were observed on hygromycin plates, which could grow even after incubation on non-selective plates for 2 months (data not shown). The integration of *hph* and *egfp* genes in the fungal genome was tested with PCR as described above, which showed that the genes were present in the transformants, but not in the wild-type strain (Fig. S6).

Overexpression of *ald1* in *T. vaccinum* increases ethanol stress tolerance

We investigated the ability of *T. vaccinum* transformants overexpressing *ald1* to circumvent ethanol and/or aldehyde stress. First, toxicity tests with the wild-type strain were carried out using different concentrations of ethanol and aldehydes to determine the optimal concentrations for this investigation. Increased fungal biomass was observed in MMNb medium containing 0.01% and 0.1% ethanol while a reduced biomass was observed in the medium without glucose but supplemented with 1% ethanol and other substrates as indicated in Table 3. Therefore, 2% ethanol was chosen as the optimal concentration for *ald1* functional analysis, since higher concentrations were detrimental to fungal growth.

Aldehyde stress mitigation by Ald1, tested with different concentrations of benzaldehyde and butyraldehyde, did not yield significant results, probably due to limited uptake (data not shown). On the other hand, observations carried out on 2% ethanol-treated fungal cultures indicated that the Ald1-overproducing transformant Tvaldh1-eGFP$^+$1 significantly reduced ethanol stress compared to the wild type (Fig. 4). Whereas ethanol stress reduced growth of the wild type by almost 50%, the growth of the transformant was only reduced by about 26–33% throughout the growth period of about 4 weeks (Fig. 4). Thus, we could link *ald1* to ethanol detoxification metabolism in this ectomycorrhizal fungus.

Discussion

To the best of our knowledge, this is the first report on cloning and functional characterization of an aldehyde dehydrogenase from a mycorrhizal fungus and one of the few to demonstrate the use of *A. tumefaciens*-mediated transformation in investigating a gene function in an ectomycorrhizal fungus. Investigation of gene function using agrotransformation in ectomycorrhizal fungi was demonstrated by heterologously overexpressing a *Paxillus involutus* metallothionein in *Hebeloma cylindrosporum* (Bellion et al. 2007) and by gene silencing experiments in *Laccaria laccata* (Kemppainen and Pardo 2010).

We isolated a functional aldehyde dehydrogenase gene, *ald1*, from *T. vaccinum*, previously shown to be differentially expressed in *T. vaccinum*-spruce ectomycorrhiza (Krause and Kothe 2006). Both NAD$^+$ and NADP$^+$ were accepted as cofactors, agreeing with the potential binding sites for both cofactors identified in the Ald1 sequence and also in the entire enzyme subfamily of fungal aldehyde dehydrogenases. This dual cofactor binding potential disagrees with the suggestion that fungal ALDHs utilize only NAD as a cofactor (Perozich et al. 1999) which was, however, based on only few fungal ALDH sequences available at that

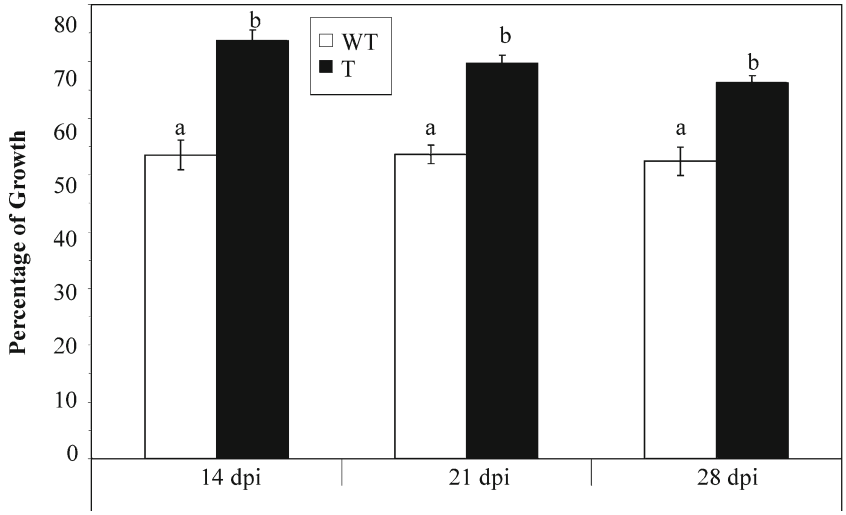

Fig. 4 Ethanol stress mitigation by *ald1*. The fungal growth, quantified using colony diameter measurements, as a result of exogenous application of 2% ethanol to fungal culture plates was compared between the wild-type *T. vaccinum* (*WT*) and a transformant overexpressing *ald1* with *egfp* fusion Tvaldh1-eGFP^{+}1 (*T*). The fungal cultures were replicated at least three times. Percentage of growth was calculated relative to a control without ethanol treatment. *Bars* denote standard error, and *letters* show significantly different fungal growths

time. Since then, several authors have revisited this topic of dual cofactor binding by ALDHs, to show that several ALDHs may bind both cofactors, albeit with different affinities (Isobe et al. 2007; Mori et al. 2002; Velasco-García et al. 2000). Our results, for the first time, suggest that the cofactor used may, indeed, depend on the substrate.

We took advantage of the growing number of fungal genome sequences to establish the relationship of Ald1 to other fungal ALDHs and to re-investigate the evolutionary relationships among the enzymes of this family. Ald1 is closely related to other fungal ALDHs with up to 74% amino acid identity. Unfortunately, none of the closely related enzymes, including the closest homolog from the ectomycorrhizal fungus *L. bicolor*, have been functionally characterized. The high similarity of Ald1 to other fungal ALDHs is also reflected in the 10 and 12 identical intron positions with *L. bicolor* and *C. cinerea* ALDHs, respectively, of the 16 introns identified, the highest number found in fungal ALDHs.

The ALDH superfamily is characterized by a large extent of homology between different enzymes. In the current fungal ALDH alignment, we observed all the highly conserved motifs and residues reported earlier (Perozich et al. 1999), although slight differences were visible in 6 of the 10 ALDH motifs (2, 3, 5, 6, 7, and 9). Loss of catalytically important sequences was found in four of the protein sequences used in the alignments, *C. cinerea* (EAU84708, EAU92000), *U. maydis* (EAK83677), and *P. graminis* (PGTG_15016), suggesting non-functional products, as has been described for *A. alternata* allergen (Achatz et al. 1995).

At the time Perozich et al. (1999) investigated ALDH phylogeny by using the 145 known amino acid sequences available by then, the fungal ALDH family was poorly represented by only nine sequences. A considerably increased number of fully sequenced fungal genomes allowed to analyze the fungal ALDH family by 53 fungal ALDHs, representing all major phyla in the kingdom of fungi. Most fungal genomes contain multiple ALDH sequences, suggesting duplication events during the course of their evolution. With this in mind, a phylogenetic reconstruction of fungal ALDHs revealed early duplication and divergence (Blackwell et al. 2009). However, ascomycete ALDHs were found to be basal to Zygomycetes, which could actually be due to the effect of long-branch attraction (reviewed in Bergsten 2005). Divergence then led to functionalization with subfamilies 1 and 2, with some fungi possessing multiple sequences in either clade. Thus, the first duplication has occurred after the split of Chytridiomycetes and another, more recent one, within the Agaricomycetes, where higher numbers of ALDH sequences are found. Assuming the current status of fungal ALDH evolution, *T. vaccinum* ALDH is suggested to have diverged relatively recently, compared to other Agaricomycetes.

Our working hypothesis was that *ald1* would mediate alcohol-, especially ethanol-, and aldehyde-related stress remediation in *T. vaccinum*, specifically in ectomycorrhizal association. This would imply that *ald1* expression would be induced under alcohol and aldehyde stress conditions. We first observed five putative STREs in the promoter region of *ald1*. In *S. cerevisiae*, STREs AGGGG and CCCCT separated by 0–60 bp were identified 100–600 bp upstream to the ORF of the stress-inducible genes *ald2* and *ald3* (Moskvina et al. 1999). In other fungal ALDHs, STREs are detectable in promoter sequences of *aldA* of *A. niger* (−212, −233, −379, −463) and of *A. nidulans* (−136, −146, −279). It is not clear whether these signals can be assigned to basidiomycete sequences, but also the promoter of ALDH (EAU87137) in *C. cinerea okayama* shows STREs (−140, −411, −453).

Our findings show that Ald1, which was predicted to be cytosolic, displayed ALDH activity only on propionaldehyde and butyraldehyde, restricted for instance by high volatility of the used substrates. *ald1* induction strongly depended on the concentration of the substrates. Our data

with competitive and real-time RT-PCR confirmed that *ald1* is, indeed, induced by aliphatic and aromatic alcohols and aldehydes. The observed discrepancy of *ald1* transcript levels reported for both RT-PCR methods, which were quantified at different time points, could be due to the fact that the fungal medium used in the real-time RT-PCR experiments contained 20 g/l of malt extract, instead of 5 g/l used in competitive RT-PCR. This could have resulted in metabolic accumulation of carbon compounds, including, but not limited to, glucose, in the fungal cultures, which could have then repressed *ald1* expression. Glucose is considered as a strong repressor of the ethanol regulon in the ascomycete model fungus *A. nidulans*, of which the aldehyde dehydrogenase gene *aldA* is part, although other carbon compounds like fructose and lactose were also identified as weak repressors (Fillinger et al. 1995). Nonetheless, the two RT-PCR methods show that ethanol and aldehydes, at relatively low concentrations that may be physiologically relevant, induce *ald1* gene expression. Since *ald1* is differentially expressed in ectomycorrhizal interaction (Krause and Kothe 2006) and its expression was highly induced by the leaf oil extracts from its host plant, spruce, we suggest that host plant signals could be involved in the in planta expression of the gene. This suggestion is partially supported by the observed induced fungal ALDH encoding genes' expression during growth of the phytopathogenic fungi *U. maydis* (Basse et al. 1996) and *Cladosporium fulvum* (Coleman et al. 1997) in their plant hosts. The hypothesized function of Ald1 of *T. vaccinum* is the reduction of the inhibitory effect of spruce root toxic alcohols and/or aldehydes during the establishment and existence of mycorrhiza.

Low amounts of some of the used ALDH substrates, isobutanol (0.01%), glutardialdehyde (0.01%), pine leaf oil (0.01%), and spruce leaf oil (0.005%), resulted in growth inhibiting or toxic effect on fungal mycelium, whereas low amounts of ethanol (0.01, 0.1%) showed growth promotion. Growth promotion on ethanol is reported for necrotrophic fungi like *Fusarium avenaceum* and *Armillaria* sp. (Asiegbu 2000). In contrast, *Heterobasidion annosum* with the host pine only shows growth promotion by ethanol in combination with saccharose, whereas growth inhibition is reported for host specificity on spruce (Asiegbu 2000).

Induction of *ald1* expression by ethanol suggests its involvement in ethanol catabolism. The role of *ald1* to circumvent ethanol stress was shown using an *ald1*-overexpressing *T. vaccinum* transformant. This function is important in reducing ethanol and/or acetaldehyde toxicity in ectomycorrhizal habitats, a critical function considering the abundance of these toxic compounds in the habitat. However, in order to fully resolve the aldehyde and alcohol stress tolerance in our ectomycorrhiza, functions of other *T.* vaccinum ALDH genes should be investigated. Now that we have developed an efficient *A. tumefaciens*-mediated transformation system and demonstrated its use in functional gene analysis, this should not only be possible for *T. vaccinum*, but also, once optimized, for other ectomycorrhizal fungi. Although agrotransformation has been demonstrated for some ectomycorrhizal fungi, this is the first report on the long-term storage of *A. tumefaciens*-transformed fungi. The genes are stable without selective pressure, as observed with *egfp*. This increases the potential for the use of transformed fungi in functional gene analysis in ectomycorrhiza, as demonstrated for *ald1*, and in long-term experiments like mycorrhization studies.

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References

Achatz G, Oberkofler H, Lechenauer E, Simon B, Unger A, Kandler D, Ebner C, Prillinger H, Kraft D, Breitenbach M (1995) Molecular cloning of major and minor allergens of *Alternaria alternata* and *Cladosporium herbarum*. Mol Immunol 32:213–227

Asiegbu FO (2000) Effects of carbohydrates, ethanol and selected cell wall phenolics on *in vitro* growth of necrotrophic fungi—*Heterobasidion annosum* & *Fusarium avenaceum*. J Basic Microbiol 40:139–148

Basse CW, Lottspeich F, Steglich W, Kahmann R (1996) Two potential indole-3-acetaldehyde dehydrogenases in the phytopathogenic fungus *Ustilago maydis*. Eur J Biochem 242:648–656

Bellion M, Courbot M, Jacob C, Guinet F, Blaudez D, Chalot M (2007) Metal induction of a *Paxillus involutus* metallothionein and its heterologous expression in *Hebeloma cylindrosporum*. New Phytol 174:151–158

Bergsten J (2005) A review of long-branch attraction. Cladistics 21:163–193

Blackwell M, Vilgalys R, James TY, Taylor JW (2009) Fungi. Eumycota: mushrooms, sac fungi, yeast, molds, rusts, smuts, etc. http://tolweb.org/Fungi. Accessed 10 Apr 2009

Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25:169–193

Cavener DR, Ray SC (1991) Eukaryotic start and stop translation sites. Nucleic Acids Res 19:3185–3192

Chen X, Stone M, Schlagnhaufer C, Romaine CP (2000) A fruiting body tissue method for efficient *Agrobacterium*-mediated transformation of *Agaricus bisporus*. Appl Environ Microbiol 66:4510–4513

Chilvers GA, Douglass PA, Lapeyrie FF (1986) A Paper-Sandwich technique for rapid synthesis of Ectomycorrhizas. New Phytol 103:397–402

Cobessi D, Tete-Favier F, Marchal S, Branlant G, Aubry A (2000) Structural and biochemical investigations of the catalytic mechanism of an NADP-dependent aldehyde dehydrogenase from *Streptococcus mutans*. J Mol Biol 300:141–152

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

Duplessis S, Courty PE, Tagu D, Martin F (2005) Transcript patterns associated with ectomycorrhiza development in *Eucalyptus globus* and *Pisolithus microcarpus*. New Phytol 165:599–611

Fillinger S, Panozzo C, Mathieu M, Felenbok B (1995) The basal level of transcription of the *alc* genes in the ethanol regulon in *A. nidulans* is controlled both by the specific transactivator AlcR and the general catabolite repressor CreA. FEBS Lett 368:547–550

Flipphi M, Mathieu M, Cirpus I, Panozzo C, Felenbok B (2001) Regulation of the aldehyde dehydrogenase gene (*aldA*) and its role in the control of the coinducer level necessary for induction of the ethanol utilization pathway in *Aspergillus nidulans*. J Biol Chem 276:6950–6958

Flipphi M, Kocialkowska J, Felenbok B (2003) Relationships between the ethanol utilization (alc) pathway and unrelated catabolic pathways in *Aspergillus nidulans*. Eur J Biochem 270:3555–3564

Gurr SJ, Unkles SE, Kinghorn JR (1987) The structure and organization of nuclear genes in filamentous fungi. In: Kinghorn JR (ed) Gene structure in eukaryotic microbes. SGM Special Publications, Oxford

Guru SC, Taranath-Shetty K (1990) Methodological aspects of aldehyde dehydrogenase assay by spectrophotometric technique. Alcohol 7:397–401

Hanif M, Pardo AG, Gorfer M, Raudaskoski M (2002) T-DNA transfer and integration in the ectomycorrhizal fungus *Suillus bovinus* using hygromycin B as a selectable marker. Curr Genet 41:183–188

Hempel J, Nicholas H, Lindahl R (1993) Aldehyde dehydrogenases: widespread structural and functional diversity within a shared framework. Protein Sci 2:1890–1900

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–755

Isobe K, Fukuda N, Nagasawa S (2007) Purification and characterization of a dehydrogenase catalyzing conversion of N α-benzyloxycarbonyl-L-aminoadipic-δ-semialdehyde to N α-benzyloxycarbonyl-L-aminoadipic acid from *Rhodococcus sp*. AIUZ-35-1. J Biosci Bioeng 104:398–402

Jakoby WB, Ziegler DM (1990) The enzymes of detoxication. J Biol Chem 265:20715–20718

Jobb G (2008) TREEFINDER version of October 2008. www.treefinder.de Accessed 1 May 2010

Jung JH, Lee SB (2006) Identification and characterization of *Thermoplasma acidophilum* glyceraldehyde dehydrogenase: a new class of $NADP^+$-specific aldehyde dehydrogenase. Biochem J 397:131–138

Katoh K, Toh H (2008) Recent developments in the MAFFT multiple sequence alignment program. Brief Bioinf 9:286–298

Kelsey RG, Joseph G (1998) Ethanol in Douglas-fir with black-stain root disease (*Leptographium wageneri*). Can J For Res 28:1207–1212

Kemppainen MJ, Pardo AG (2010) pHg/pSILBA gamma vector system for efficient gene silencing in homobasidiomycetes: optimization of ihpRNA-triggering in the mycorrhizal fungus *Laccaria bicolor*. Microb Biotechnol 3:178–200

Kotchoni SO, Jimenez-Lopez JC, Gao DY, Edwards V, Gachomo EW, Margam VM, Seufferheld MJ (2010) Modeling-dependent protein characterization of the rice aldehyde dehydrogenase (ALDH) superfamily reveals distinct functional and structural features. PLoS One 5:e11516

Kottke I, Guttenberger M, Hampp R, Oberwinkler F (1987) An *in vitro* method for establishing mycorrhiza on coniferous seedlings. Trees 1:191–194

Krause K, Kothe E (2006) Use of RNA fingerprinting to identify fungal genes specifically expressed during ectomycorrhizal interaction. J Basic Microbiol 46:387–399

Kreuzwieser J, Papadopoulou E, Rennenberg H (2004) Interaction of flooding with carbon metabolism of forest trees. Plant Biol 6:299–306

Kreuzwieser J, Hauberg J, Howell KA, Carroll A, Rennenberg H, Millar AH, Whelan J (2009) Differential response of gray poplar leaves and roots underpins stress adaptation during hypoxia. Plant Physiol 149:461–473

Liu Y-G, Mitsukawa N, Oosumi T, Whittier RF (1995) Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J 8:457–463

Liu Z-J, Sun Y-J, Rose J, Chung Y-J, Hsiao C-D, Chang W-R, Kuo I, Perozich J, Lindahl R, Hempel J, Wang BC (1997) The first structure of an aldehyde dehydrogenase reveals novel interactions between NAD and the Rossmann fold. Nat Struct Mol Biol 4:317–326

Mankel A, Krause K, Kothe E (2002) Identification of a hydrophobin gene that is developmentally regulated in the ectomycorrhizal fungus *Tricholoma terreum*. Appl Environ Microb 68:1408–1413

Morel M, Kohler A, Martin F, Gelhaye E, Rouhier N (2008) Comparison of the thiol-dependent antioxidant systems in the ectomycorrhizal *Laccaria bicolor* and the saprotrophic *Phanerochaete chrysosporium*. New Phytol 180:391–407

Mori N, Fuchigam S, Kitamoto Y (2002) Purification and properties of Betaine aldehyde dehydrogenase with high affinity for NADP from *Arthrobacter globiformis*. J Biosci Bioeng 93:130–135

Moskvina E, Imre EM, Ruis H (1999) Stress factors acting at the level of the plasma membrane induce transcription *via* the stress response element (STRE) of the yeast *Saccharomyces cerevisiae*. Mol Microbiol 32:1263–1272

Müller T, Avolio M, Olivi M, Benjdia M, Rikirsch E, Kasaras A, Fitz M, Chalot M, Wipf D (2007) Nitrogen transport in the ectomycorrhiza association: the *Hebeloma cylindrosporum–Pinus pinaster* model. Phytochem 68:41–51

Nakazono M, Tsuji H, Li Y, Saisho D, Arimura S, Tsutsumi N, Hirai A (2000) Expression of a gene encoding mitochondrial aldehyde dehydrogenase in rice increases under submerged conditions. Plant Physiol 124:587–598

Nehls U, Gohringer F, Wittulsky S, Dietz S (2010) Fungal carbohydrate support in the ectomycorrhizal symbiosis: a review. Plant Biol 12:292–301

Osundina MA (1998) Nodulation and growth of mycorrhizal *Casuarina equisetifolia* J.R. and G. First in response to flooding. Biol Fertil Soils 26:95–99

Pardo AG, Hanif M, Raudaskoski M, Gorfer M (2002) Genetic transformation of ectomycorrhizal fungi mediated by *Agrobacterium tumefaciens*. Mycol Res 106:132–137

Perozich J, Nicholas H, Wang B, Lindahl R, Hempel J (1999) Relationships within aldehyde dehydrogenase extended family. Protein Sci 8:137–146

Rambout A (2009) FigTree v1.2.3. Tree figure drawing tool. http://tree.bio.ed.ac.uk/software/figtree/. Accessed 1 May 2010

Rambout A, Drummond AJ (2007) Tracer v1.4. http://beast.bio.ed.ac.uk/Tracer. Accessed 1 May 2010

Rao RP, Hunter A, Kashpur O, Normanly J (2010) Aberrant synthesis of indole-3-acetic acid in *Saccharomyces cerevisiae* triggers morphogenic transition, a virulence trait of pathogenic fungi. Genetics 185:211–220

Reineke G, Heinze B, Schirawski J, Buettner H, Kahmann R, Basse CW (2008) Indole-3-acetic acid (IAA) biosynthesis in the smut fungus *Ustilago maydis* and its relevance for increased IAA levels in infected tissue and host tumour formation. Mol Plant Pathol 9:339–335

Rutto KL, Mizutani F, Kadoya K (2002) Effect of root-zone flooding on mycorrhizal and non-mycorrhizal peach (*Prunus persica* Batsch) seedlings. Sci Hortic 94:285–295

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Scheidegger C, Brunner I (1993) Freeze-fracturing for low-temperature scanning electron microscopy of Hartig net in synthesized *Picea abies–Hebeloma crustiliniforme* and *–Tricholoma vaccinum* ectomycorrhizas. New Phytol 123:123–132

Singer R (1986) The agaricales in modern taxonomy. 4th ed. Koeltz Scientific Books, Königstein/Ts

Stamatakis A (2006) RaxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinf 22:2688–2690

Swofford DL (2002) PAUP* 4.0 b10. Phylogenetic analysis using parsimony (and other methods). Sinauer Associates, Sunderland

Tagu D, Martin F (1995) Expressed sequence tags of randomly selected cDNA clones from *Eucalyptus globus–Pisolithus tinctorius* ectomycorrhizae. Mol Plant Microbe Interact 8:781–783

Tagu D, Python M, Crétin C, Martin F (1993) Cloning symbiosis-related cDNAs from eucalypt ectomycorrhizas by PCR-assisted differential screening. New Phytol 125:339–343

Tanaka E, Tanaka C (2008) Phylogenetic study of clavicipitaceous fungi using acetaldehyde dehydrogenase gene sequences. Mycoscience 49:115–125

Velasco-García R, González-Segura L, Muňz-Clares RA (2000) Steady-state kinetic mechanism of the $NADP^+$- and NAD^+-dependent reactions catalysed by betaine aldehyde dehydrogenase from *Pseudomonas aeruginosa*. Biochem J 352:675–683